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Volume 2

Akadémiai Kiadó, Budapest

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 2 (1), pp. 3—9 (1967)

Structure and Function of Erythrocytes

II. Relation between Potassium Transport and Morphology

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(Received July 29, 1966)

The relations between maintenance of the biconcave shape and K transport, these two energy-requiring processes of erythrocytes have been studied. Experiments on the metabolic regulation of K-outflow have shown that in a number of cases the morphological changes do not run parallel with K-loss. This may be attributed mainly to the excessive NaF sensibility of the mechanism responsible for the maintenance of the biconcave shape, furthermore to the Ca requirement of the rapid K-outflow and its connection with the breakdown of 2,3-DPG*. Investigations concerning the inhibition of active K transport have revealed that the mechanism responsible for the maintenance of the biconcave shape is not ouabain sensitive, it actually utilizes the ATP excess produced by the inhibition of active K transport in the presence of ouabain for the formation of the biconcave shape. Thus the relation between the two mechanisms underlying the maintenance of biconcave shape and K transport may be characterized as two processes competing for the utilization of ATP.

* * *

The two essential energy-requiring functions of erythrocytes are the maintenance of the biconcave shape and of unequal ion distribution. According to Nakao et al. (1961) the maintenance of biconcave shape is based on the interaction between ATP and membrane proteins, while the phenomenon of the disk-sphere transformation is directly dependent on the decrease in ATP content. Whittam (1964), however, in connection with Nakao's theory suggests to determine first the actual ion concentrations before deciding whether or not ATP has a direct action on the membrane structure. ATP may namely act on the shape of the cell indirectly as well, by changing the ion and water concentrations. Ponder (1948) showed that parallel with the disk-sphere transformation induced by sublytic amounts of hemolysins, a K-loss occurs likewise. Other known shape transformations are similarly accompanied by K-loss e.g. those elicited by glycolytic inhibitors or those which appear in the course of the storage of preserved blood.

In our previous work (Gárdos et al., 1966) we have reported that the morphological change follows but slowly, with some delay, the changes in ATP level.

*Abbreviations: ATP = adenosine 5'-triphosphate; 2,3-DPG = 2,3-diphosphoglycerate; IA = iodoacetate (sodium salt); EDTA = ethylenediaminetetraacetate; I_m = morphological index.

A similar lag period can be observed in case of K-efflux in substrate-deficient erythrocytes or in those treated with glycolytic inhibitors (Davson, 1941; Ponder, 1949; Gárdos, Straub, 1957). All these data point to the necessity of a closer study of the relation between unequal ion distribution and maintenance of the biconcave shape.

Experimental

In the experiments fresh defibrinated human blood was used. 75 per cent of the experimental mixture consisted of normal blood, the active agents formed the remaining 25 per cent. In this way we worked at an average 30 per cent hematocrit value. The changes occurring in ATP-level, morphology and K-content of erythrocytes were followed in experimental systems incubated at 37 °C.

Morphology was evaluated according to our own method (Gárdos et al., 1966). ATP was estimated in two different ways: *a)* by spectrophotometric analysis of the fractions separated on a Dowex-1-Cl ion exchange column, according to the method of Deutsch and Nilsson (1953); *b)* enzymically, by using glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, by the aid of the Boehringer-test (Bücher, 1947). The values measured were expressed as percentages of the initial values.

2,3-DPG was determined subsequent to separation by ion exchange chromatography by the chromotropic acid reaction, following the method of Bartlett (1959). K determinations were performed by flame photometry.

Results

Our experiments performed with glycolytic inhibitors pointed to a striking similarity between the kinetics of morphological degeneration and of K-outflow (Fig. 1). When, however, glycolytic inhibitors were combined with some other active agents a number of differences concerning their effect on these two phenomena were established. Thus IA (1 mM) and adenosine (10 mM) when applied simultaneously, may produce an increased rate of K-outflow (Gárdos, 1956, 1960) and morphological degeneration, too. However, while this rapid K-outflow can be inhibited by $NaHSO_3$ (3 mM), Na_2HAsO_4 (2.5 mM) and EDTA (2 mM), the same agents affect but slightly the morphological degeneration (Table 1). If in addition to IA – which was found to fully inhibit glycolysis – NaF is being applied in a concentration of 2 and 5 mM, respectively, K-efflux will be far more increased than the degeneration of morphology. At the same time 2 mM NaF is known to have a 50 per cent, and 5 mM NaF a still higher inhibitory effect on the decomposition of 2,3-DPG (Gárdos, 1966). This inhibitory action, evidently, manifests itself besides IA likewise on the ATP synthesis brought about by the breakdown of 2,3-DPG.

$NaHSO_3$ (15 and 30 mM) applied together with IA only slightly inhibits K-outflow for 120 and 90 min, respectively, but subsequently it induces a K-efflux of great velocity. The beginning of this rapid K-outflow occurs simultaneously with the breakdown of 2,3-DPG. The effect exerted on morphology is of a different nature, it corresponds to the shifts taking place in the ATP-level as a response to $NaHSO_3$ (Fig. 2).

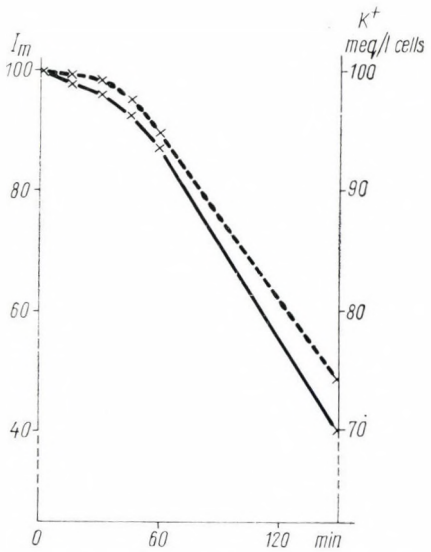

Fig. 1. Kinetics of K^+-loss and morphological degeneration induced by 16 mM NaF. The broken line indicates the morphological degeneration, the solid line marks K^+-loss

Table I

The effect of different agents on the morphological degeneration and K^+ loss of human erythrocytes in the presence of 1 mM IA

Agents added	Morphological degeneration ΔI_m/180 min	K^+ loss ΔK^+/180 min in meq/l cells
1 mM IA	27	4.8
1 mM IA + 10 mM adenosine	59	34.0
1 mM IA + 10 mM adenosine + 2 mM EDTA	57	5.2
1 mM IA + 10 mM adenosine + 2.5 mM Na_2HAsO_4	56	9.5
1 mM IA + 10 mM adenosine + 3 mM $NaHSO_3$	57	5.2
1 mM IA + 2 mM NaF	36	21.0
1 mM IA + 5 mM NaF	40	39.4

16 mM NaF is found to accelerate the K-outflow which can be inhibited by 10 mM adenosine or 2 mM EDTA, whereas 2.5 mM Na_2HAsO_4 will evoke a further increase. On the other hand, morphological degeneration induced by 16 mM NaF is not affected by adenosine, it is slightly reduced by EDTA and not very appreciably increased by Na_2HAsO_4 (Table 2).

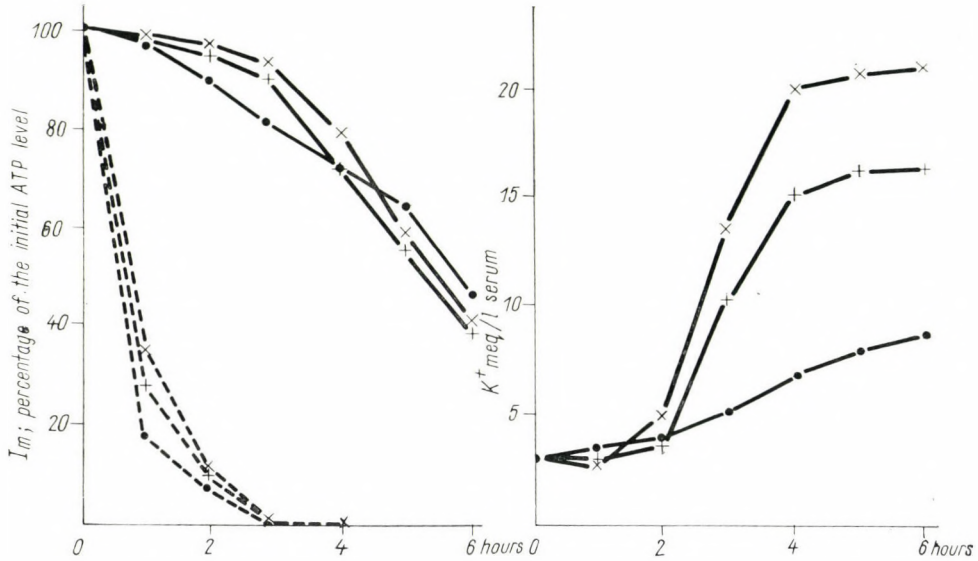

Fig. 2. Effect on ATP breakdown, morphological degeneration and K^+-outflow of $NaHSO_3$ applied together with IA. ●——● 1 mM IA, +——+ 1 mM IA + 15 mM $NaHSO_3$, ×——× 1 mM IA + 30 mM $NaHSO_3$. In the figure on the left the broken lines mark ATP breakdown, the solid lines indicate the morphological degeneration

Table 2

The effect of different agents on the morphological degeneration and K^+ loss of human erythrocytes in the presence of 16 mM NaF

Agents added	Morphological degeneration ΔI_m/120 min	K^+ loss ΔK^+/120 min in meq/l cells
16 mM NaF	45	36.0
16 mM NaF + 10 mM adenosine	45	3.0
16 mM NaF + 2 mM EDTA	36	3.0
16 mM NaF + 2.5 mM Na_2HAsO_4	52	50.5

If the so-called transport ATPase is inhibited by 10 μM ouabain, the K-outflow elicited by IA will be slightly accelerated while the rate of morphological degeneration will show a slow decrease. For example: in the presence of 1 mM IA the ΔI_m per 180 min = 27 whereas with 1 mM IA + 10 μM ouabain the same value amounts to 16 only. The reverse process, morphological regeneration, is likewise influenced in the same sense by ouabain. If the cells are incubated with 8

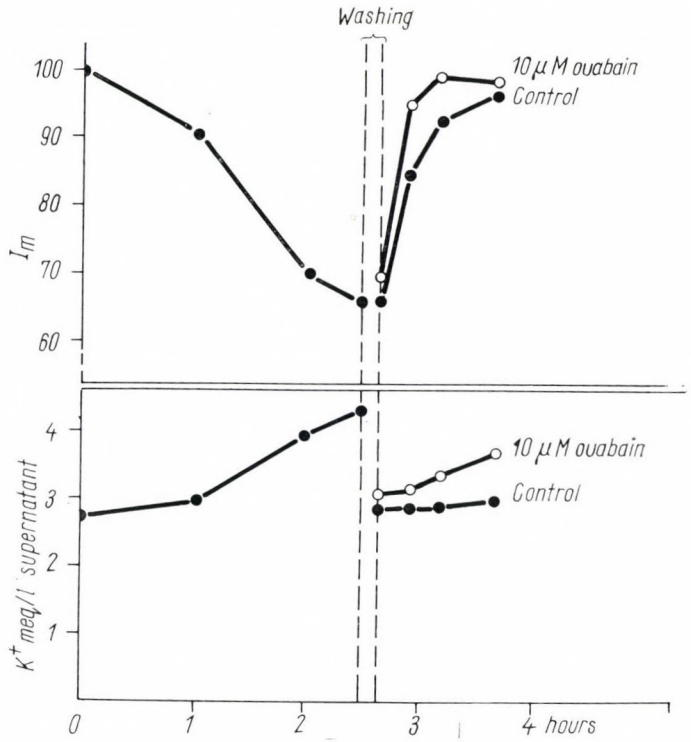

Fig. 3. Effect of ouabain on the morphological regeneration occurring during the fermentative resynthesis of ATP, and on K^+-transport. For the details see the text

mM NaF for 150 min, after the removal of NaF by washing ATP is resynthesised and as a result of this K-accumulation will be resumed. The presence of ouabain prevents the development of the state of dynamic equilibrium which leads to a further net loss in K. By contrast, the rate of morphological regeneration is more pronounced in the presence of ouabain than in the ouabain-free control (Fig. 3).

Discussion

In the present experiments we have produced two changes of different types in the processes of K-transport. The first type was the induction of an excessive K-outflow by treatment with 16 mM NaF, or by the combination of IA and

adenosine. In these cases the cells have lost their K-content at a rate of 12–25 meqs per l erythrocytes per hour without an equivalent Na-uptake (Dunker, Passow, 1950; Passow, 1961). According to Wilbrandt (1940) under these conditions the cells display a decrease in volume as well, and become more resistant to glycerol-induced hemolysis. As a further characteristic property the K-efflux requires the presence of Ca ions in the medium (Gárdos, 1958, 1958a, 1959, 1961) which means that the rapid K-outflow can be stopped by EDTA. On the other hand, changes of morphology are but slightly affected by EDTA. $NaHSO_3$ bringing about a high-rate decomposition of 2,3-DPG (Mányai, Várady, 1956) and Na_2HAsO_4 – producing the arsenolysis of inosine – influenced, in the presence of IA, the ATP-level and morphology only to a negligible extent while they significantly affected the rapid K-outflow. Similarly, the action on the morphology of compounds applied together with NaF greatly differs from their effect on K-efflux. Thus, for example, adenosine applied together with NaF besides its significant action on the ATP-level and K-transport, has no influence on the maintenance of biconcave shape.

Our experiments characterized by high rates of K-outflow suggest that this kind of K-outflow does not run parallel with the changes in morphology. The discrepancies can be summarized as follows:

1. The cessation or marked decrease of the excessive K-efflux was not accompanied by the improvement of the morphological picture.

2. The binding of Ca ions had a slighter influence on the morphological changes than on the K-outflow.

3. 2,3-DPG acted on the morphology only in as much as ATP was formed of it whereas it considerably influenced the extent of K-efflux.

In the second part of our experiments active K-transport was inhibited. This inhibition took place on treatment of the cells with IA or 4–5 mM NaF following the breakdown of 70–90% of the ATP-content of the cell, i.e. subsequent to a lag period of 40–60 min needed for the exhaustion of the energy source of active transport (Gárdos, Straub, 1957). The same thing happened, without a lag period, when active transport was inhibited by ouabain. When glycolytic inhibitors and ouabain were applied simultaneously, active transport was inhibited without a lag period. This K-outflow occurring earlier elicited a somewhat higher K-level compared to the medium of cells treated merely with glycolytic inhibitors. Dunham (1957) and Whittam (1958) reported that the ATP-content of substrate-free erythrocytes and of those treated with glycolytic inhibitors decreases slower in the presence of ouabain than in the ouabain-free controls. Thus the ATP which has not been utilized for active transport occurs in the cells as excess ATP. Our experiments indicate that the cell is able to utilize this excess energy, the rate of morphological degeneration was slowed down in the presence of ouabain. This means that in contrast to active ion transport, the mechanism maintaining the biconcave shape is not sensitive to ouabain, and while ouabain damages the maintenance of unequal ion distribution, it has a very favourable effect on the

maintenance of biconcave shape. This proves that the two mechanisms are not identical. Our findings are in a good agreement with the experiments of Nakao et al. (1963) who succeeded in isolating a ouabain-sensitive and a ouabain-insensitive erythrocyte membrane ATPase.

Acknowledgements

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Die Einkörperung des ^{35}S-Methionins in Proteine des Zentralnervensystems bei jungen und ausgewachsenen Ratten

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(Eingegangen am 8. August, 1966)

Die Verfasser untersuchten bei 7 bzw. 10 Tage alten und ausgewachsenen Ratten den Einbau des i. p. injizierten ^{35}S-Methionins in die Eiweiße von 13 verschiedenen Gebieten des Gehirns mit autoradiographischer Methode. Bei den jungen Tieren war der Einbau in allen geprüften Gebieten ausgeprägter als bei den ausgewachsenen. Dies wurde mit der Verschiedenheit der Blut-Hirnschranke und der größeren Menge der Proteine kurzer Halbwertzeit in jungen Tieren erklärt. Das Verhältnis der in die einzelnen Abschnitte des Gehirns eingebauten markierten Aminosäure zu der in die Großhirnrinde eingebauten ist bei jungen und ausgewachsenen Tieren im wesentlichen dasselbe. Ausnahme ist das Kleinhirnmark, in dem der Einbau beim jungen Tier wesentlich größer ist als beim ausgewachsenen. Die Abhängigkeit dieses Unterschieds von der Markentwicklung wird erwogen.

Clouet und Gaitonde (1956) haben in biochemischen Untersuchungen gezeigt, daß der Einbau von ^{35}S-Methionin in die Proteine des Zentralnervensystems bei neugeborenen Ratten bedeutend höher ist als bei ausgewachsenen. Sie fanden die höchste Markierung in der separierten Mikrosomen-Fraktion. Die isolierten Proteine gehörten zu den Liponucleoproteinen.

Für eine vergleichende Untersuchung von Hirnregionen bzw. Zellgruppen sind die autoradiographischen Schnittmethoden den biochemischen überlegen, da diese nur an kleinen und morphologisch nicht immer mit Sicherheit bestimmbaren Hirnteilen ausgeführt werden können.

Cohn, Gaitonde u. Richter (1954) haben an Ratten autoradiographisch (stripping film-Technik) gefunden, das die Einkörperung von ^{35}S-Methionin in die Gehirnproteine in der grauen Substanz bedeutend höher war als in der weißen Substanz. Bei 1 Tag alten Tieren war allerdings die Einkörperung »relativ diffus«.

Es wurden mit Hilfe der von uns ausgearbeiteten autoradiographischen Methode, die *quantitativen* Untersuchungen auch an großen Serien von Tieren ermöglicht (Mérei, Gallyas, 1964a), der Einbau von ^{35}S-Methionin in die Proteine des Zentralnervensystems der Ratte studiert (Mérei, Gallyas, 1964b). Die morphologisch verschiedenen Gehirngebiete wurden hinsichtlich der relativen Isotopenkonzentration in 7 Gruppen eingeteilt.

In vorliegender Arbeit wird über den Einbau des ^{35}S-Methionins in verschiedene Gehirnabschnitte von neugeborenen und ausgewachsenen Ratten berichtet.

Methode

Die Untersuchungen wurden an 98 weißen Ratten aus unserer Hauszüchtung ausgeführt: 56 junge (je 28–7 bzw. 10 Tage alte, von 12 g bzw. von 19 g Körpergewicht), ferner 28 ausgewachsene (Durchschnittsgewicht von 150 g). Jeder Ratte wurden i. p. 150 μC in $2{,}02 \times 10^2$ μg/100 g Körpergewicht ^{35}S-Methionin injiziert. Nach der Injektion wurden je 4 Tiere jeder Gruppe zu verschiedenen Zeitpunkten (nach 5, 10, 15, 30 und 60 Min, ferner nach 8 und 24 Stunden) durch Entblutung getötet. Von sämtlichen Tieren wurden bei der Entblutung Blutproben genommen, um die spezifische Radioaktivität des Blutes zu bestimmen. Gehirn und zervikaler Teil des Rückenmarks, sowie Leber, Herz und Nieren wurden gewogen. Das Gehirn wurde in 10%igem Formalin fixiert. Nach 24-stündiger Fixierung wurden 5 Horizontalscheiben des Gehirns zusammen mit den anderen Gewebsstücken in Paraffin eingebettet. 15 μ dicke Schnitte wurden verfertigt. Nach Entfernung des Paraffins mit Chloroform wurden die Schnitte in eine 0,5%ige Acrylat-Chloroform-Lösung getaucht; Eintrocknen der Chloroform-Acrylat-Lösung, Autoradiographie.

Die Autoradiogramme und die photometrische Bestimmung der relativen Isotopenkonzentration wurden mit unserer oben erwähnten Methode ausgeführt. Der Fehler des Verfahrens bleibt in Gebieten mit relativ niedriger Aktivität zwischen 8–10%, bei relativ höherer Isotopenkonzentration der Gewebe zwischen 4–6%.

Die relative Konzentration des ^{35}S-Methionins wurde in 13 funktionell und morphologisch verschiedenen Gebieten des Zentralnervensystems bestimmt.

4 weiteren ausgewachsenen Ratten wurden 150 μC/100 g Körpergewicht ^{35}S-Methionin i. v. injiziert und mit der von uns beschriebenen Methode (Gallyas, Mérei, 1965) Blutproben gesammelt. Die Blutproben (10 μl) wurden aus der rechten Herzkammer entnommen.

Die Blutproben, die wir durch den Kunststoffkatheter oder bei der Entblutung gewonnen haben, wurden gewogen und mit Zugabe von 0,5 ml destilliertem Wasser hämolysiert. Nach Eintrocknen der Präparate wurde die spezifische Radioaktivität der Proben bestimmt.

Bei 10 weiteren Tieren verschiedenen Alters und Körpergewichts bestimmten wir das Gewicht der erwähnten inneren Organe und des Gehirns in bezug auf das Körpergewicht.

Ergebnisse

Das Verhältnis zwischen Körpergewicht und dem Gewicht von Leber, Herz, Niere und Zentralnervensystem wird in Abb. 1 dargestellt. Während das Gehirngewicht bei einem 15 g schweren Tier etwas mehr als 5% des Körpergewichts beträgt, macht es bei ausgewachsenen, 150 g wiegenden Tieren um 1% aus. Dagegen verändert sich das Verhältnis des Gewichts von Herz und Niere zum Körpergewicht während der Entwicklung des Tieres kaum. Im Verhältnis des Lebergewichts zum Körpergewicht sehen wir einen Abstieg bei den 20 g und 35 g

schweren Tieren, später aber einen Anstieg bis 5% als endgültigen Wert. Das Gehirngewicht bezogen auf das Körpergewicht sinkt vom Zeitpunkt der Geburt an ständig.

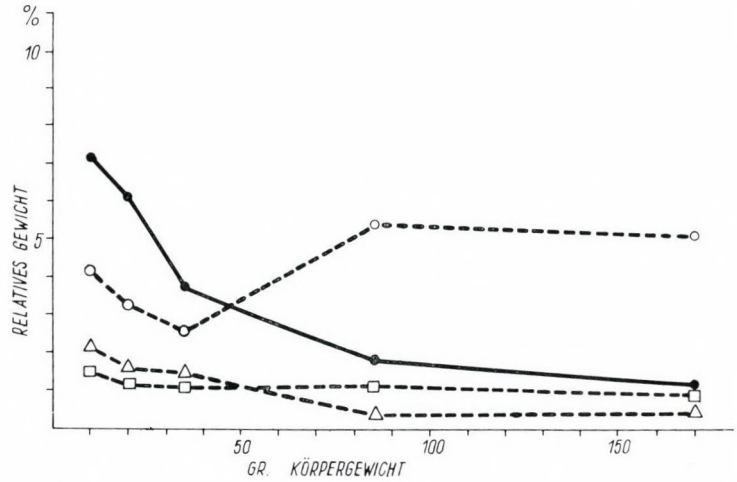

Abb. 1. Graphische Darstellung des Verhältnisses von Körpergewicht zum Gewicht von Hirn (●), Leber (○), Herz (△) und Niere (□). Das relative Gehirngewicht nimmt während der Reifung des Tieres allmählich ab; dagegen ändert sich das Verhältnis des Gewichtes von Herz und Nieren zum Körpergewicht kaum

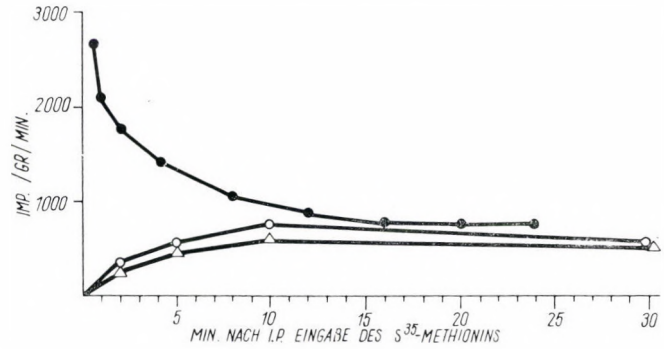

Abb. 2. Zeitkurve des Eintritts des Plasma-Gewebegleichgewichtes von ^{35}S-Methionin bei Ratten (1/2 St). ● intraarterielle, ○ und △ intraperitoneale Einspritzung; ○ ausgewachsene, △ 10 Tage alte Tiere

Abb. 2 zeigt, daß bei der Konzentration des markierten Stoffes im Plasma und Gewebe ein Gleichgewicht zustandekam. Beim ausgewachsenen Tier ist die Radioaktivität des Blutes nach intraarterieller Verabreichung naturgemäß am Anfang maximal, und es erfolgt nach etwa 10 Min ein Absinken auf ein Niveau,

das zum mindesten für 1/4 St beibehalten wird. Ungefähr das gleiche Niveau wird nach intraperitonealer Verabreichung ebenfalls in 10 Min erreicht und mindestens für weitere 20 Min gehalten. Bei jungen Tieren ist die spezifische Aktivität des Blutes nach intraperitonealer Verabreichung dieselbe wie bei den ausgewachsenen.

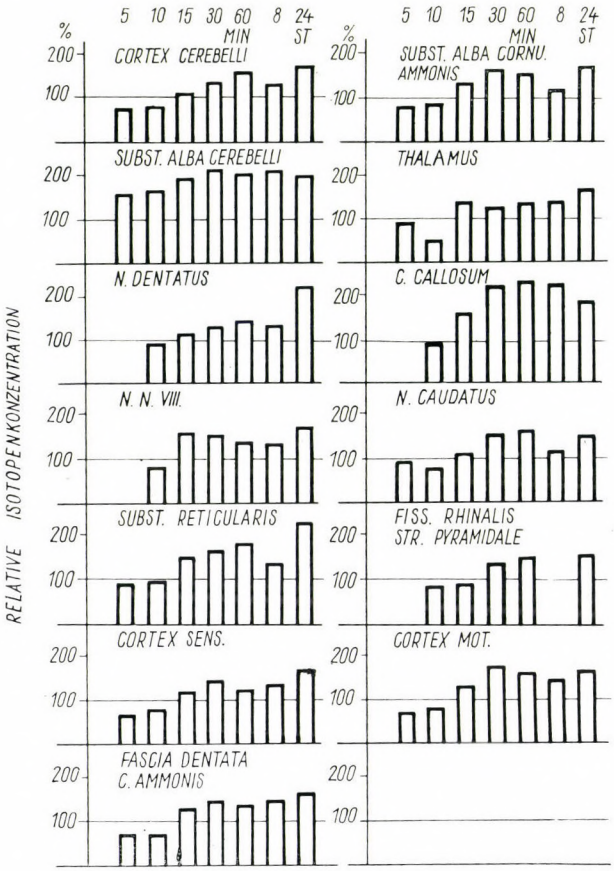

Abb. 3. Die relative Isotopenkonzentration des Gehirns in 13 verschiedenen Gehirnregionen von jungen Ratten; die bei den ausgewachsenen Ratten in demselben Gebiet festgestellten Werte werden als 100% angenommen

Es wurde früher festgestellt, daß die relative Isotopenkonzentration des Gehirngewebes in geradem Verhältnis zu der spezifischen Aktivität der Aminosäure steht (Mérei, Gallyas, 1964b).

Der Einbau des ^{35}S-Methionins in 13 verschiedene Gehirngebiete der neugeborenen und erwachsenen Tiere wird in Abb. 3 auf Grund der relativen Isotopenkonzentrationen dargestellt.

Die bei den ausgewachsenen Ratten gefundenen Werte wurden als 100% angenommen, die bei den jungen Tieren gefundenen hierauf bezogen und in Prozente umgerechnet. Es ist ersichtlich, daß bei den 5–10 Min nach i. p. Eingabe getöteten neugeborenen Tieren die relative Isotopenkonzentration, mit Ausnahme der weißen Substanz des Kleinhirns, bedeutend niedriger ist als bei den ausgewachsenen. Zu der Zeit, zu der ein Plasma-Isotopengleichgewicht eintritt (10–15 Min nach der Injektion), liegt die relative Isotopenkonzentration in allen 13 Gebieten bedeutend höher als bei den ausgewachsenen Tieren. Der Unterschied beträgt in der weißen Substanz des Kleinhirns +95%, im Vestibularis-Kerngebiet +68%, und in der Rinde +57%. Etwas niedriger ist der Unterschied in der weißen Substanz des Ammonshorns (+34%).

Ungefähr gleich ist die relative Isotopenkonzentration bei den neugeborenen Tieren in der Kleinhirnrinde (+2%), im Nucleus dentatus (+4%) und Nucleus caudatus (+7%), etwas niedriger im Rhinencephalon (−13%).

30 Min nach Eingabe des ^{35}S-Methionins ist die relative Isotopenkonzentration in der weißen Substanz des Kleinhirns bei jungen Tieren +30%, im Corpus callosum +20%. Auch in den übrigen Gebieten ist der Unterschied sehr ausgeprägt.

Nach weiteren 30 Minuten bleiben die Verhältnisse unverändert.

Bei den nach 8 Stunden getöteten Tieren ließ sich in manchen Gebieten eine erhebliche Verringerung des Unterschiedes zwischen den beiden Altersgruppen feststellen. Besonders ausgeprägt war diese Verringerung im Kleinhirn (31%), Nucleus dentatus (43%) und Nucleus caudatus (53%).

Die nach 24 Stunden getöteten jungen Tiere zeigen wieder eine Erhöhung der relativen ^{35}S-Methionin-Konzentration im Gehirn im Vergleich mit den ausgewachsenen, besonders in der Substantia reticularis (+97%), im Nucleus dentatus (+99%) und in der Rinde (+44%).

Besprechung

Die Frage, ob das i. p. verabreichte und durch das Gehirn aufgenommene ^{35}S-Methionin tatsächlich in Eiweiße eingebaut wird, wurde durch Untersuchungen von Cohn, Gaitonde und Richter, sowie Clouet und Gaitonde geklärt.

Wie aus unseren Untersuchungen hervorgeht, ist die ^{35}S-Methionin-Aufnahme der Proteine bei jungen Tieren bedeutender als bei den ausgewachsenen.

Diese Erscheinung könnte man mit den folgenden Möglichkeiten erklären. Bei jungen Tieren 1. wird das Methionin durch das Peritoneum leichter resorbiert; 2. ist der Einbau des Methionins in die Eiweiße des Zentralnervensystems schneller; 3. bei neugeborenen Tieren fehlt entweder die sog. »Blut-Hirnschranke«, oder sie ist unvollständig entwickelt.

Die erste Möglichkeit ist auszuschließen, da unsere Experimente zeigten, daß die Resorption des i. p. verabreichten Methionins bei jungen und ausgewachsenen Tieren übereinstimmend ist. Clouet und Gaitonde (1956) wiesen darauf hin, daß das Verhältnis von Proteinen mit kurzer Halbwertzeit bei jungen Tieren höher ist

als bei ausgewachsenen, und der Einbau des Methionins in Eiweiße mit kurzer Halbwertzeit bedeutender ist als in Proteine mit längerer Halbwertzeit. Es ist möglich, daß die sog. »Blut-Hirnschranke« bei jungen Tieren unvollkommener entwickelt ist als bei ausgewachsenen.

Die Aminosäureaufnahme des Gehirns wurde oft mit der von anderen Organen vergleichen; es wurde festgestellt, daß sich das Gehirn von den anderen Geweben verschieden verhält. Die Aufnahme verschiedener Stoffe, einschließlich die des Methionins, ist in der Leber, Niere und quergestreiften Muskulatur unterschiedlich. Das andersartige Verhalten des Zentralnervensystems bedeutet also nicht unbedingt, daß dieses über eine einzigartige, alleinstehende metabolische Eigenschaft verfügto der daß das Zentralnervensystem durch einen morphologisch oder physiologisch genau definierbaren Mechanismus Stoffe selektiv aufnimmt. Bezüglich der Problematik verweisen wir auf die zusammenfassende Arbeit von Dobbing.

Thompson und Ballou (1956) haben die Eiweißstabilität in verschiedenen Organen (Leber, Niere, Muskel, Gehirn, Knochen und Bindegewebe) vergleichend untersucht. Es hat sich gezeigt, daß es in allen Organen sowohl Proteine von sehr langer biologischer Halbwertzeit (130 – 1000 Tage) als auch Proteine gibt, deren Austausch mit den gleichen oder präkursorischen Molekülen des zirkulierenden Blutes sehr schnell erfolgt, d. h. deren Halbwertzeit in den verschiedenen Geweben zwischen 4,5 – 22 Tagen liegt. Aus den Untersuchungen von Thompson und Ballou geht ferner hervor, daß im Gehirn ungefähr die Hälfte der Eiweiße (54%), dagegen in der Leber und Niere nur 8% eine lange Halbwertzeit besitzen.

Die Tatsache, daß das Gehirngewicht, verglichen mit dem Körpergewicht, beim jungen Tier ungefähr fünfmal so groß ist wie beim ausgewachsenen, bietet eine Erklärung dafür, daß in der Aufnahme von Stoffen durch das Gehirngewebe zwischen jungen und ausgewachsenen Tieren ein Unterschied vorhanden ist.

Der Unterschied der relativen Isotopenkonzentration der verschiedenen Hirngebiete bei jungen und ausgewachsenen Tieren ist gleich. In Abb. 4 wird die relative Isotopenkonzentration von den 13 gemessenen Gebieten mit der relativen Isotopenkonzentration der Hirnrinde verglichen. Die für die Rinde gewonnenen Werte haben wir als 100% angenommen; die in der Abbildung angegebenen Werte widerspiegeln den Quotienten zwischen der relativen Isotopenkonzentration der Rinde und der einzelnen Hirnteile. Aus dem Vergleich der Werte geht klar hervor, daß zwischen den jungen und ausgewachsenen Tieren kein wesentlicher Unterschied besteht.

Die hohe relative Isotopenkonzentration der weißen Substanz des Kleinhirns hängt wahrscheinlich mit dem erhöhten Stoffwechsel der Markentwicklung im Kleinhirn (7 – 9 Tage nach der Geburt) zusammen (Almeida, Pearse).

Die Frage, ob der Unterschied der relativen Isotopenkonzentration zwischen jungen und ausgewachsenen Tieren durch Fehlen bzw. minderwertige Leistungsfähigkeit (Lajtha, Tóth) der sog. »Blut-Hirnschranke« oder durch Verschiedenheiten im Stoffwechsel verursacht wird, bleibt offen.

Aus unseren Untersuchungen ergeben sich folgende Ergebnisse. Die relative Isotopenkonzentration ist im Gehirn bei jungen Tieren höher als bei ausgewachsenen. Der Unterschied ist in der weißen Substanz am meisten ausgeprägt,

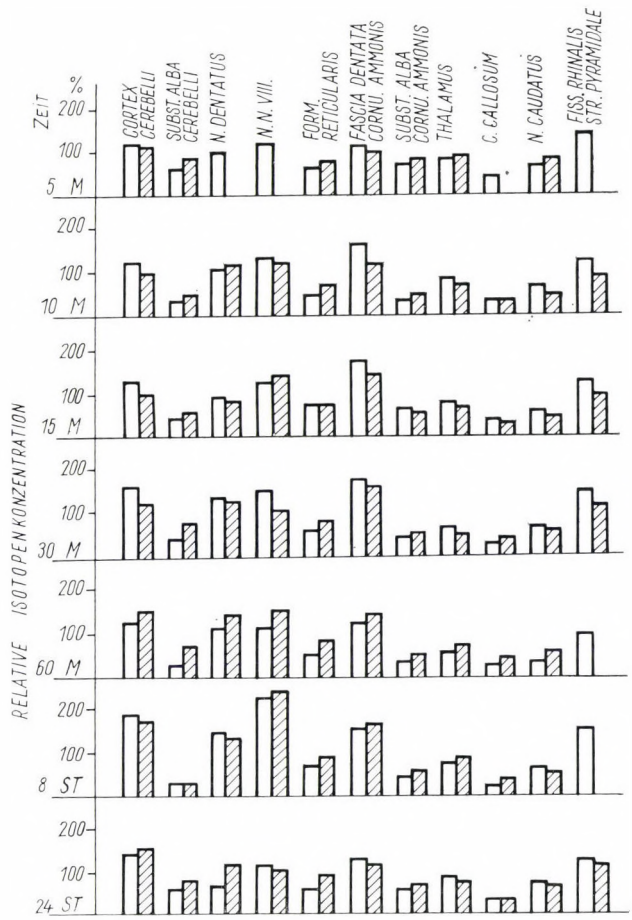

Abb. 4. Veränderung der relativen Isotopenkonzentration in verschiedenen Hirngebieten von jungen und erwachsenen Ratten; die relative Isotopenkonzentration der Großhirnrinde wird als 100% angenommen

was wahrscheinlich durch die fortschreitende Markentwicklung erklärt werden kann. Die hohe relative Isotopenkonzentration in den übrigen Gebieten dürfte darauf beruhen, daß sich das Gehirn junger Tiere sowohl morphologisch als auch physiologisch von dem der ausgewachsenen unterscheidet.

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Effect of Diethylpyrocarbonate on Proteins

I. Reaction of Diethylpyrocarbonate with Amino Acids

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It has been shown by means of ultraviolet and infrared spectrophotometry, electrometric titration and specific photometric measurements that the amino groups of amino acids, the N-1 of the imidazole ring of histidine, the guanidino-group of arginine, the phenolic hydroxyl-group of tyrosine, and the sulfhydrylgroup of cysteine are carbethoxylated by diethylpyrocarbonate. The reaction is favoured by alkaline pH, except for that of the imidazole group. As a result of carbethoxylation, the pK of the dissociation of the carboxylic group is displaced towards alkaline pH, while that of imidazole towards acidic pH. The N-carbethoxy imidazole proved to be labile in both acid and alkali, while the O-carbethoxy tyrosine was labile only in alkali. The N-carbethoxyguanidine arginine failed to give the Sakaguchi-reaction characteristic of free guanidine groups even after 72 hours of incubation in acid or alkali.

Introduction

Diethylpyrocarbonate (DEP) has recently been widely used in food industry as a preservative, due to its bactericidal effect (Hecht, 1961). The application of this substance is especially advantageous because DEP decomposes spontaneously in aqueous media to ethanol and CO_2, and in 24 hours practically no DEP can be detected in the solution.

DEP inhibits the activity of several enzymes, among others that of proteases and nucleases (Hullán et al., 1965; Fedorcsák, Ehrenberg, 1966). DEP also exhibits phage-inhibitory effects (Fedorcsák, Turtóczky, 1965); in this too it acts through the protein, as it has been shown that the transformational properties of DNA remain unaffected.

Data in the literature concerning the mechanism of the effect of DEP on proteins are scanty. Recent studies have shown (Larroquere, 1964; Rosnati, 1964) that DEP reacts with the NH_2-groups of amino acids and primary amines in neutral or weakly alkaline media forming N-carbethoxy compounds with the liberation of CO_2:

$$R-NH_2 + C_2H_5-O-CO-O-CO-O-C_2H_5 \longrightarrow$$
$$R-NH-CO-O-C_2H_5 + CO_2 + C_2H_5OH$$

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The aim of the present investigations was to obtain further data concerning the reaction of DEP with natural amino acids to have some information on the suitability of this substance for side chain modification in protein research.

Materials and Methods

DEP. A Bayer commercial preparation (Baycovin) was used in 10 per cent solution.

5-*amino tetrazole* was purchased from "Serva".

All other chemicals were obtained from "Reanal" (Budapest), and were of reagent grade.

The DEP-treatment of amino acids was carried out in acetate buffer, pH 6, or in borate buffer, pH 7.6 and 9, respectively, in the presence of at least a 4–5-fold excess of DEP. Reaction mixtures were kept at 2 °C for minimum 24 hours. During this period the unreacted DEP completely decomposed to ethanol and CO_2. According to Török et al. (1958) the half-time of DEP-decomposition is 16.8 minutes in aqueous media at 20 °C, thus the unreacted DEP was practically eliminated from the reaction medium before the subsequent examinations.

Histidine was determined by the imidazole-assay of Horinoshi et al. (1963) or Sluyterman (1960), based on a modified Pauly-reaction, with diazo 1–H-tetrazole and diazosulphanylic acid, respectively.

Tyrosine was iodinated by the method of Hughes and Streassle (1950) at pH 10. Excess I_2 was reduced with sodium arsenite.

The guanidino-group of arginine was determined by the Sakaguchi-reaction as modified by Ceriotti and Spandrio (1957).

Sulfhydryl groups were assayed by Ellman's method (1958).

Ultraviolet spectra were taken in a Spektromom 201 type spectrophotometer, at 5 mμ intervals.

The infrared spectra of the potassium salts of DEP-treated amino acids were taken in a Zeiss (Jena) UR 10 type double-beam spectrometer in KBr pastil. Amino acid derivatives were prepared in aqueous solution. The solutions of amino acids were adjusted to pH 8–9 with KOH after the addition of DEP, and after 24 hours of incubation they were acidified to pH 5 with HBr and lyophilized. The freeze-dried material was dissolved in 96 per cent ethanol or chloroform, then precipitated by petrolether and dried. In this way crystalline, though highly hygroscopic, material was obtained.

Electrometric titrations were performed in a Radiometer TTT 1 titrator equipped with a SBR 2 titrigraph. Amino acids were dissolved in 0.02 M $NaHCO_3$ to 0.01 M end-concentration, then the solution was made 0.04 M with respect to DEP and incubated for 24 hours at 2 °C.

The pH of solutions to be titrated was adjusted to 2.1, then the dissolved CO_2 was driven out by bubbling nitrogen through the solution for 30 minutes.

Titrations were made with 0.5 N NaOH. The alkali uptake was corrected for that of the amino acid-free control ($NaHCO_3$ + DEP).

In the figures, the ordinate shows the amount of neutralized H^+ ions from pH 2 up to the pH value indicated on the abscissa.

Results and Discussion

According to Larroquere (1964) and Rosnati (1964) DEP reacts with the amino-groups of amino acids in neutral or slightly alkaline media. The reaction leads to the respective N-carbethoxy derivatives.

As the amino-groups, with the exception of the N-terminal amino group, are masked as peptide bonds in the proteins, and the proteins still react with DEP, we have to consider the possibility of the reaction of other functional groups.

Electrometric titration of amino acids reacted with DEP

The electrometric titration curves of DEP-treated glycine, lysine, histidine and tyrosine (Figs 1 a, b, c and d) show that the shoulder due to the amino group is absent, and the pK of the carboxylic group is considerably displaced in the

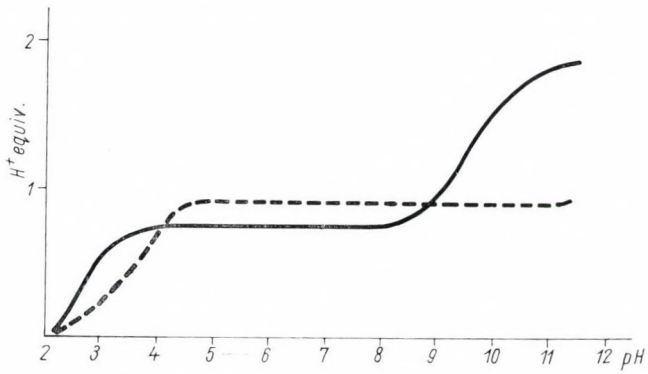

Fig. 1a. Electrometric titration curve of glycine: —— glycine; — — — DEP-treated glycine

alkaline direction. The displacement of pK is caused by the fact that the protonation of nitrogen is hindered owing to the formation of the N-carbethoxy group. Thus the strongly electron-withdrawing NH_3-group cannot be formed, and this results in a lower pK of the carboxylic groups of amino acids.

In case of lysine the disappearance of 2 equivalents of prototropic groups was observed in the alkaline region as a result of carbethoxylation, i.e. both the α- and ε-amino group reacted with DEP.

Similar results were obtained when DEP-treated tyrosine was titrated. The two prototropic groups correspond to the amino and phenoxy groups. Consequently, it can be established that the phenolic OH of tyrosine also reacts with DEP.

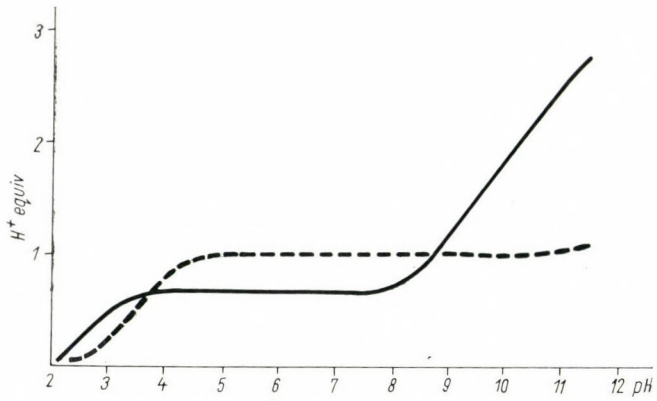

Fig. 1b. Electrometric titration curve of lysine: ——— lysine; — — — DEP-treated lysine

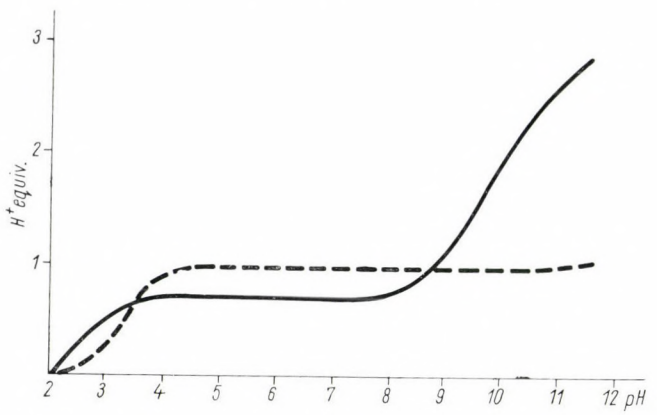

Fig. 1c. Electrometric titration curve of tyrosine: ——— tyrosine; — — — DEP-treated tyrosine

A characteristic titration curve was obtained with DEP-treated histidine. In addition to the disappearance of the shoulder due to the amino group, the fusion of the shoulders of two equivalents, corresponding to carboxylic and imidazole groups, could be observed. It is obvious that the displacement of the pK of carboxylic group in the alkaline direction is accompanied by the shift of the pK of imidazole N-3 in the acidic direction. The decrease in the basicity of nitrogen is brought about according to all probability by the strong electron-withdrawing effect of the carbethoxy group bound to imidazole.

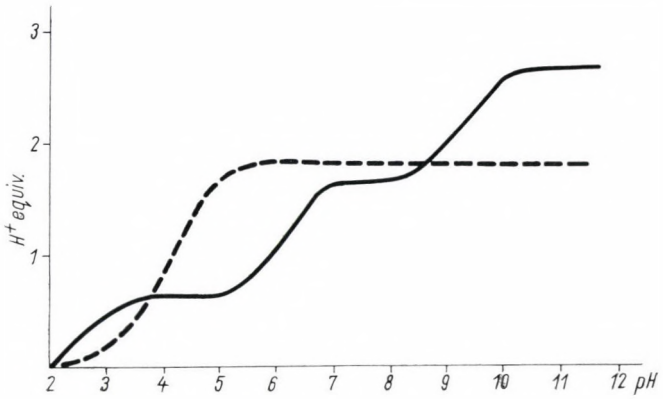

Fig. 1d. Electrometric titration curve of histidine: histidine; — — — DEP-treated histidine

Effect of DEP on the ultraviolet spectra

To examine the reaction which takes place between DEP and amino acids in a greater detail, the ultraviolet spectra of histidine and tyrosine treated with DEP were studied.

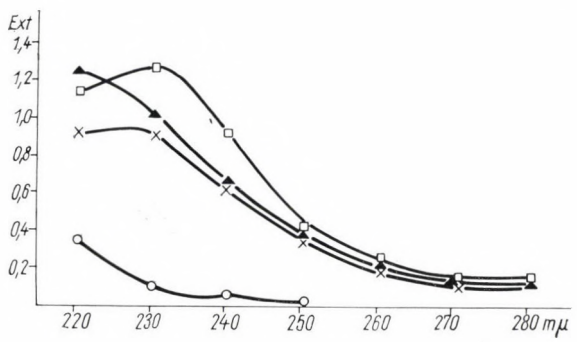

Fig. 2. Ultraviolet spectra of DEP-treated histidine. Histidine, 2×10^{-4} M, was treated with 1.7×10^{-2} M DEP for 24 hours at 2 °C, at pH 6.0, 7.6 and 9.0, respectively. The spectra were taken at pH 7.6. ▲——▲ histidine treated at pH 6.0, □——□ histidine treated at pH 7.6, ×——× histidine treated at pH 9.0, ○——○ untreated control

The light absorption of histidine, treated with DEP at different pH-values, strongly increases as compared to untreated controls (Fig. 2). The increase in absorption is most pronounced in the 220 – 240 mμ wavelength region. This change in absorption in the ultraviolet region is due to the reaction of the imidazole ring.

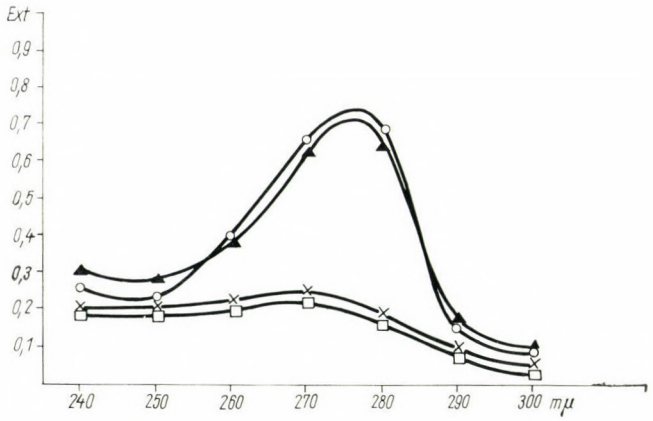

Fig. 3. Ultraviolet spectra of DEP-treated tyrosine. Tyrosine, 5×10^{-4} M, was incubated with 1.7×10^{-2} M DEP for 24 hours at 2 °C, at pH 6, 8 and 9, respectively. Spectra were taken at pH 8. ▲——▲ tyrosine treated at pH 6, ×——× tyrosine treated at pH 8, □——□ tyrosine treated at pH 9, ○——○ untreated control

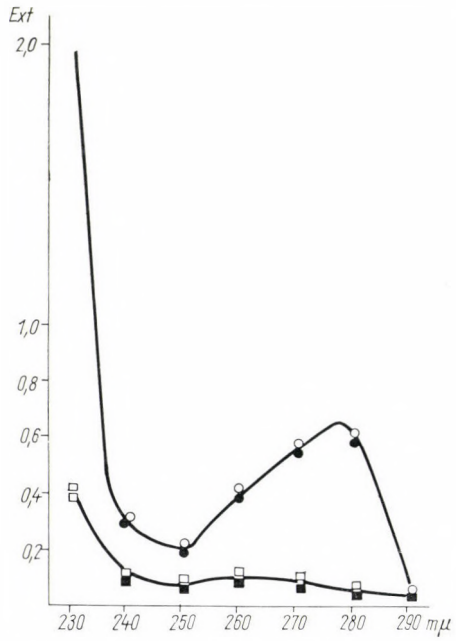

Fig. 4. Ultraviolet spectra of DEP-treated tyrosine and N-acetyl tyrosine ethylester. Tyrosine, 5×10^{-4} M, and the same amount of N-acetyl tyrosine ethylester, respectively, were incubated with 1.7×10^{-2} M DEP for 24 hours at 2 °C, at pH 7.6. The spectra were taken at pH 7.6. □——□ DEP-treated tyrosine, ■——■ DEP-treated N-acetyl tyrosine ethylester, ○——○ untreated tyrosine control, ●——● untreated N-acetyl tyrosine ethylester control

Imidazole reacts with DEP already at pH 6 in contrast to the reaction of NH_2-groups which occurs only above pH 7 as shown by data in the literature (Larroquere, 1964) and also by our own experiments.

The ultraviolet spectrum of tyrosine is also altered on treatment with DEP (Fig. 3); the absorption decreases between 250 and 290 mμ. The reaction is promoted by high pH-values (pH 8 – 10). At pH 6 practically no reaction takes place.

As the spectral change after DEP-treatment is the same both with tyrosine and N-acetyl tyrosine (Fig. 4) the alteration in the spectrum can be attributed to the phenoxy group.

Effect of DEP on the infrared spectra

A good deal of information can be gained concerning the changes in DEP-treated amino acids from the analysis of infrared spectra of the modified amino acids (Figs 5 a, b, c, d, e, f, g).

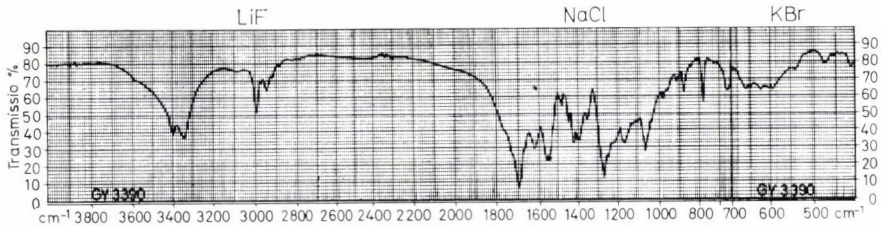

Fig. 5a. Infrared spectrum of DEP-treated glycine

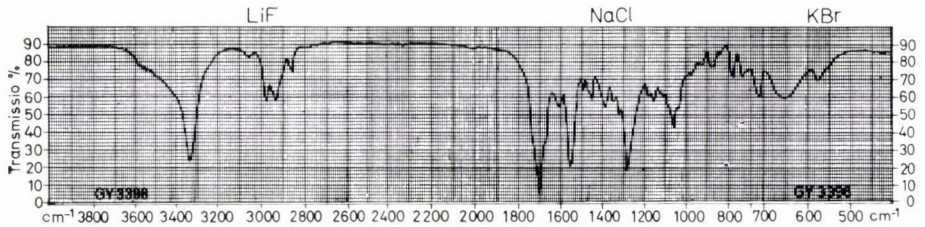

Fig. 5b. Infrared spectrum of DEP-treated lysine

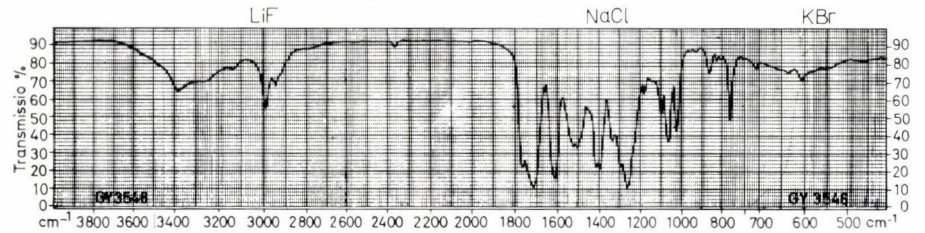

Fig. 5c. Infrared spectrum of DEP-treated histidine

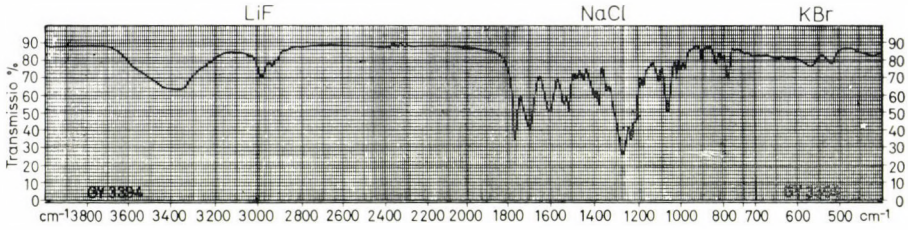

Fig. 5d. Infrared spectrum of DEP-treated tyrosine

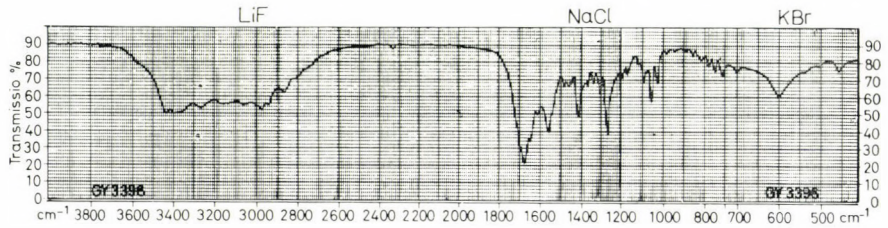

Fig. 5e. Infrared spectrum of DEP-treated arginine

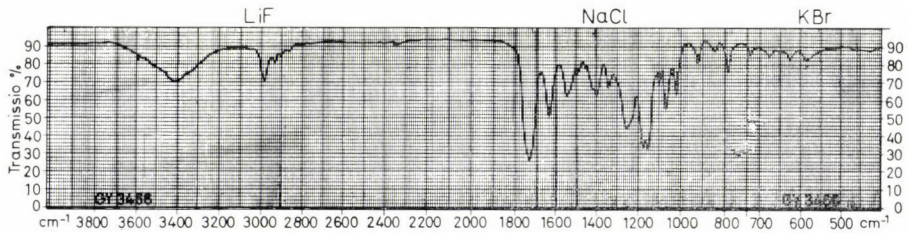

Fig. 5f. Infrared spectrum of DEP-treated cysteine

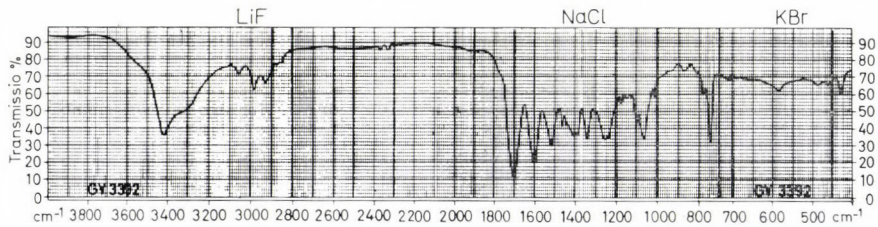

Fig. 5g. Infrared spectrum of DEP-treated tryptophane

The very intensive $\nu C=O$ band, which can be assigned to the N-carbethoxy group, appears in the spectrum of every amino acid. This indicates the reaction of the α-amino group. With amino acids possessing other functional groups the spectrum is modified in the following way.

In case of lysine the 1700 cm^{-1} band is twice as intensive suggesting the carbethoxylation of the ε-amino groups as well (Fig. 5b).

In the infrared spectrum of DEP-treated histidine (Fig. 5c), in addition to the very strong $\nu C=O$ frequency at 1715 cm^{-1}, which can be assigned to the C=O bond of the carbethoxy group bound to the α-amino group, a strong band appears also at 1765 cm^{-1}. This latter band is the $\nu C=O$ frequency of the carbethoxy group bound to the imidazole ring. This $\nu C=O$ band appears at such a high wave number because the C=C and C=N bonds adjacent to the N atom increase the bond-order by the I-effect.

Our hypothesis as to the structure of DEP-treated histidine is supported by the ethoxy assay carried out according to Zeisel (Pregl, Roth, 1958). The ethoxy content, as determined by the above method, was found to be 26.94 per cent (theoretical value: 26.71 per cent).

In the infrared spectrum of DEP-treated tyrosine (Fig. 5d), in addition to the frequency of N-carbethoxy group, strong absorption was observed also at 1770 cm^{-1}. This observation unequivocally proves that the phenolic OH also reacted and a phenol-ester was formed.

In the infrared spectrum of DEP-treated arginine (Fig. 5e) a very broad and intensive C=O band appears at 1680 cm^{-1}. The intensity of the band indicates that a carbethoxy group was bound to the guanidine group as well.

In the infrared spectrum of DEP-treated cysteine (Fig. 5f) the ν C=O bands belonging to the α-amino carbethoxy and S-carbethoxy C=O bond appear in an overlapping manner at 1715 cm^{-1}.

In the infrared spectrum of DEP-treated tryptophane (Fig. 5g) only the reaction of the NH_2-group could be detected. The acidic imino group of the imidazole ring did not react with DEP, as shown by the presence of the ν NH band at 3420 cm^{-1} which belongs to the indole ring.

The lack of a reaction of the indole moiety of tryptophane with DEP is supported also by the finding that the ultraviolet spectrum of tryptophane treated with DEP at different pH-values (between pH 7 and 10) does not differ from that of the untreated control.

Although the fluorescence of DEP-treated tryptophane in 0.1 M borate buffer (pH 7.6) increases by 120 per cent (excitation at 280 mμ, emission measured at 342 mμ) this increase cannot be due to the reaction of the indole moiety, as in N-acetyl tryptophane, where the carbethoxylation of the α-amino group is prevented, no increase in fluorescence could be observed under the same conditions.

Further evidence of the reaction of the individual amino acids with DEP was provided by the lack of the following specific side chain reactions after DEP-treatment.

If histidine is treated with DEP the Pauly reaction characteristic of the imidazole group does not develop. Similarly, the Sakaguchi reaction characteristic of the guanidine group of arginine and the Ellman reaction characteristic of the SH-groups were also found to be negative.

On blocking of the phenolic hydroxyl group of tyrosine the iodination of the ring is prevented (Table I).

Table I

Iodination reaction of tyrosine after DEP-treatment

DEP M	pH of DEP-treatment	Iodinated tyrosine M
0	—	10.0×10^{-4}
4.2×10^{-3}	acetate, pH 6	7.6×10^{-4}
1.7×10^{-2}	acetate, pH 6	7.6×10^{-4}
4.2×10^{-3}	borate, pH 9	2.5×10^{-4}
1.7×10^{-2}	borate, pH 9	0.4×10^{-4}

Tyrosine, 10^{-3} M, was incubated with DEP at 0 °C for 24 hours in the medium indicated in the Table. DEP-treated tyrosine was then iodinated in borate buffer, pH 10.

Stability of the carbethoxy amino acids

The stabilities of the products are different. As indicated by the changes in light absorption at 240 mμ of DEP-treated histidine, the imidazole N-carbethoxy bond is relatively labile, especially in acidic media. In 1 N HCl at 37 °C or in 0.1 N HCl the increase in absorption characteristic of N-carbethoxy imidazole group is halved in 1 or 3.5 hours, respectively (Fig. 6).

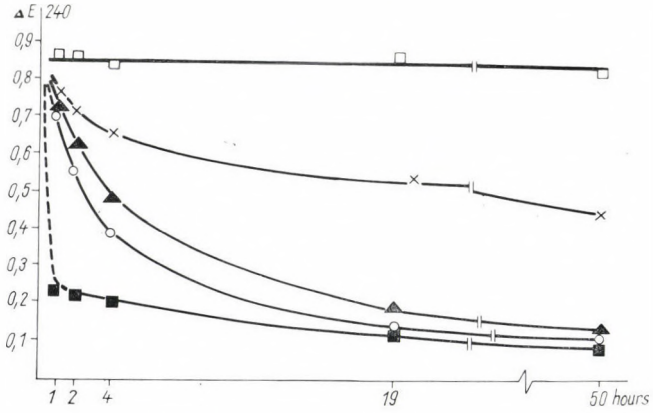

Fig. 6. Stability of N,N-dicarbethoxy histidine as measured on the basis of spectral changes at 240 mμ. N,N-dicarbethoxy histidine, 2×10^{-4} M, was incubated at 37 °C in the extreme acidic and alkaline media indicated below. For the calculation of absorption difference histidine incubated in the same manner served as control: ×——× 0.1 N HCl, ■——■ 1.0 N HCl, ▲——▲ 0.1 N NaOH, ○——○ 1.0 N NaOH, □——□ borate buffer, pH 7.6.

Table II

Stability of N,O-dicarbethoxy tyrosine as measured on the basis of spectral changes at 280 mμ

Time of incubation hr	Absorption difference at 280 mμ				
	0.1 N HCl	1.0 N HCl	0.1 N NaOH	1.0 N NaOH	0.2 M borate buffer pH 7.6
0.03	0.475	0.49	0.00	0.00	0.54
1	0.48	0.49	0.00	0.00	0.51
2	0.48	0.485	0.00	0.00	0.48
4	0.49	0.485	0.00	0.00	0.45
19	0.48	0.46	0.00	0.00	0.25
26	0.475	0.455	0.00	0.00	0.21

N,O-dicarbethoxy tyrosine, 5×10^{-4} M, was incubated in the extreme acidic and alkaline media at 37 °C. For the calculation of absorption difference tyrosine incubated in the same manner served as control.

Studying the stability of N,O-dicarbethoxy tyrosine we observed that the phenolester is split instantaneously at 37 °C in 0.1 or 1.0 N NaOH. On the other hand, in 0.1 or 1.0 N HCl it is almost unaffected even after 50 hours, while in borate buffer at pH 8.8 about 80 per cent is split in 50 hours (Table II).

DEP-treated arginine failed to give the Sakaguchi reaction characteristic of free guanidino group even after 50 hours of incubation at 37 °C in 1 N HCl or 1 N NaOH, or in the same media after a 1-hour incubation at 100 °C.

Studies on the application of DEP in protein chemistry are in progress.

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Effect of Steric Changes in the Protein on the Kinetics of Enzymic Reactions

I. Rapid Equilibrium Treatment of Reactions with One Substrate

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The theory, the equations of initial velocity, the general equation and the Haldane relationship of reactions with one substrate are elaborated, if the steric changes in the enzyme influence essentially as well as non-essentially the rate of enzymic activity. One of the elementary steps is assumed to be rate limiting.

In these cases the constant in the kinetic equation does not equal the dissociation constant of enzyme-substrate complex, but is a combination of the latter and of different equilibrium constants of the preliminary elementary steps.

More than fifty years ago Michaelis and Menten (1913) developed a very successful mathematical formulation for the original assumption of Brown (1902) and Henri (1902) on enzyme-substrate complex formation during the enzyme catalyzed reactions. They described the rapid equilibrium treatment of enzymic reactions with one substrate. In this kinetic equation the constant equals the dissociation constant of the enzyme-substrate complex.

Ample experimental evidence confirmed both the assumption of an enzyme-substrate complex formed during the reaction and the validity of the Michaelis–Menten equation, if one of the elementary steps is rate determining (Cf. Dixon, Webb, 1964; Laidler, 1958; Reiner, 1959; Webb, 1963, etc.).

During the last decade, however, experimental evidence accumulated to show that some enzymes may undergo steric changes during catalysis and thereby influence the kinetics of the reaction. The induced fit theory of Koshland (1960) was very successful in stimulating experimental research on substrate-induced steric changes of the enzyme molecule. According to this assumption the substrate is bound by the enzyme and the binding of the substrate induces steric changes in the enzyme. Only the sterically "changed" enzyme molecule is able to catalyze the breakdown of the enzyme-substrate complex, i.e. the formation of the product.

A different theory, developed by Straub and Szabolcsi (1964), is based on the motility of the proteins. They proposed that different conformations of the same enzyme are in dynamic equilibrium with each other and only one of these is able to bind the substrate. Thus the formation of the enzyme-substrate complex shifts the equilibrium in the direction of this conformation. In this case only the "changed" enzyme is able to form the enzyme-substrate complex (since the shift of the dynamic equilibrium between the different conformations of the enzyme changes

the previous conformation of the majority of the molecules) and only the "changed" enzyme is able to catalyze the breakdown of the complex.[1]

These possibilities are not taken into account in the original mathematical formulation of Michaelis and Menten. If steric changes in the enzyme take place during catalysis the constant in the kinetic equation does not equal the dissociation constant of enzyme-substrate complex, but will be a combination of this and of the equilibrium constants of the step: enzyme $\rightleftarrows$ changed enzyme and/or of the step: enzyme-substrate $\rightleftarrows$ changed enzyme-substrate.

Table I

*Essential or non-essential steric changes in the enzyme during catalysis**

The substrate forms complex with	The breakdown of the enzyme-substrate complex is catalyzed by		
	the enzyme	the changed enzyme	both the enzyme and the changed enzyme
the enzyme	IIa (1)	Ib (2)**	IIa (2)
the changed enzyme	Ia (2)***	Ia (1)****	Ia (3)
both the enzyme and the changed enzyme	IIb (1)	Ib (3)	IIb (2)

* The models marked with I mean essential steric changes and those marked with II mean non-essential steric changes in the enzyme.

** Induced fit theory.

*** Only a rechanged enzyme is able to catalyze the formation of the product ("induced fit theory", since the binding of substrate induces steric changes in the enzyme). The case when other type of "changed" enzyme, i.e. E″ is formed is not taken into account in this paper.

**** Straub–Szabolcsi-theory.

This paper deals with the reaction kinetics of enzymes which undergo steric changes during the reaction (assuming that only the forms enzyme and changed enzyme are present) and catalyze reactions with one substrate, assuming one of the elementary steps as rate determining.

The steric changes in the enzyme may be: I) essential for the reaction[2] or II) may not be essential.[3]

[1] In the following "enzyme" means a population of enzyme molecules which do not suffer such steric changes which influence their *kinetic* properties; "changed enzyme" means a population of enzyme molecules which underwent such steric changes which influence their *kinetic* properties (i. e. enzyme molecules which suffer steric changes without any alterations in their kinetic properties are called "enzyme").

[2] By essential steric changes is meant that only the changed enzyme is able to form complex with the substrate or to catalyze the breakdown of the enzyme-substrate complex.

[3] By non-essential steric changes is meant that the enzyme or both the enzyme and the changed enzyme are able to form complex with the substrate and to catalyze the breakdown of the enzyme-substrate complex.

If the steric changes are not essential for the reaction, these changes may

A) not alter the kinetics, since

A_1) they influence neither the dissociation of the complex nor its breakdown (Michaelis–Menten kinetics), or

A_2) influence both the dissociation and the breakdown of the complex but to the same extent and in opposite directions. (This case is theoretically different from the Michaelis–Menten kinetics, but experimentally – kinetically – are indistinguishable from each other.)

B) Inhibit the reaction[4] because of

B_1) an increase in the dissociation of the complex,

B_2) a decrease in the rate of breakdown of the complex,

B_3) an increase in the dissociation and decrease in the rate of breakdown of the complex.

C) Activate the reaction because of

C_1) a decrease in the dissociation of the complex,

C_2) an increase in the rate of breakdown of the complex,

C_3) a decrease in the dissociation and increase in the rate of breakdown of the complex.

Equations of initial velocity

In all cases E=enzyme, E'=changed enzyme, S=substrate, P=product. The following dissociation and equilibrium constants are used:
$K_1 = [E]/[E']$, $K_2' = [E'][S]/[E'S]$, $K_2 = [E][S]/[ES]$, $K_3 = [E'S]/[ES]$, $K_4' = [E'][P]/[E'P]$, $K_4 = [E][P]/[EP]$, $K_5 = [E'P]/[EP]$ and K_{eq} = equilibrium constant of the over-all reaction ($= [P]/[S]$), V_f = maximum velocity of the forward reaction, V_r = maximum velocity of the reverse reaction, V' = maximum velocity of the breakdown of ES complex if enzymically active $E'S$ complex is also formed during the reaction, V'' = maximum velocity of the breakdown of

[4] In the following, inhibition or activation does not refer to real, measurable inhibition or activation but to a theoretical one, with respect to the original Michaelis–Menten kinetics. If steric changes in the enzyme take place during the reaction, either essential or non-essential for the enzyme activity, it is not possible to measure the same enzyme action without steric changes in the enzyme, i. e. to compare the "inhibited" (or "activated") reaction with the "original" one.

$E'S$ complex if enzymically active ES complex is also formed during the reaction. It is to be noted that;

$$K_1 = K_2/K_3K_2' = K_4/K_4'K_5.$$

In all cases the intramolecular transformation of enzyme-substrate complex in enzyme-product complex is assumed to be the rate limiting step.

The general mechanism which takes into consideration all possibilities of essential and non-essential steric changes in the enzyme which affect the catalytic activity is as follows:

(1)	$E \rightleftarrows E'$
(2)	$E+S \rightleftarrows ES$
(3)	$E'+S \rightleftarrows E'S$
(4)	$ES \rightleftarrows E'S$
(5)	$ES \rightleftarrows EP$
(6)	$E'S \rightleftarrows E'P$

Tables II and III summarize, in the case of essential and non-essential steric changes, respectively, the elementary steps of the general mechanism functioning in the different mechanisms.

Table II

Elementary steps of the general mechanism functioning in the different mechanisms if essential steric changes in the enzyme influence the kinetics

Ia (1)		Ia (2)	Ia (3)	Ib (2)	Ib (3)	
α	β				α	β
(1), (3), (6)	(1), (3), (4) (6)	(1), (3), (4), (5)	(1), (3), (4), (5), (6)	(1), (2), (4), (6)	(1), (2), (3), (6)	(1), (2), (3), (4), (6)

If the dissociation constant of each enzyme-substrate complex equals K_S, i.e. $K_2 \equiv K_S$ and $K_2' \equiv K_S$ we obtain the general equation of enzymic reactions with one substrate when the steric changes in the enzyme are essential or non-essential:

$$1/v_0 = mK_S/[S] + n$$

The values of m and n in the different mechanisms are summarized in Table IV.

The Haldane equations, i.e. the relationship between the equilibrium constant

Table III

Elementary steps of the general mechanism functioning in the different mechanisms if non-essential steric changes in the enzyme influence the kinetics

IIa (1)				IIa (2)*	IIb (1)		IIb (2)	
α**	β***	γ***	δ***		α+	β++	α+++	β++++
(2), (5)	(2), (4), (5)	(1), (2), (5)	(1), (2), (4), (5)	(1), (2), (4), (5), (6)	(1), (2), (3), (4), (5)	(1), (2), (3), (5)	(1), (2), (3), (5), (6)	(1), (2), (3), (4), (5), (6)

* If $V'' > V'$ and/or $K_3 > 1/K_1$ the reaction becomes activated. If $V' > V''$ and/or $1/K_1 > K_3$ the reaction becomes inhibited. If $V' = V''$ and $K_3 = 1/K_1$ or $|V''-V'| = |1/K_1 - K_3|$ the steric changes will not influence the rate of the reaction.

** Michaelis–Menten kinetics, i.e. no steric changes in the enzyme.

*** In the cases of IIa (1) β) to δ) the changes in the steric structure "inhibit" the reaction. The inhibition is uncompetitive in the case β), competitive in the case γ) and mixed in the case δ). In the case δ) the inhibition may be non-competitive if $1/K_1 = K_3$.

\+ In this case the steric changes in the enzyme inhibit the reaction and the inhibition is of a mixed type or — if $1/K_1 = K_3$ — of a non-competitive type.

++ The steric changes inhibit the reaction and the inhibition is of a mixed type, or — if $K_2' = K_2$ — of a non-competitive type.

+++ If $V' > V''$ and/or $K_2 > K_2'$ the reaction is inhibited. If $V'' > V'$ and/or $K_2' > K_2$ the reaction is activated. If $V' = V''$ and $K_2 = K_2'$ or $|V' - V''| = |K_2' - K_2|$ the steric changes in the enzyme do not influence the rate of the reaction.

++++ The general mechanism if steric changes in the enzyme do influence the kinetics of the catalysis. If $V' > V''$ and/or $K_1 > 1/K_3$ the reaction is inhibited. If $V'' > V'$ and/or $1/K_3 > K_1$ the reaction is activated. If $V' = V''$ and $K_1 = 1/K_3$ or $|V' - V''| = |1/K_3 - K_1|$ the steric changes in the enzyme do not influence the rate of the reaction.

of the over-all reaction and the kinetic constants (Haldane, 1930) are summarized in Tables V and VI (p. 37) for the cases of essential and of non-essential steric changes in the enzyme, respectively.

It is to be noted that kinetically it is not possible to differentiate between an enzyme which follows the regular Michaelis–Menten kinetics and another which suffers steric changes influencing the kinetics. By measuring the kinetic constants, with kinetic methods only, it is not possible to differentiate K_S from mK_S as well as V from $1/n$. In all cases where steric changes in the enzyme do influence the kinetics, the usual determinations of the Michaelis constant and of maximum velocity will be in error by factor dependent on the value of the equilibrium constants of the preliminary elementary steps.

If the dissociation constant of the enzyme-substrate complex (in cases when it is possible to determine it without the influence of the preliminary equilibria) differs from that measured kinetically, we may suppose that some steric changes in the enzyme influence the kinetics, if the kinetics of the reaction follows the rapid equilibrium suggestion.

Table IV

The values of the factors in the general equation of initial velocity of reactions in which steric changes in the enzyme influence the kinetics

Mechanism	m	n
Ia (1) α)*	$(1 + K_1)/V$	$1/V$
Ia (1) β)	$(1 + K_1)/V$	$(1/V)(1 + 1/K_3)$
Ia (2)**	$K_3(1 + K_1)/V$	$(1 + K_3)/V$
Ia (3)*	$K_3(1 + K_1)/(V''K_3 + V')$	$(1 + K_3)/(V''K_3 + V')$
Ib (2)**	$(1/VK_3)(1 + 1/K_1)$	$(1/V)(1 + 1/K_3)$
Ib (3) β)*	$(1 + K_1)/V$	$(1/V)(1 + 1/K_3)$
Ib (3) α)*	$(1 + K_1)/V$	$(1/V)(1 + K_1K_2'/K_2)$
IIa (1) α)	$1/V$	$1/V$
IIa (1) β)	$1/V$	$(1 + K_3)/V$
IIa (1) γ)*	$(1 + K_1)/VK_1$	$1/V$
IIa (1) δ)	$(1 + K_1)/VK_1$	$(1 + K_3)/V$
IIa (2)*	$(1 + K_1)/K_1(V' + V''K_3)$	$(1 + K_3)/(V' + V''K_3)$
IIb (1) α)*	$(1 + K_1)/VK_1$	$(1 + K_3)/V$
IIb (1) β)*	$(1 + K_1)/VK_1$	$(1/V)(1 + K_2/K_1K_2')$
IIb (2) α)	$K_2(1 + K_1)/(K_1K_2'V' + K_2V'')$	$(K_1K_2' + K_2)/(K_1K_2'V' + K_2V'')$
IIb (2) β)	$K_3(1 + K_1)/(V' + V''K_3)$	$(1 + K_3)/(V' + V''K_3)$

* Ia (1) α) is apparently the mirror image of IIa (1) γ), Ia (3) of IIa (2), Ib (3) α) of IIb (1) β), and Ib (3) β) of IIb (1) α). But since — as assumed — E is the non-changed enzyme and E' is the changed one, the models with I mean essential steric changes and those with II mean non-essential steric changes.

** Ia (2) and Ib (2) are the mirror images of each other.

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Table V

The Haldane relationship in reactions in which the steric changes in the enzyme are essential

forward \ reverse	Ia (1) α), Ia (1) β) Ib (3) α), Ib (3) β)	Ia (2)	Ia (3)	Ib (2)
Ia (1) α), Ia (1) β), Ib (3) α), Ib (3) β)	$V_f K_4'/V_r K_2'$	$V_f K_4' K_5/V_r K_2'$	$V_f K_4' K_5(V_r'' K_5 + V_r')$	$V_f K_4/V_r K_1 K_2' K_5$
Ia (2)	$V_f K_4'/V_r K_2' K_3$	$V_f K_4' K_5/V_r K_2' K_3$	$V_f K_4'/K_2' K_3(V_r'' K_5 + V_r')$	$V_f K_4/V_r K_1 K_2' K_3 K_5$
Ia (3)	$K_4'(V_f'' K_3 + V_f')/V_r K_2' K_3$	$\dfrac{K_4' K_5(V_f'' K_3 + V_f')}{V_r K_2' K_3}$	$\dfrac{K_4' K_5(V_f'' K_3 + V_f')}{(V_r'' K_5 + V_r') K_2' K_3}$	$K_4(V_f'' K_3 + V_f')/V_r K_1 K_2' K_3 K_5$
Ib (2)	$V_f K_1 K_3 K_4'/V_r K_2$	$V_f K_1 K_3 K_4' K_5/V_r K_2$	$V_f K_1 K_3 K_4' K_5/(V_r'' K_5 + V_r') K_2$	$V_f K_3 K_4/V_r K_2 K_5$

Table VI

The Haldane relationship in reactions in which the steric changes in the enzyme are non-essential

forward \ reverse	IIa (1) β), IIa (1) γ)* IIa (1) δ), IIb (1) α) IIb (1) β)	IIa (2), IIb (2) β)	IIb (2) α)
IIa (1) β), IIa (1) γ), IIa (1) δ), IIb (1) α), IIb (1) β)	$V_f K_4/V_r K_2$	$V_f K_4/(V_r' + V_r'' K_5) K_2$	$V_f K_1 K_4 K_4'/(K_1 K_4' V_r + K_4 V_r'') K_2$
IIa (2), IIb (2) β)	$(V_f' + V_f'' K_3) K_4/V_r K_2$	$(V_f' + V_f'' K_3) K_4/(V_r' + V_r'' K_5) K_2$	$(V_f' + V_f'' K_3) K_1 K_4 K_4'/(K_1 K_4' V_r' + K_4 V_r'') K_2$
IIb (2) α)	$(K_1 K_2' V_f + K_2 V_f'') K_4/V_r K_2 K_2' K_1$	$\dfrac{(K_1 K_2' V_f' + K_2 V_f'') K_4}{(V_r' + V_r'' K_5) K_1 K_2 K_2'}$	$\dfrac{(K_1 K_2' V_f' + K_2 V_f'') K_1 K_4 K_4'}{(K_1 K_4' V_r' + K_4 V_r'') K_2 K_2'}$

* If the forward reaction is IIa (1) β), the reverse reaction must be the same since step $E \rightleftarrows E'$ is absent only from this model. IIa (1) γ) or δ or IIb (1) α) and β may be the forward or reverse reaction in the over-all one without any restriction.

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Complex of D-glyceraldehyde-3-phosphate Dehydrogenase with NAD. I.

The pH-dependence of the Absorption Spectrum and Stability of the Complex

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The light absorption of the GAPD–NAD complex is pH-dependent. Maximum absorption was found between pH 7 and 10. The stability of the complex is not significantly lower in slightly acidic solution than in the neutral — slightly alkaline range. Therefore, the chromophore itself must be changed in the slightly acidic region. It is suggested that the pH can affect the chromophore either directly through the acid-base dissociation of a functional group in the chromophore or indirectly through altering the steric structure of the protein. The decreasing absorption in the alkaline range is due to the dissociation of the enzyme–NAD complex.

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Taylor et al. (1948) have found that GAPD [D-gylceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating) E. C. 1.2.1.12.] isolated from mammalian muscle contains bound NAD. Racker and Krimsky (1952), and Velick (1953) have later shown that the enzyme-NAD complex has a characteristic absorption band in the near u.v. region. Yeast GAPD does not contain bound NAD but a similar absorption band appears when NAD is added to the yeast enzyme (Stockel, 1959). Making use of this absorption band Stockel has studied the stability of the yeast GAPD – NAD complex. The nature of the binding of coenzyme to enzyme, that is the structure of the chromophore, is not yet known, though attempts have been made to interpret the absorption band of the complex. In this series of papers results of further investigations on the nature of this bond are presented. This paper contains studies on pH-dependence of the absorption spectrum and stability of the NAD-complex of the enzyme from pig muscle.

Materials and Methods

Pig muscle GAPD was isolated by the procedure of Elődi and Szörényi (1956). Four times recrystallized enzyme preparations were used. We used a molecular weight of 140 000 in the calculations (Elődi, 1958).

Enzyme solutions were gel filtered before use. 5 g columns of Sephadex G-100 were prepared. The columns were equilibrated with a buffer containing 0.005 M EDTA and 1 % ammonium sulphate and used at 5 °C.

Bound NAD was removed from GAPD with charcoal (Velick et al., 1953). The enzyme solution was stirred under cooling for 20 minutes with charcoal and then centrifuged. The amount of adsorbent added was roughly equal to the total weight of protein. Removal of bound NAD was checked in two ways. First, the optical density of the solution was determined at pH 7.0 at 280 and 260 mμ. (The ratio E_{280}/E_{260} is 1.15 for the GAPD–NAD complex, and 1.9 for the free enzyme.) Second, we checked whether the optical density of the enzyme solution remained constant at 360 mμ after the addition of 8 mole equivalents of p-chloromercury-benzoate (PCMB). Concentration of the enzyme was determined by reading the optical density of dilutions in 0.1 N NaOH at 280 mμ. The molar absorbancy values used were 1.4×10^5 for the enzyme–NAD complex and 1.27×10^5 for NAD–free GAPD.

Spectra were determined at 5 °C, by using a thermostated cuvette compartment in a Unicam SP-500 spectrophotometer. 1 cm cuvettes were used for the spectral measurements and the concentration of the enzyme was kept at 10^{-4} M. In the case of determination at pH values of 9 or higher, the pH was adjusted by the addition of a small known volume of a strong base in the cuvette. The pH of the solutions was checked at the end of the spectral measurements.

The dissociation constant of the enzyme–NAD complex was determined by the procedure of Stockel (1959). In these experiments 10^{-5} M enzyme was used in 4 cm cuvettes except in the alkaline pH range where 10^{-4} M enzyme was used in 1 cm cuvettes.

The chemicals used were reagent grade commercial preparations. PCMB was obtained from Reanal, and 88 per cent pure preparations of NAD from Sigma.

Results

A) *The pH-dependence of the light absorption of the enzyme–NAD complex*

We determined the spectrum of the enzyme–NAD complex at different pH values in the 300 to 450 mμ range. In these studies we used GAPD–NAD as it is obtained with the procedure of Előd i and Szörényi (1956) from pig muscle. Fig. 1 shows that markedly lower optical densities were found at acidic or very alkaline pH values than in the neutral and slightly alkaline pH range. The spectrum shows a plateau between 340 and 370 mμ at pH 5–6. In the neutral to slightly alkaline range (pH 7–10) there is a minimum at 325 mμ and a well defined maximum at 360 mμ. The optical density was not constant above pH 10. At these high pH values readings were taken 5 minutes after the addition of alkali. Only a small decrease in optical density occurred during the measurements. The alkaline spectra which were obtained in this way no longer showed a maximum. When the optical density at 360 mμ, which was read 30″ after pH adjustment, is plotted against pH, a maximum curve is obtained. The upward slope of the curve falls into the range pH 5.2 to pH 7, and it levels off in the range from pH 7.0 to 10.5.

We also determined the effect of hydrogen ion concentration on the spectral change (Velick, 1953) which is due to the addition of PCMB to GAPD. 8 mole equ. of PCMB were used at pH values lower than 8.5, and 20 equ. at pH values above 8.5. It was found that these concentrations of PCMB were sufficient to cause a maximum decrease in light absorption. The spectrum obtained in the presence of PCMB was subtracted from the spectrum of untreated enzyme. Velick has shown

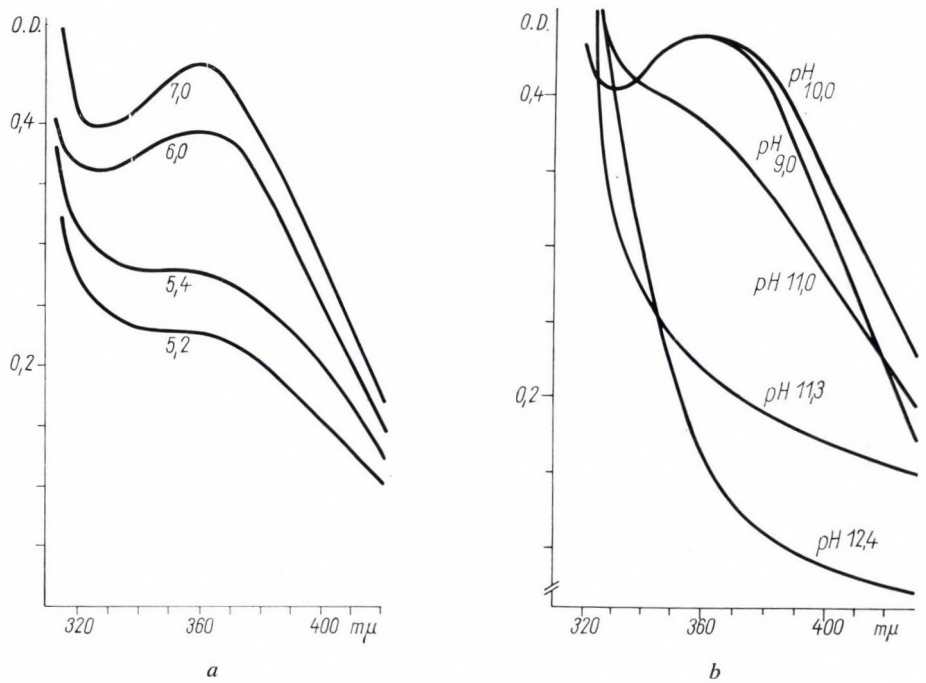

Fig. 1. Absorption spectrum of the GAPD–NAD complex at some selected pH values. *a)* Spectra in the range pH 5.2—7. *b)* Spectra at pH values above 9.

Absorption spectra were determined at 5 °C in a solution containing 14 mg/ml enzyme, 0.005 M EDTA, and 1 per cent ammonium sulphate. pH values above 9 were adjusted in the cuvettes. Absorption spectra were determined 5 minutes after pH adjustment

that the addition of PCMB results in the dissociation of the enzyme–NAD complex. Therefore, the effect of pH on the difference spectra in the near u.v. region can be ascribed to pH effects on the enzyme–NAD chromophore.

The difference spectra show a maximum at 365 mμ, except in the pH range above 11, where the curves become flattened. The effects of pH on the absorption at 365 mμ of untreated enzyme and on the difference spectra are very similar: both curves go upwards as the pH is increased to 7.0, both run parallel with the abscissa in the pH range 7—10, and both have a downward slope above pH 10.

In a third series of experiments we have first removed the bound NAD from the enzyme–NAD complex and determined the spectrum of the NAD-free enzyme at different pH values. These measurements were repeated with NAD-free enzyme after the addition of increasing concentrations of NAD. The results of these measurements indicated that the enzyme–NAD complex is immediately formed when NAD is added to GAPD, and that the complex thus formed is stable if the pH is lower than 10. Alkali and NAD were added to the enzyme at the same time, and the spectrum was assayed after 5 minutes. The variation of the spectrum of NAD with pH was also determined under comparable conditions. From the optical density of

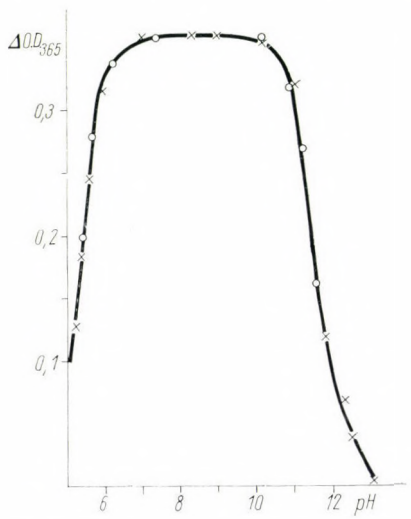

Fig. 2. The pH dependence of the characteristic light absorption at 365 mμ of the GAPD–NAD complex. ΔO.D.$_{365}$ values were obtained by subtracting the optical density of each component of the mixture. × decrease in O.D.$_{365}$ due to the addition of PCMB to GAPD–NAD. ○ increase in O.D.$_{365}$ due to the addition of a saturating concentration of NAD to charcoal-treated GAPD. When working in the pH range 9—12.5, the pH of the neutral enzyme solution was adjusted in the cuvette by the simultaneous admixture of the required volume of a KOH solution, and PCMB or NAD. Optical density was read 30 seconds after mixing the solutions

the reconstructed enzyme–NAD complex we have subtracted the corresponding figures for both NAD-free enzyme and NAD. The difference spectra thus obtained are practically identical with the difference spectra obtained by addition of PCMB to the GAPD—NAD complex.

Fig. 2 shows the difference in optical density at 365 mμ which was obtained 30 seconds after the addition of NAD + alkali.

The pH-dependence of the dissociation of the enzyme–NAD complex

We have found in earlier experiments (Boross, 1965) that part of the bound NAD can be removed from GAPD by Sephadex filtration. It can be clearly seen from the change in the ratio of the optical densities at 260 and 280 mμ in the fractions of the filtrate that the release of NAD is a continuous process during gel filtration. By carrying out Sephadex filtration of the GAPD–NAD complex at different pH values it was found that a somewhat greater fraction of bound NAD can be removed at pH 5.6 than at pH 7–8, and that more than half of the total amount of bound NAD can be separated at alkaline pH values (Table I). These observations clearly indicate that the stability of the GAPD–NAD complex is dependent on pH.

Table I

Stability and light absorption of the GAPD—NAD complex at three pH values

pH	per cent NAD* separated by gel filtration	molar absorbance** at 365 mμ 10^3	apparent dissociation constant of the complex(K_{NAD})***
5.6	7 ± 3	0.75 ± 0.03	2.2×10^{-6} M
7.8	5 ± 3	1.00 ± 0.03	9.0×10^{-7} M
11.0	60 ± 10	0.8 ± 0.06	7.0×10^{-5} M

All experiments were carried out at 5 °C.

* Figures are expressed in percentage of total — enzyme-bound — NAD. Released NAD was calculated from the E_{260}/E_{280} ratio of the fractions eluted after the protein peak.

** Calculated for 1 M enzyme-bound NAD from the difference spectrum.

*** Determined after Stockel (1959).

In the following experiments we added increasing amounts of NAD to charcoal-treated GAPD. Fig. 3 shows that the optical densities at 365 mμ when plotted against the concentration of NAD show a more or less sharp breaking point when the experiment is carried out at pH 7 or 5.4. In the case of alkaline solutions saturation of the enzyme can be attained at higher concentrations of NAD and there is no sharp breaking point on the titration curve. The maximal absorption values, attainable at saturating concentrations of NAD, are lower on both the acidic and alkaline side than at pH 7. The dissociation constant of the complex at three selected pH values was calculated with Stockel's equation (Stockel, 1959), and these are shown in Table 1. It is seen that the stability of the enzyme–NAD complex is highest at neutral pH.

We have stated above that in strongly alkaline solutions the optical density of the enzyme–NAD complex was not constant. In the pH range 10 – 12 the readings taken at 365 mμ decreased for at least 40 minutes. In even more alkaline solutions an increase of optical density with time was observed. Since, however, a practically identical increase was obtained with NAD in the absence of enzyme, the observed change in optical density above pH 12 must be due to the chemical instability of NAD in strongly alkaline solutions (Burton, Kaplan, 1963).

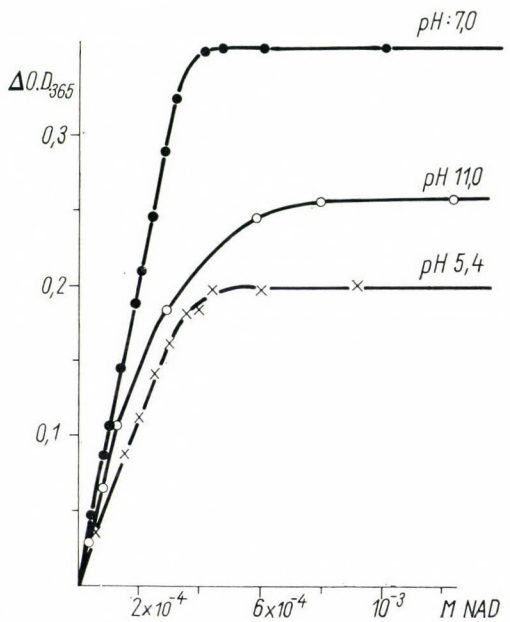

Fig. 3. The recombination of charcoal-treated GAPD with NAD at three pH values. The curves were obtained by plotting ΔO.D.$_{365}$ against the concentration of NAD. The concentration of GAPD was 14 mg/ml. The enzyme solution was gel filtered before treatment with charcoal. Sephadex G-100 was used with a solution which contained 0.01 M EDTA and 1 per cent ammonium sulphate. pH 11 was adjusted by the simultaneous mixing of the required amounts of KOH and NAD, the neutral solutions of the NAD-free enzyme. Optical density was read 30 seconds after mixing the solutions

The spectrum of NAD-free GAPD obtained by charcoal treatment was practically constant over 1 hour at pH 11. In further experiments we added NAD to charcoal-treated enzyme after preincubation of the latter for increasing periods of time at pH 11. By determining the optical density at 365 mμ immediately after the addition of NAD to preincubated NAD-free enzyme lower residual concentrations of the GAPD – NAD complex were obtained than with GAPD – NAD incubated for the same time at pH 11.

Discussion

A marked pH-dependence of light absorption in the near u.v. region by solutions of pig muscle GAPD is shown by the results presented. This pH-dependence of light absorption indicates the pH-sensitivity of a chromophore which is formed when NAD is bound to GAPD. Convincing evidence for this conclusion comes from our studies of difference spectra which were obtained by treating GAPD–NAD with PCMB and from experiments in which NAD was added to charcoal-treated enzyme. The pH-dependence of the light absorption by the NAD complex of the pig muscle enzyme in our studies was more pronounced than that of yeast GAPD described by Stockel (1959).

It was shown that the difference spectrum due to the binding of NAD to the enzyme had a maximum at 365 mμ. From the curve obtained by plotting ΔO.D.$_{365}$ against pH (Fig. 2) it can be concluded that the interaction between enzyme and NAD is maximal in the pH range 7–10. Theoretically, this effect of pH can be interpreted in three ways:

a) the enzyme–NAD complex is fully formed only in this pH range

b) the enzyme–NAD complex is formed in a wider range of pH but the steric structure of the enzyme is affected by pH in such a way that the groups which take part in the formation of the chromophore cannot sufficiently approach each except in this pH range

c) a definite dissociation state of certain functional groups is essential for the formation of the chromophore and this state(s) is present in the pH range 7–10.

In the same pH range where the ΔO.D.$_{365}$ versus pH curve shows a maximum, there is a sharp breaking point in the ΔO.D.$_{365}$ versus NAD concentration curve obtained when the charcoal-treated enzyme was titrated with NAD (Fig. 3). From the position of this breaking point, the binding of 3.5 ± 0.2 equivalents of NAD by mole of GAPD can be calculated when 140.000 is taken for the molecular weight of the enzyme. In other words 39,000–43,000 is the equimolar weight of the protein which binds 1 mole of NAD. This is in a good agreement with Friedrich's (1965) results and with the figures published by Velick (1958). The apparent dissociation constant in the neutral pH range of the enzyme–NAD complex was found by Stockel's method (1959) to be 9×10^{-7} M. This value is somewhat higher than the previously published ones for mammalian GAPD-s. With the pig muscle enzyme Friedrich (1965) found a dissociation constant of 4×10^{-7} M and Keleti and Batke (1965) gave 3.2×10^{-8} for the Michaelis constant of the same enzyme. With the rabbit muscle enzyme Velick (1958) obtained a dissociation constant of 6×10^{-8} M in spectrofluorometric studies. According to Stockel (1959) the dissociation constant for the NAD complex of yeast GAPD is 4.5×10^{-6} M.

The stability of the enzyme–NAD complex is slightly lower in the range of pH 5–7 than between pH 7 and 10. The apparent dissociation constant of the enzyme–NAD complex at pH 5.4 was found by Stockel's method (1959) to be 2.2×10^{-6} M. This slight increase in the dissociation of the enzyme–NAD complex

cannot be responsible for the decreased light absorption of the complex even when excess NAD is used. The effect of pH on the formation of the chromophore, which may or may not follow the binding of NAD to the enzyme, can be explained in two ways. *Either* the steric structure of the enzyme is changed by lowering the pH, *or* we have to assume the essential dissociation of a functional group in the eventual chromophore.

The enzyme–NAD complex is much less stable above pH 10 than it is in either the neutral or in the slightly acidic region. This was demonstrated in the gel filtration experiments as well as by titrating the enzyme with NAD. The apparent dissociation constant of the complex in the alkaline pH region was found to be markedly higher than at lower pH values. It is a difficult task to obtain precise estimations of any characteristics of the enzyme at elevated pH values because the enzyme itself is unstable above pH 10. The presented figures for the optical density of the enzyme–NAD complex in alkaline solutions must be regarded as approximations. From the lability of the complex it follows that the decreasing optical densities above pH 10.0 are due to an increasing decomposition of the GAPD – NAD complex in this pH range and not to the inhibition of the formation of the chromophore structure. In contrast to the chromophore in the slightly acidic region, the downward slope of the pH-curve in the alkaline region cannot reflect the dissociation of a functional group which would be essential for the formation of the chromophore structure.

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Complex of D-glyceraldehyde-3-phosphate Dehydrogenase and NAD. II.

Investigations on the Role of Thiol Groups

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The residues involved in the chromophore part of the GAPD–NAD complex have been investigated. The absorption spectrum of the GAPD–NAD complex was found to be more similar to that of mercaptan–NAD complex than to the charge-transfer spectrum of tryptophane and NAD both as far as the λ_{max} and the pH-dependence of the spectra are concerned. The apparent dissociation curve of the reactive thiol group of the enzyme coincided with the pH-dependence curve of the absorption of the enzyme-coenzyme complex with a pK value of 5.4. The spectrum of the ternary Ag–GAPD–NAD complex develops even in slightly acidic solution where the absorption of the binary enzyme-coenzyme complex is markedly reduced. The experiments support the assumption that the active thiol group of the enzyme plays a role in the formation of the chromophore of GAPD–NAD complex.

There have been several attempts to interpret the light absorption of the GAPD*–NAD complex. Racker and Krimsky (1952) who have first described this spectrum explained the properties of the complex assuming a bond between a thiol group of the enzyme and NAD. Later Kosower (1956) supposed a charge-transfer interaction between the enzyme and the coenzyme. In model experiments Cilento and Giusti (1959), Cilento and Tedeschi (1961) and Shifrin (1964) have shown that indole derivatives, such as tryptophane, are able to enter into charge-transfer interaction with pyridinium compounds. On the other hand, the enzyme contains a strongly nucleophylic thiol group which has been suggested to act as electron donor (Polgár, 1964a).

In our earlier experiments we have shown that the absorption spectrum of the GAPD–NAD complex changes according to a maximum curve as a function of pH (Cseke, Boross, 1967). The absorption at 365 mμ of the enzyme-coenzyme complex increases between pH 5 and 7, while the dissociation of the complex practically does not change. Therefore, it has been suggested that the formation of the chromophore part of the complex requires either a specific conformation of the protein or a dissociated, or undissociated, state of a functional group involved in the complex. This paper presents our recent experiments concerning this problem.

* Abbreviation: GAPD = D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating) EC 1.2.1.12

Materials and Methods

GAPD was isolated from pig muscle by the method of Elődi and Szörényi (1956). Four times recrystallized preparations were used. The spectrum of the GAPD–NAD complex was measured as described previously (Cseke, Boross, 1967). The molecular weight of the enzyme was taken for 140,000 (Elődi, 1958).

The difference spectrum of the tryptophane–NAD charge-transfer complex was measured in a tandem cell system (Remily, Wolfe, 1960; Laskowski et al. 1960). In the reference light path one cell contained NAD, while the other cell contained tryptophane, both in 0.2 M glycine buffer of various pH values. In the sample light path one cell contained NAD, tryptophane, and the same buffer as in the reference cells, while the other cell contained buffer only. The pH of solutions was checked after taking the spectra. The charge-transfer spectrum of indole and NAD was measured in the same way. The spectrophotometric measurements were carried out using a Unicam SP-500 spectrophotometer. The experiments at pH 2 and 7 were repeated in an OPTON spectrophotometer equipped with double monochromator in order to reduce errors due to the stray light.

The experiments with mercaptoethanol were performed in solutions containing 0.01 M EDTA. The pH of solutions was adjusted in the cells with 0.1 M Na_3PO_4. As in the alkaline region the complex as well as its components undergo chemical changes, it appeared to be more convenient to calculate the difference spectra *via* subtracting the absorption of the components from that of the complex.

The acidic dissociation of mercaptoethanol was measured in a Radiometer automatic titrator. The required amount of mercaptoethanol was added to a 0.1 M KCl solution previously freed of CO_2 by nitrogen flow. The mercaptoethanol, 0.1 M, was titrated with 1 N NaOH. During titration no nitrogen bubbling was applied. If distilled water was used instead of KCl the same titration curve was obtained.

The rate constant of the alkylation reaction of mercaptoethanol with iodoacetate was determined by two independent methods:

1. The reaction was followed by measuring the decrease in absorption at 265 mμ on the basis of the change in the spectrum of iodoacetate on alkylation (Finkle, Smith, 1958). Iodoacetate and mercaptoethanol, both in a final concentration of 10^{-3} M, were added to a 0.01 M EDTA solution, adjusted to different pH values with 0.1 M Na_3PO_4, and the decrease in absorption at 265 mμ was measured at 25 °C. The pH of mixtures was checked 5 minutes after starting the reaction. The reference cells contained buffer mixtures of the same composition and pH as the sample cell.

2. Mercaptoethanol and iodoacetate, both in a final concentration of 4×10^{-4} M, were added to 0.2 M glycine buffer solution of different pH values. After various intervals aliquots were taken from the mixture and the reaction was stopped by the addition of excess PCMB simultaneously adjusting the pH to 8.5. The amount of unreacted mercaptoethanol was calculated from the absorption at 255 mμ according to Boyer (1954).

In both cases the rate constant of the reaction, k, was calculated on the basis of equation $kt = \frac{x}{a(a-x)}$, valid for bimolecular reactions under equimolar conditions, where a is the initial concentration of the compounds, x is the concentration of compound reacted within time t.

The rate of the reaction of GAPD with iodoacetate was measured at an enzyme concentration of 10^{-4} M at 25 °C in solutions containing 0.4 M ammonium sulphate. The pH of enzyme solution was adjusted in the cells with appropriate mixtures of 0.1 M EDTA and 0.1 M sodium acetate solutions. The mixture contained 3.5×10^{-4} M iodoacetate. This concentration equals that of the active thiol groups, since in solutions containing ammonium sulphate the addition of 3.5 moleequivalents of PCMB results in the complete abolition of the absorption characteristic of GAPD–NAD complex. The reaction was followed by measuring the decrease in absorption at 360 mμ, since carboxymethylation of the active thiol groups abolishes the absorption of GAPD–NAD complex around this wavelength (Racker, Krimsky, 1952). The maximum difference in absorption was calculated from the absorption of the untreated enzyme and that of charcoal-treated enzyme at the corresponding pH value. The rate constant of the reaction was determined on the basis of the above equation for bimolecular reactions. The plot $\frac{x}{a(a-x)}$ versus t, was found to be linear up to 2 minutes, corresponding to about 30–60 per cent of the over-all reaction. The value of k was calculated from this part of the curve.

The spectrum of the Ag–GAPD–NAD complex was read by using undialyzed enzyme solutions diluted with 1 M ammonium sulphate to a concentration of 14 mg enzyme protein per ml. Measurements were taken at various pH values at 5 °C. The pH of solutions was checked after measurements. The change in absorption at 335 mμ was followed after the addition of 1 to 5 moleequivalents of $AgNO_3$ and the spectra were taken when no further increase in absorption occurred (2 to 3 minutes).

Results

A) Model experiments

It has been shown that NAD enters into charge-transfer interaction with indole derivatives (Cilento, Giusti, 1959; Cilento, Tedeschi, 1961; Shifrin, 1964) and, on the other hand, with mercaptans it forms complexes which absorb in the near ultraviolet region (Van Eys, Kaplan, 1957). For this reason we have examined whether the tryptophane–NAD charge-transfer spectrum shows a pH-dependence similar to that of the GAPD–NAD complex and, in addition, we repeated the experiments of Van Eys and Kaplan with some mercaptan–NAD complexes.

Fig. 1 shows the tryptophane–NAD charge-transfer spectra in the 300 to 400 mμ region at different pH values. The spectra exhibit absorption maximum be-

tween 300 to 305 mμ. The absorption decreases between pH 2 and 7 with increasing pH. The pH-dependence of the absorption values at 305 mμ is shown in Fig. 2, curve A.

A similar curve was obtained if indole was used instead of tryptophane. This indicates that the pH-dependence of the charge-transfer spectrum is not influenced by the carboxylic group of tryptophane.

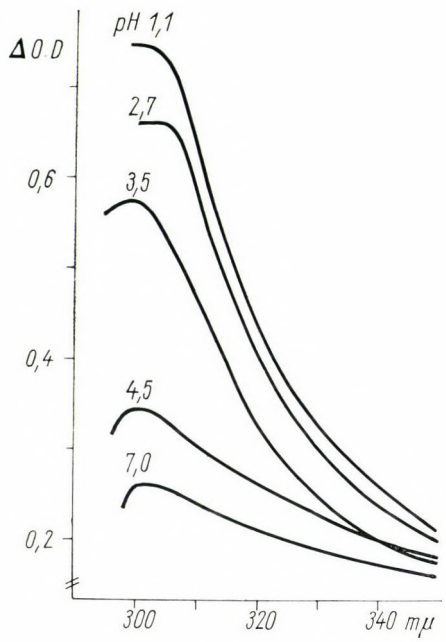

Fig. 1. The pH-dependence of the tryptophane–NAD charge-transfer complex. Spectrum measurements were carried out in 0.2 M glycine buffer of the indicated pH, in a tandem cell system, at room temperature. Tryptophane, 4×10^{-3} M; NAD, 1.8×10^{-2} M

The pH-dependence as well as the λ_{max} of the tryptophane–NAD charge-transfer spectrum considerably differ from those of the GAPD–NAD spectrum. On the basis of Van Eys and Kaplan's experiments (1957) the respective properties of the mercaptan–NAD complexes were expected to be more similar.

The spectra of solutions containing mercaptoethanol and NAD were measured in the 300 to 400 mμ range at different pH values. Difference spectra were derived by subtracting the absorption of the ingredients measured separately. In agreement with Van Eys and Kaplan (1957) we found that mercaptide ions are required for the development of the spectrum. The differences in absorption measured at λ_{max}, when plotted as a function of pH, resulted in a curve similar to the dissociation curve of the thiol group (Fig. 2, curve C). In case of mercapto-

ethanol the pK of acidic dissociation was found to be 9.8±0.1, while the half-value of the maximum absorption difference of the mercaptan–NAD complex, determined in strongly alkaline medium, was obtained at pH 9.9±0.1. In case of ethylmercaptan and thioglycolic acid the pK values were 9.8 and 10.5, respectively. The pH values, at which the half-maximum absorption differences were observed, agreed within 0.2 pH unit with the pK values for the respective mercaptan.

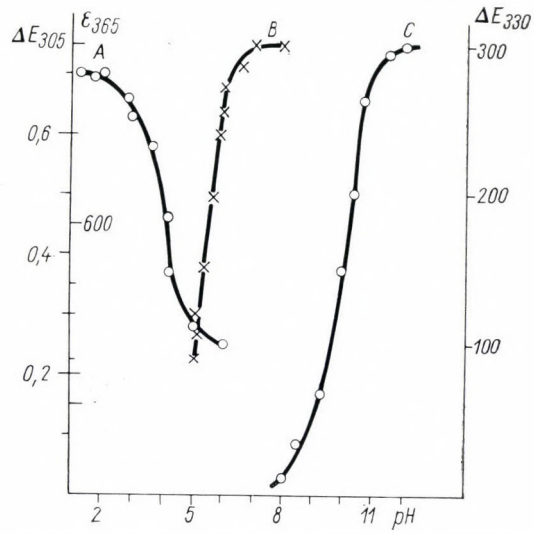

Fig. 2. The pH-dependence of spectra of the complexes of tryptophane, mercaptoethanol and GAPD with NAD. A) pH-dependence of the tryptophane–NAD charge-transfer spectrum. The curve shows the absorption differences measured at 305 mμ under condition described in Fig. 1. B) pH-dependence of the absorption of GAPD–NAD complex. The curve shows the molar extinction values at 365 mμ, calculated for 1 NAD binding site from the increase in absorption when NAD was added to charcoal-treated GAPD. C) pH-dependence of the absorption of the mercaptoethanol–NAD complex. The curve shows the difference absorption values at 330 mμ of the mercaptoethanol–NAD complex. Measurements were carried out in 0.1 M Na_3PO_4—0.1 M EDTA buffer at room temperature. Mercaptoethanol, 0.01 M; NAD 0.001 M

If iodoacetate was added to the mercaptoethanol–NAD complex, the light-absorption around 330 mμ of the solution decreased in time. This finding suggests that alkylation of the thiol group leads to the disintegration of the complex.

B) Apparent dissociation constant of the active thiol group of GAPD

The results of model experiments have shown that the spectrum of GAPD–NAD complex resembles that of the mercaptan–NAD complexes, more than the tryptophane–NAD charge-transfer spectrum, as far as the λ_{max} and pH-dependence

are concerned. As mercaptans form complexes with NAD only in the mercaptide ion state, we examined whether GAPD contains thiol groups which dissociate between pH 5 and 7, i.e. in the pH range where the absorption of the GAPD complex sharply changes. On the basis of published data (Genevois, Larroquere, 1962; Polgár, 1964a, 1964b) it could be assumed that the so-called active thiol groups of the enzyme which form a thiolester with substrate during the enzymic process, may exhibit marked acidic dissociation differing from that of aliphatic mercaptans.

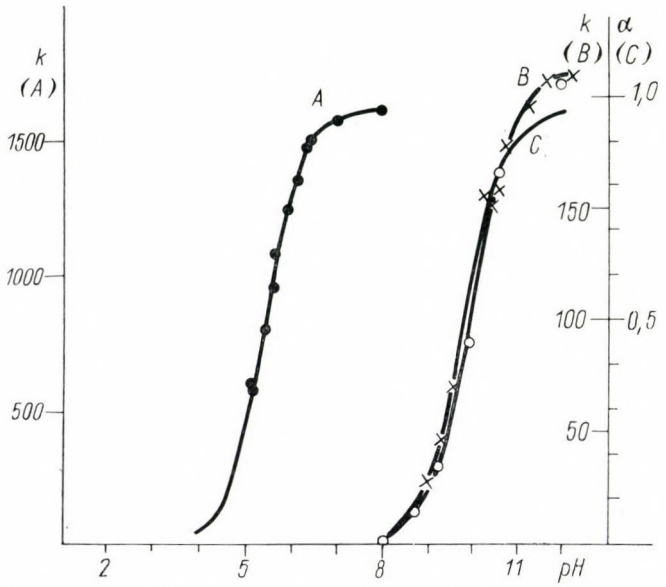

Fig. 3. The pH-dependence of the reaction of GAPD and mercaptoethanol with iodoacetate A) pH-dependence of the apparent rate constant of alkylation reaction of the active thio groups of GAPD with iodoacetate at 25 °C. B) pH-dependence of the alkylation reaction of mercaptoethanol. × = points obtained by measuring the decrease in absorption at 265 mμ; ○ = points obtained by back-titration with PCMB of remaining thiol groups. C) Acidic dissociation of mercaptoethanol. The curve shows the titration of 0.1 M mercaptoethanol with 1 N NaOH, in 0.1 M KCl or in distilled water. For further details see Methods

The dissociation of the active thiol group of the enzyme was determined by using the alkylation reaction since iodoacetate reacts with mercaptide ions (cf. Cecil, 1963). The pH-dependence curve of the apparent rate constants of the alkylation reaction of mercaptoethanol or thioglycolic acid with iodoacetate coincided very well with the dissociation curve of the corresponding mercaptan (Fig. 3).

The alkylation with iodoacetate of the active thiol group of GAPD was followed spectrophotometrically at 360 mμ since it has been shown that this reaction abolishes the characteristic absorption band of the GAPD–NAD complex (Racker, Krimsky, 1952). The curve obtained by plotting apparent rate

constants, determined at different pH values, as a function of pH coincides with the pH-dependence curve of absorption of the GAPD – NAD complex (Fig. 3). Half maximum value was obtained at pH 5.4. The apparent rate constant values fit in well with the dissociation curve of a hypothetic residue of pK 5.4.

C) pH-dependence of the formation of Ag – GAPD – NAD ternary complex

It has been shown earlier (Boross, 1965) that native GAPD is able to form a ternary Ag – GAPD – NAD complex with silver ions, where Ag is bound by the active thiol groups of the enzyme. We examined how the formation of the ternary

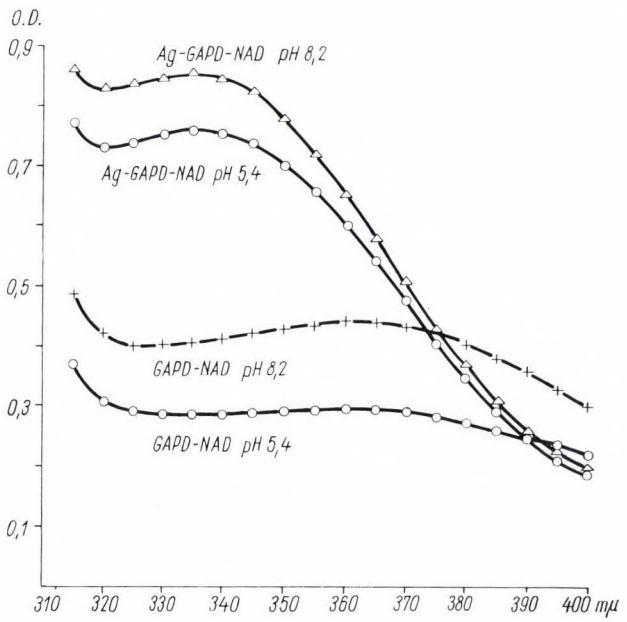

Fig. 4. Spectrum of the Ag–GAPD–NAD and GAPD–NAD complexes in acidic and slightly alkaline media. The measurement of spectra was carried out with 14 mg per ml enzyme concentration at 5 °C, at the pH values indicated

complex depends on pH. Silver ions were added to solutions containing GAPD and NAD at different pH values and the absorption spectra were measured. Fig. 4 shows the spectra of the ternary complex at pH 5.4 and 8.2. The isosbestic point, which lies in neutral or slightly alkaline solution at 375 mμ, is shifted towards the longer wavelengths on decreasing the pH. At the same time, the absorption curve of the ternary complex measured in acidic medium intersects around 375 mμ that of the enzyme-coenzyme complex measured at pH 8.2. The experiments indicate that the ternary Ag – GAPD – NAD complex is formed in

approximately 85 per cent even under conditions when chromophore portion of the enzyme-coenzyme complex can be formed only in 50 per cent.

Discussion

Two theories have been suggested for the interpretation of the absorption characteristic of the GAPD–NAD complex. According to Racker and Krimsky (1952) a bond is formed between a thiol group of the enzyme and a carbon atom of the nicotinamide moiety of coenzyme, this bond is split by the substrate resulting in the formation of reduced coenzyme and enzyme-thiolester. The other theory assumes a charge-transfer interaction between the enzyme and coenzyme which manifests itself in the characteristic absorption band. This theory was first advanced by Kosower (1956). It has been proven experimentally that NAD is able to form charge-transfer complex with tryptophane, where tryptophane serves as electron donor, while the pyridinium ring of NAD is an electron acceptor (Cilento, Giusti, 1959; Cilento, Tedeschi, 1961; Shifrin, 1964). Polgár (1964a) assumed in his paper on the mechanism of the catalytic process that a nucleophilic thiol group of the enzyme, in the mercaptide ion form, would serve as electron donor.

As shown by our experiments, the λ_{max} of the spectrum of GAPD–NAD complex and that of the tryptophane–NAD charge-transfer spectrum are relatively far from each other, similarity can be found only in their broad, protracted shape. Moreover, the pH-dependence curves of the two spectra run in an opposite manner. However, we cannot exclude the possibility that in spite of the model experiments, a tryptophane residue, localized in a special environment on the enzyme surface, would serve as electron donor and this results in an absorption band with λ_{max} at higher wavelengths and with greater molar extinction coefficient than those of the tryptophane–NAD charge-transfer spectrum.

Van Eys and Kaplan's experiments (1957) with various mercaptans have shown that mercaptide ions and pyridinium compounds can form complexes with absorption maxima around 330–340 mμ. We could confirm these observations. The absorption maximum of the GAPD–NAD complex, 365 mμ, lies much nearer to these wavelengths than to the maximum of the tryptophane–NAD charge-transfer spectrum. The absorption band, however, is not so broad in case of the mercaptide–NAD complexes as with the GAPD–NAD complex.

The structure of mercaptan–NAD complexes is unknown, the only fact that could be established is that mercaptide ions are required for their formation. It is possible that in this case we also deal with a charge-transfer interaction, where the mercaptide ion serves as an electron donor, similarly to the complex of OH^-, J^-, and SO_2^{2-} with NAD (Burton, Kaplan, 1963; Kosower, 1960). At any rate, on the addition of iodoacetate the mercaptide ions become alkylated and the significant absorption of the complex disappears. Thus the absorption of the complex can be attributed to a labile, dissociable bond rather than to the formation of a covalent C–S–C system.

GAPD contains a markedly reactive thiol group per active center (Racker, Krimsky, 1958; Genevois, Larroquere, 1962; Harris et al. 1963; Polgár, 1964b) the reactivity of which in neutral solution is comparable to that of mercaptide ions. As shown by our results, the apparent dissociation of this residue takes place between pH 5 and 7, in the range where the absorption of the enzyme-coenzyme complex appears. The apparent pK value of this thiol group was found to be 5.4, as calculated from the pH-dependence curve of the rate constant of the alkylation reaction. At the same pH the absorption of the enzyme-coenzyme complex amounts to one-half of that measured in neutral solutions. The pK value of the aliphatic mercaptans is higher by 4 – 5 units than the value measured for the active thiol group of GAPD. Thus, in the neighbourhood of the active thiol groups the enzyme must have a special distribution of charges – a special interaction of functional groups – which renders the electronic structure of the sulfur atom similar to that of mercaptide ions even at neutral pH. The pK value determined for this thiol group may then be the manifestation of these interactions. It is perhaps related to the dissociation of another functional group of the protein which can interact with the cysteinyl side chain. An interaction of this type has been suggested by Rabin and his co-workers for creatinophosphokinase (Rabin, Watts, 1960) and for yeast alcohol dehydrogenase (Rabin, Whitehead, 1962) where the mercaptide ion-like character of thiol groups is explained by postulating an imidazole-thiol H-bonding. It does not seem probable that the mercaptide ion-like character of this thiol is due to interaction with the coenzyme, since this residue shows nucleophilic character even in the absence of the coenzyme (Polgár, 1964b). Racker and Krimsky (1958) have shown that this thiol group reacts with iodoacetate faster if the coenzyme is bound to the enzyme, i.e. the presence of the coenzyme may increase the nucleophilic character of the sulfur atom.

The coincidence of the apparent dissociation curve of the thiol group of the enzyme and of the pH-dependence curve of the absorption of the enzyme-coenzyme complex supports the hypothesis that the absorption of the complex is due to an interaction between the mercaptide ion and pyridinium ring. On this basis it can be readily explained why the absorption of the complex disappears on PCMB-blocking (Velick, 1953) or on the alkylation of thiol groups with iodoacetate (Racker, Krimsky, 1952) even when the coenzyme is not split off from the enzyme (Friedrich, 1965). There are experimental findings to show that the active thiol group of GAPD need not be in the free state for the development of an absorption band in the near ultraviolet. On modifying the active thiol group with certain alkylating reagents, such as iodoacetamide or bromacetone (Boross et al. 1966) the absorption band of the GAPD – NAD complex is not abolished but rather increased and shifted towards longer wavelengths. Moreover, on the addition of certain heavy metal ions (Ag and Cd) ternary metal-enzyme-coenzyme complexes are formed which show altered absorption (Boross, 1965). Cupric ion does not shift the absorption band, only the shape of the spectrum curve is slightly changed (Boross, Szabadi, 1967). Kaplan et al. (1957) have found that the absorption of

the enzyme–acetylpyridine–NAD complex does not disappear, only somewhat decreases, on treatment with iodoacetate. These experimental findings can be reconciled with hypothesis of mercaptide ion-NAD interaction if we assume that not only the mercaptide ions but also the free electron pair of sulfur atoms involved in special linkages can come into interaction with the pyridinium ring which may be sterically fixed by other bonds of the enzyme-coenzyme complex.

This assumption is supported by the observation that the spectrum of the Ag–GAPD–NAD complex can almost fully develop even in acidic medium, where the absorption of the GAPD–NAD complex is considerably reduced. At pH 5.4 only 50 per cent of the active thiol groups of the untreated enzyme is in the "dissociated", mercaptide ion-like state, while the S–Ag is practically undissociated. This suggests that the S–Ag group can replace the mercaptide ion in the complex. The small difference in the height of the spectrum of the ternary complex between pH 5.4 and 8.2, which amounts to about 15 per cent, is probable due to the structural change of the protein induced by pH (Előd i et al. 1960).

The experiments presented support only the hypothesis that the active thiol group of GAPD is directly involved in the chromophore part of the enzyme-coenzyme complex. It cannot be excluded, however, that the changes in absorption caused by different modifications of the thiol group are secondary phenomena due to alteration of the structure of GAPD.

The elucidation of the absorption of the enzyme-coenzyme complex is of interest not only from the point of view of physical chemistry. The spectrum of the complex lies very near to that of NADH formed during the enzymic reaction. It is tempting to assume that the electronic structure of the coenzyme in the complex is similar to that of NADH, i.e. it exists in an intermediate state between the oxidized and reduced form. This would be of special importance even if this state is elicited only by the excitation energy of a photon, i.e. if the absorption of the GAPD–NAD complex is a charge-transfer spectrum. It is conceivable that the electron can be transferred from the enzyme to the coenzyme not only by the photon-energy but also as a result of the oxidation of substrate, when the newly formed electronic structure becomes stabilized. It is to be expected that the final clarification of the absorption of the enzyme-coenzyme complex will promote the understanding of enzymic catalysis.

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Protein Denaturation by the Blocking of Thiol Groups

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The kinetics of the blocking of the thiol groups of glyceraldehyde-3-phosphate dehydrogenase* with PMB was studied. It was found that, in agreement with earlier data, 8 thiol groups of the enzyme react instantaneously with PMB, while blocking of the remaining 8 thiol groups requires prolonged incubation. The blocking of the latter masked thiol groups obeys first order kinetics, with rate constant $k_{SHg} = 0.131 \pm \pm 0.010\ min^{-1}$. The structural change induced by mercaptide bond formation can be followed by measuring the hypsochromic shift in the absorption spectrum of the protein, which is due to the exposure of aromatic chromophores. The structural change accompanying the blocking of masked thiol groups, as measured at 295 mμ, obeys first order kinetics, with rate constant $k_{st} = 0.136 \pm 0.008\ min^{-1}$. In the light of the close agreement of the two first order rate constants, the mechanism of protein denaturation induced by mercaptide bond formation is discussed.

Muscle GAPD consists of four identical subunits (Harris, Perham, 1965; Harrington, Karr, 1965). It contains no disulphide bridges and has four cysteinyl residues per subunit, i.e. 16 residues per mole of enzyme (Segal, Boyer, 1953; Szabolcsi et al. 1960; Harris, Perham, 1965). [Molecular weight 145,000 (Elődi, 1958; Harrington, Karr, 1965)].

The thiol groups of GAPD have been extensively studied by the aid of various blocking agents. With iodoacetate only one thiol group per subunit can be blocked in the native state, and this results in the loss of enzymic activity, since this thiol binds the substrate through the formation of an S-acyl enzyme (Racker, Krimsky, 1952; Racker, Krimsky, 1958; Harris et al. 1963). On the other hand, PMB is able to react gradually with all thiol groups of the enzyme and this leads to the inactivation of the enzyme as well as to profound changes in its conformation: the protein structure is shifted towards denaturation. The loosening of the steric structure induced by mercaptide bond formation has been demonstrated by the increased tryptic digestibility (Szabolcsi, 1958; Szabolcsi et al. 1959) and levorotation (Elődi, 1960) of the enzyme. From these studies and those performed on aldolase (Szabolcsi, Biszku, 1961) it was suggested that the

* *Abbreviations:* GAPD, glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating), (EC 1.2.1.12); PMB, p-chloromercurybenzoate.

unmasking of buried thiol groups was due to the conformational changes induced by PMB treatment of the most reactive cysteines of the proteins. These methods were, however, not suitable to follow rapid changes, and owing to this, no time-correlation could be established between mercaptide bond formation and the structural change.

The aim of the present work was to elucidate the above relationship in the case of GAPD and to provide thereby an insight into the mechanism of protein denaturation induced by thiol-group blocking.

The structural alterations were followed by difference spectrophotometry, by making use of the well-known hypsochromic effect that occurs in the absorption spectra of proteins on denaturation ("Denaturation blue shift", Glaser et al. 1957; Bigelow, Geschwind, 1960; Yanary, Bovey, 1960). The decrease in absorption in the near ultraviolet region as a result of PMB-treatment provided us with a convenient and flexible tool in the tracing of structural changes and allowed us to perform the comparative kinetic analysis of mercaptide bond formation and the unfolding phenomenon.

Materials and Methods

Three times recrystallized swine muscle GAPD (Elődi, Szörényi, 1956) was used. Enzyme solutions were freshly prepared in 0.05 M Tris-buffer, pH 8.5, and the solution was freed of ammonium sulphate by gel-filtration on a Sephadex G-50 column, equilibrated with the same buffer. All manipulations with the enzyme were carried out in the cold (0 – 5 °C).

Protein concentration was determined spectrophotometrically on the basis of molar extinction coefficients at 280 mμ, ε_{280} being 1.45×10^5 and 1.32×10^5, for the native and charcoal-treated enzyme, respectively. [Molecular weight 145,000 (Elődi, 1958; Harrington, Karr, 1965)].

PMB (British Drug Houses, Ltd.) content was determined spectrophotometrically, based on a molar extinction coefficient $\varepsilon_{234} = 1.7 \times 10^3$ (Boyer, 1954).

Mercaptide bond formation was measured according to the method of Boyer (1954) by following the increase of absorption at 250 mμ. The number of thiol groups was determined also by the aid of dithiobisnitrobenzoic acid (Ellman, 1959).

Difference spectra were measured in a tandem-cuvette system (Laskowski et al. 1960; Remily, Wolfe, 1960) at 5 ± 0.5 °C in a Beckman DU spectrophotometer. The measurements were confined to an optical density range where errors due to stray light (Mehler, 1954) and fluorescence (Mehler et al. 1957) were negligible.

The native enzyme (containing firmly bound NAD) was freed of coenzyme by charcoal treatment (Taylor et al. 1948), in the following way. Crystals of GAPD were dissolved in 0.05 M Tris-buffer, pH 8.5 (30 – 40 mg/ml), then charcoal was added to the solution in a 1 : 1 protein/charcoal weight ratio and the mixture was stirred by a magnetic stirrer for 15 minutes in the cold. After the addition of anoth-

er, equal amount of charcoal the stirring was repeated. The mixture was filtered and gel-filtered on a Sephadex G-50 column equilibrated with 0.05 M Tris-buffer, pH 8.5. The enzyme solution thus obtained (15–20 mg/ml) had an E_{280}/E_{260} ratio of 1.85–1.95.

Results

1. *Kinetics of the formation of mercaptide bonds*

Fig. 1 shows the semilogarithmic plot of the disappearance of thiol groups, i.e. the formation of mercaptide bonds, when a slight excess (17 moleequivalents per 145,000 molecular weight) of PMB was added to native GAPD. The reaction is

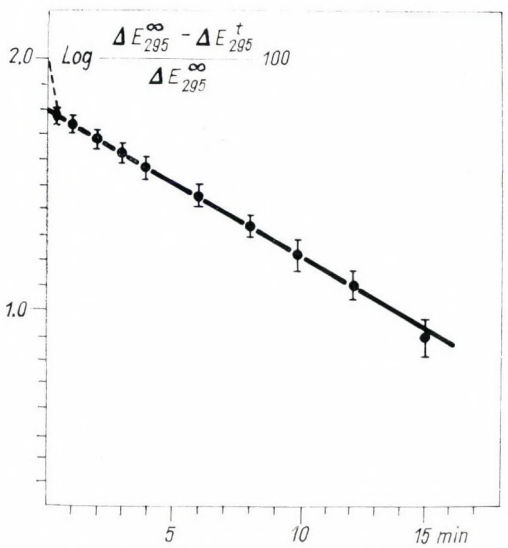


Fig. 1. Semilogarithmic plot of the disappearance of thiol groups in the reaction of GAPD with PMB. Reaction mixture: GAPD, 6×10^{-3} μmoles; PMB, 1.02×10^{-1} μmoles; in 0.05 M Tris-buffer, pH 8.5, final volume 3.0 ml. The reference cell contained 1.02×10^{-1} μmoles of PMB in 3.0 ml of 0.05 M Tris-buffer, pH 8.5. The reaction was started by the addition of PMB to the reaction mixture. Light path 1 cm, temperature 5 °C. The maximum increase in absorption at 250 mμ, ΔE^{∞}_{250}, corresponded to the formation of 15.6 ± 0.7 mercaptide bonds per mole of enzyme. [In parallel measurements with dithiobisnitrobenzoic acid (Ellman, 1959) 15–16 moles of thiol group per mole of protein were found]. ΔE^{t}_{250} is the increase in absorption at time t

completed when all the thiol groups of GAPD are blocked. As can be seen in Fig. 1, the initial very rapid phase of the reaction, which was practically instantaneous under the experimental conditions, was followed by a much slower one. Since the velocity of the slow reaction depends only on the concentration of GAPD and is

independent of the concentration of PMB above 16 moleequivalents (measured up to 90 moleequivalents) and the reaction gives a straight line in the semilogarithmic plot, it follows first order kinetics with a rate constant $k_{SHg} = 0.131 \pm 0.010$ min^{-1}. By extrapolation to 0 time, the intercept on the ordinate gives the amount of fast and slow reacting thiol groups, 50 – 50 per cent, which corresponds to 7.8 fast and 7.8 slow reacting thiol groups.

These results agree with earlier data, according to which about 7 thiol groups of GAPD react instantaneously with PMB, while blocking of the rest of thiol groups takes place in a measurable time period (Segal, Boyer, 1953; Szabolcsi, 1958; Szabolcsi et al. 1960).

2. *Difference spectra in the near ultraviolet due to mercaptide bond formation*

We have found that PMB treatment of GAPD alters the absorption of the enzyme around 300 mμ. The decrease in absorption of native* and charcoal-

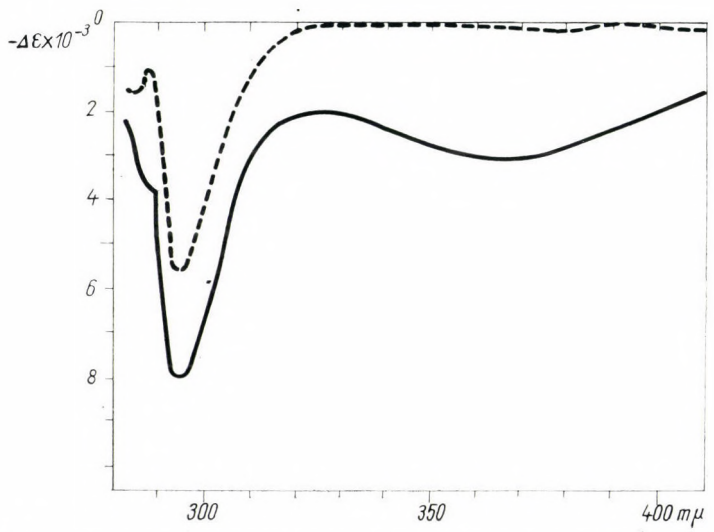


Fig. 2. Difference spectra in the near ultraviolet induced by mercaptide bond formation in native and charcoal-treated GAPD. Two cells (each of 1 cm light path) were applied in both sample and reference light paths (S, S' and R, R', respectively). Cells S and R contained $3.0 \times \times 10^{-2}$ μmoles of GAPD in 0.05 M Tris-buffer, pH 8.5, final volume 2.9 ml. Cells S' and R' contained 2.9 ml of 0.05 M Tris-buffer, pH 8.5. Temperature 5 °C. The spectrum of sample light path was taken against the reference one to obtain the base line. Then 0.51 μmoles of PMB, in 0.1 ml of 0.05 M Tris-buffer, pH 8.5, was added to cells S' and R, while 0.1 ml of buffer to cell S. The mixtures were incubated at 5 °C for 40 minutes, then the spectrum of sample light path was read against the reference one and the base line was subtracted. Solid curve: native GAPD; dotted curve: charcoal-treated GAPD

* In the following the designation of native enzyme is used for GAPD with its full complement of coenzyme, i. e. about 4 moles of firmly bound NAD per 145,000 g protein.

treated GAPD upon PMB-blocking is shown in Fig. 2. With the native enzyme two minima were found: the minimum around 360 mμ is the result of the abolition of the absorption band characteristic of enzyme-coenzyme complex (Racker, Krimsky, 1952), while that around 294–5 mμ is due to the unfolding of the protein, i.e. to the exposure of aromatic chromophores (mainly tryptophane) imbedded in the interior of the native protein (Glaser et al. 1957; Yanari, Bovey, 1960). With charcoal-treated (NAD-free) GAPD, as expected, only the latter minimum was found. The difference at 295 mμ increased with the amount of PMB added up to 16 moleequivalents; on the addition of more PMB no further increase in the difference occurred. The molar difference extinction coefficient, $-\Delta\varepsilon_{295}$, was then 8000 and 5500 for the native and charcoal-treated enzyme, respectively.

The difference between the two values can be explained by the fact that PMB splits off tightly bound NAD from the protein (Velick, 1953). In fact, a hyperchromic effect has been observed in this wavelength region upon binding of NAD to GAPD (Friedrich, 1965). This increase in absorption might be due to some specific interaction between enzyme and coenzyme or to a conformational change (increased folding) of the protein induced by NAD-binding (Elődi, Szabolcsi, 1959; Bolotina et al. 1966; Racker, Krimsky, 1958; Furfine, et al. 1965). On this basis it is to be expected that upon depletion of bound coenzyme by PMB-treatment a hypochromic effect will occur.

Libor et al. (1965) have shown that the absorption of GAPD decreases between 270 and 300 mμ on urea, acid, or detergent treatment; they found minima at 286–7 and 293–4 mμ. The latter minimum very probably corresponds to that found by us on PMB-treatment. The shorter wavelength minimum, however, could not be reliably measured in our case owing to the disturbing effect in this region of the absorption of mercaptide bonds.

3. *Kinetics of the development of the 295 mμ difference spectrum*

Fig. 3 shows the semilogarithmic plot of the decrease in absorption at 295 mμ due to the reaction of GAPD with PMB. Reaction conditions were identical to those applied in the measurement of mercaptide bond formation (cf. Fig. 1). As it can be seen in Fig. 3, after a rapid fall the absorption at 295 mμ decreased at a slower rate. Identical curves were obtained if more than 16 moleequivalents of PMB were used (measured up to 90 moleequivalents of PMB). If charcoal-treated GAPD was used, only the second slow phase could be observed. This finding suggests that the rapid phase in case of native enzyme is due to the release of tightly bound NAD (cf. point 2), and that the blocking of surface-exposed thiol groups in the NAD-free enzyme does not result in a burst-like structural change. The slow reaction, which corresponds to the unmasking of aromatic chromophores, follows first order kinetics with a rate constant $k_{st} = 0.136 \pm 0.008$ min^{-1}, which agrees well with the rate constant of the slow phase of mercaptide bond formation ($k_{SHg} = 0.131 \pm 0.010$ min^{-1}).

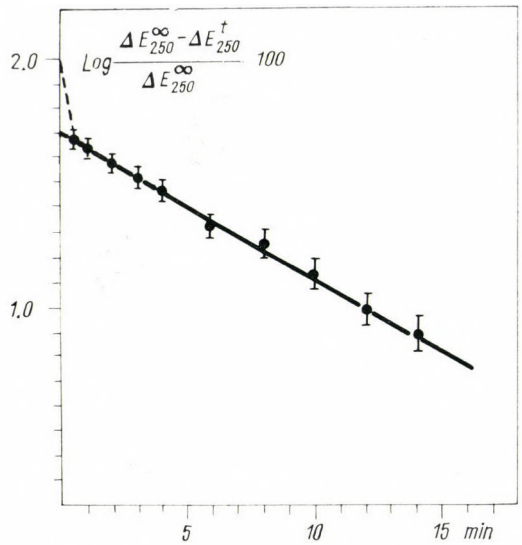


Fig. 3. Semilogarithmic plot of the decrease in absorption at 295 mμ in the reaction of GAPD with PMB. Cells of 4 cm light path were used. Reaction mixture: GAPD, 2.4×10^{-2} μmoles; PMB, 0.408 μmoles in 0.05 M Tris-buffer, pH 8.5, end volume 12.0 ml. The reference cell contained 0.408 μmoles of PMB in 12.0 ml of 0.05 M Tris-buffer, pH 8.5. The reaction was started by the addition of PMB to the reaction mixture. Temperature 5 °C. The maximum decrease in absorption, ΔE^{∞}_{295}, was attained in about 30 minutes. ΔE^{t}_{295} is the decrease in absorption at time t

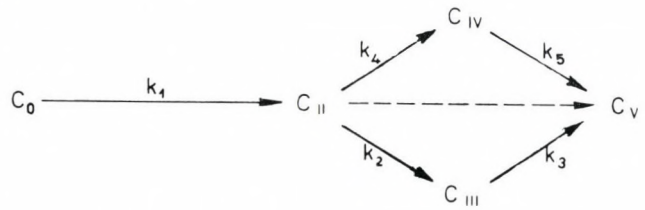
Discussion

It has been known for a long time that, as to their localization in the tertiary structure of the protein, the thiol groups of GAPD can be divided into two groups: 1. surface exposed, instantaneously reacting ones, and 2. masked, slow reacting ones (Koeppe et al. 1956; Szabolcsi et al. 1960). The present investigations confirm these earlier data and give the exact number of cysteinyl residues for both groups as 1.95 per subunit, i. e. there are round 8 fast and 8 slow reacting thiol groups in the tetramer.

The PMB-blocking of masked thiol groups follows first order kinetics, its rate depending on protein concentration only. Since the formation of mercaptide bond is obviously a bimolecular reaction, first order kinetics can be obtained only if *i)* one of the reagents is in infinite excess, or *ii)* if the overall reaction is limited by a first order reaction. The first alternative can be ruled out at once, since the first order character is preserved under equimolar conditions as well. Taking the second alternative, we can rightly assume that the over-all reaction is determined by an unfolding process which might follow first order kinetics The alternative is

supported by the fact that the structural changes induced by PMB treatment follow first order kinetics with a rate constant which agrees well with that of mercaptide bond formation ($k_{st} = 0.136 \pm 0.008\ \text{min}^{-1}$ and $k_{SHg} = 0.131 \pm 0.010\ \text{min}^{-1}$). The phenomenon is readily conceivable by considering that the rate of unfolding is much too slow as compared to the blocking reaction itself; once a thiol group becomes unmasked it is immediately blocked by PMB.

The structural changes taking place in the protein can be outlined by the following scheme:



where C_0 is the conformation of the native enzyme, C_{II} the conformation after blocking SH_I^* and SH_{II}, while C_{III}, C_{IV}, and C_V designate conformations where SH_{III}, SH_{IV}, and $SH_{III} + SH_{IV}$, respectively, become unmasked. (There may be assumed the existence of a C_I state, between C_0 and C_{II}, representing the conformation after blocking of one fast reacting thiol group. This, however, has no bearing on the present consideration.)

If the reaction of masked thiol groups with PMB is *consecutive*, i. e. the blocking of SH_{III} precedes the unmasking of SH_{IV}, the process follows the lower reaction line. In this case, a single straight line can be obtained throughout the reaction of masked thiol groups in Figs 1 and 3 only, if *i)* ($k_2 = k_3$ or *ii)* $k_2 \ll k_3$, with the restriction in *i)* that the same amounts of aromatic chromophores become exposed to the solvent in transitions $C_{II} \rightarrow C_{III}$ and $C_{III} \rightarrow C_V$. Since interpretation *i)* requires two deliberate, less probable postulates, the following mechanism is suggested instead.

If a molecule reaches C_{III}, it is rapidly transformed into C_V, i. e. $k_2 \ll k_3$, the protein molecule "collapses". An extreme case of this is when there is no discrete C_{III} or C_{IV}, i. e. SH_{III} and SH_{IV} are unmasked in one step. (Indicated by a dotted arrow in the scheme.) The protein molecules in solution, due to their different fluctuational states (Linderstrøm-Lang, Schellman, 1959), undergo this "collapse" at various times, but for the whole set of molecules the process can be characterized by the average k_2 constant. By this *one-by-one mechanism* the kinetics of the exposure of aromatic chromophores is also readily interpreted.

The possibility cannot be excluded that the blocking of thiol groups is not consecutive, i.e. the unmasking of SH_{III} and SH_{IV} proceeds independent of each

* The following designations are introduced: SH_I: acylenzyme-forming thiol group; SH_{II}: second thiol group reacting fast with PMB; SH_{III} and SH_{IV}: the two masked thiol groups.

other, with the rate constants k_2 and k_4, respectively. However, also in this case, the most plausible explanation of the simple, first order kinetics is the assumption of a one-by-one mechanism in thiol-group blocking.

GAPD fully blocked with PMB is not a completely denatured protein. This is shown by the smaller molar difference extinction coefficient, $\Delta\varepsilon_{295} = 8000$, found after PMB-treatment, than that obtained upon urea or acid treatment, $\Delta\varepsilon_{294} = 20{,}000$ (Libor et al. 1965). The same conclusion can be drawn from optical rotation and digestibility studies (Elődi, 1960; Szabolcsi et al. 1959). The blocking of thiol groups of GAPD thus results in the partial unfolding of the polypeptide chains, with the preservation of a good deal of ordered protein structure.

It has been reported that on denaturing ovalbumin and conalbumin in urea solutions of high molarity, the decrease in absorption at 287–292 mμ follows first order kinetics, but caution has been suggested in the interpretation of such spectral changes (Glaser et al. 1963; McKenzie, 1963; Leach, Scheraga, 1960). In the reaction of GAPD with PMB, however, where the point of attack of the denaturing agent is known, the inferences set forth above appear to be sound.

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Microwave Investigation of Biological Substances

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The present paper describes a microwave method for the determination of the attenuation coefficient of substances of great loss. As a control, the results of measurements made on distilled water and KCl solution were compared with pertaining literary data. According to the measurements made on KCl solutions the conductivity corresponding to the ion content in a frequency range from 1.8 Gc/s to 4.0 Gc/s does not show perceivable changes.

Introduction

Ultra-short radiowaves have been used in the research of biological structures since Höber's first investigation (Höber, 1910). Radio-frequency measurements in our Institute have shown the presence of bound K in muscle (Ernst, 1935; Masszi, 1957; Örkényi, Masszi, 1961; Masszi, Tigyi-Sebes, 1962; Ernst, 1963). Measurements on gelatine sol and gel and also on native or coagulated egg-white solutions pointed to a correlation between the dielectric behaviour and the structural state of protein solutions (Koczkás, 1938; Masszi, Örkényi, 1963). The development of the microwave technique made it possible to use it for studying biological structures (Masszi, Örkényi, 1966). The subject of the recent work is to describe a method which makes it possible to determine microwave parameters in a relatively wide frequency range.

Methods

The methods to measure microwave attenuation were applied to the investigation of solutions of great loss with good results (Honerjäger, Meckbach, 1950; Hasted, 1961). A schematic diagram of the equipment designed by us to measure attenuation can be seen in Fig. 1. The TEM wave (transversal electric and magnetic) coming from the signal generator and modulated by the rectangle pulse of 1 kc/s gets into the cell matched with a double stub tuner. Here by passing through the sample, the amplitude of the electric field strength exponentially decreases:

$$E = E_0 e^{-\alpha l} \tag{1}$$

E_0 is the field strength entering into the cell, E is the field strength at the end of the cell, l is the length of the cell, and α is the attenuation coefficient. The indicator is connected to the cell and shows proportionate voltage (U) to the second power of the outcoming amplitude, therefore:

$$U = kE_0^2 e^{-2\alpha l} \tag{2}$$

According to equation (2) the attenuation coefficient (α) of the sample is determined in the following way:

a) At constant entrance field strength (E_0) at lengths l_1 and l_2 of the sample from voltage U_1 and voltage U_2 measured according to equation (2) we obtain:

$$\alpha = \frac{\ln \dfrac{U_1}{U_2}}{2(l_1 - l_2)} \tag{3}$$

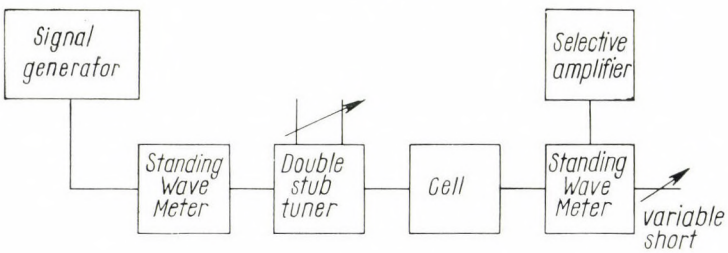

Fig. 1. The scheme of the equipment

b) At constant E_0 and l, with the help of a substance of known attenuation, we derive from equation (2):

$$\alpha = \frac{\ln \dfrac{U_1}{U}}{2l} + \alpha_1 \tag{4}$$

where α_1 and U_1 refer to the known substance, U is the value of the sample. This method is mainly suitable for the demonstration of the change of the attenuation coefficient. Distilled water can be used as a standard.

c) In the case of constant E or U, by the application of the formula $\ln \dfrac{E_{01}}{E_{02}}$ which can be determined with the power meter of the signal generator we obtain from equation (1):

$$\alpha = \frac{\ln \dfrac{E_{01}}{E_{02}}}{l_1 - l_2} \qquad \text{or} \qquad \alpha = \frac{\ln \dfrac{E_{01}}{E_0}}{l} + \alpha_1, \tag{5}$$

where indexes 1 and 2 have the same meaning as in *a)* and *b)*. This equation is applied to control the method described in *a)* and *b)*.

For the measurement an EMG signal generator, type TZA-101, was used, which operated in a band of 1.8 Gc/s – 4.0 Gc/s. The maximum value of the power output is 1 mW which can be varied by a calibrated power divider with an accuracy of ± 1 dB. The role of the double stub tuner is to adjust the input impedance of the cell to the generator. In the case of proper adjustment, the indicator shows maximum voltage, and the standing wave ratio measurable in the direction

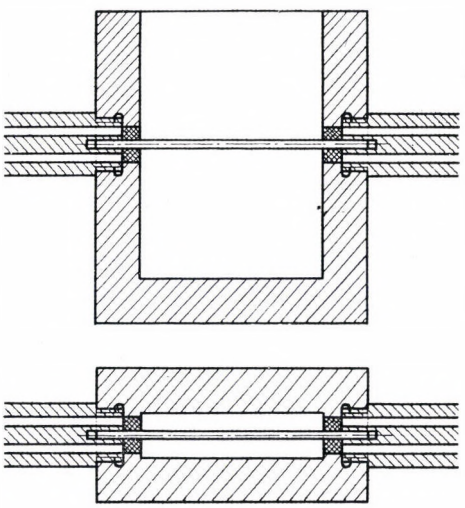

Fig. 2. The cell

of the cell agrees with that of the signal generator (Almássy, 1961). According to the experience, the degree of adjustment is reliably indicated by the maximum voltage appearing on the indicator. The proper adjustment ensures that E_0 is a constant value during the measurement, and makes it possible to determine $\ln \frac{E_{01}}{E_{02}}$ with the help of the power divider of the signal generator.

The conductor part of the cell (Fig. 2) consists of two metal sheets 60 mm wide which are fixed parallel to each other at a distance of 7 mm, with a cylindric inner conductor 2 mm in diameter passing between them. An arrangement like this corresponds to a coaxial line from an electric point of view (Wholey, Eldred, 1950). The electromagnetic field concentrates around the central conductor and the path of wave motion is not influenced by the more distant parts. This made it possible to shape the cell into cavity open at the top to hold the sample. In this way other measurements could be made on the sample without any disturbing effect (e. g. temperature measurement). The central line is fixed by a teflon plug

which is placed in the eyelet of the metal sheet 1 cm wide which fastens the two ends of the cell. The coaxial line conducting into, respectively, conducting out is connected to this. Three vessels were made 2 cm, 4 cm, and 8 cm long, respectively. Measurement of type *a* can be carried out by changing the vessels. The vessels were made of red copper and were coated by a galvanic procedure with a layer of palladium 0.5 μm thick, another layer of silver, 5.0 μm thick and a third layer of palladium, 0.5 μm thick. The good microwave conductance is ensured by the silver layer, and the electrolytic effect of the solution is prevented by palladium. In a cell constructed in this way, the attenuation resulting from the finite conductivity of the walls can be neglected as compared to the attenuation appearing in the sample (Hoffmann, 1963, 1964). The cell is enclosed in a plexi coating in which water of a constant temperature is circulating. The temperature of the water was kept constant with an accuracy of ± 0.1 °C.

An Orion-FMV 1963 type standing wave meter with a changeable short at the end was used as an indicator. A selective amplifier of 1 kc/s is connected to the crystal detector. The admittance which is represented by the probe was matched to the output of the cell by the repeated change of the changeable short, the position of the probe carrier, and the depth of the probe. The indicator shows maximum voltage in the case of correct matching and the standing wave ratio, which can be measured by a second standing wave meter probe carrier in the direction of the uncoupling probe, corresponds to the standing wave ratio which can be measured in the direction of the cell. Similarly to the matching of the input of the cell, looking for the maximum suffices here, too. The length of the cell was chosen such that the energy passing through the cell was still enough for the indication. Because the energy reflected from the end of the cell becomes negligible by the time it reaches the beginning of the cell, the tuning can be carried out independently at the two sides of the cell. The characteristic of the "IN21 Silvania" crystal detector used for indication is square-law and so in the case of correct matching equation (2) is valid.

Results

To check the equipment, measurements were made on distilled water and on KCl solution at $f = 3$ Gc/s in the first group of measurements.

Table 1

Attenuation coefficient of distilled water

T °C	α measured	α literature
25	0.411 ± 0.008	0.412*
35	0.309 ± 0.007	0.324*
45	0.247 ± 0.006	0.242**

* Weiss et al. (1965)
** Hasted (1961)

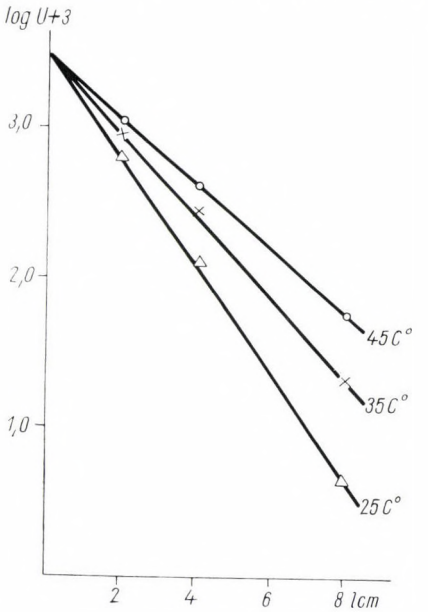

Fig. 3. Relation between the length of the cell (1) and the measured voltage (U)

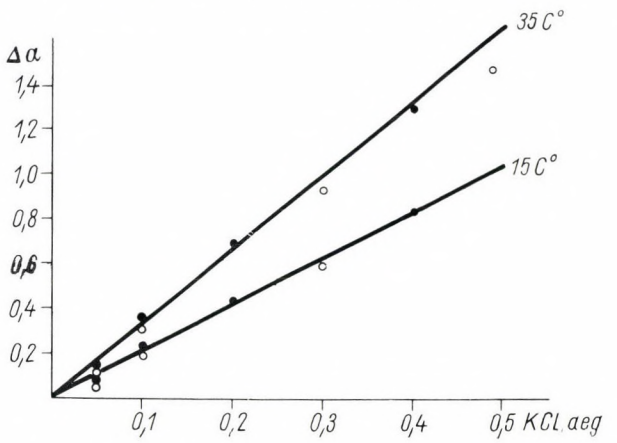

Fig. 4. Relation between the increase of the attenuation coefficient ($\Delta\alpha$) and the KCl concentration. ●——● own measurements, ○——○ from the data of Weiss et al. (1965)

Fig. 3 shows the data obtained with distilled water at 25 °C, 35 °C, and 45 °C, respectively. It can be seen that the U values measured decrease exponentially according to equation (2). In Table 1 the values of the attenuation coefficients calculated on the basis of the measurements are compared with the literary. In Fig. 4 the increase of the attenuation of a KCl solution is plotted against the KCl

content. These data are in good agreement with the data of Weiss et al. (1965). The Figure shows in addition that in the case of dilute solutions the attenuation varies linearly with the concentration.

The frequency dependence of the attenuation of KCl solution at frequencies of 1.8 Gc/s, 2.0 Gc/s, 3.0 Gc/s and 4.0 Gc/s, respectively, was studied in the second group of measurements (λ = 16.7 cm, 15.0 cm, 10.0 cm, and 7.5 cm). In Fig. 5 $\log \frac{U_{H_2O}}{U}$ proportional to $\Delta\alpha = \alpha - \alpha_{H_2O}$ is plotted against the concentration. The

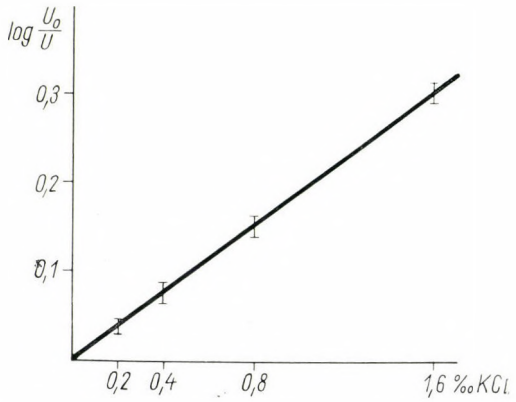

Fig. 5. Attenuation coefficient of KCl solutions plotted against the concentration at different frequencies. The vertical segments connect the smallest and the greatest values measured at the concentration shown

temperature of the solution is 40 °C. The values measured at different frequencies but at the same concentration are in a good agreement; the error is less than 3 per cent.

Discussion

According to the transmission-line theory, in the case of TEM waves, the relation between the attenuation coefficient and the dielectric characteristics of the sample is expressed by the following equation:

$$\alpha = \frac{\sqrt{2}\,\pi}{\lambda_0} \left(\sqrt{\varepsilon_r'^2 + \varepsilon_r''^2} - \varepsilon_r'\right)^{\frac{1}{2}} \tag{6}$$

where λ_0 is the wavelength in vacuum, ε_r' and ε_r'' determine the relative complex dielectric constant of the sample:

$$\varepsilon_r^+ = \varepsilon_r' - j\varepsilon_r'' \tag{7}$$

$$\operatorname{tg}\delta = \frac{\varepsilon_r''}{\varepsilon_r'} \qquad \varepsilon_r'' = \frac{\sigma}{\varepsilon_0 \omega} \tag{8}$$

where tg δ stands for the loss tangent, σ for the conductivity, ε_0 for the dielectric constant of the vacuum, and ω for the angular frequency of the electromagnetic oscillations.

Equation (6) can be simply interpreted on the basis of the theorem of Pythagoras according to Fig. 6, where

$$\alpha_0^2 = \sqrt{\varepsilon_r'^2 + \varepsilon_r''^2} - \varepsilon_r' \tag{9}$$

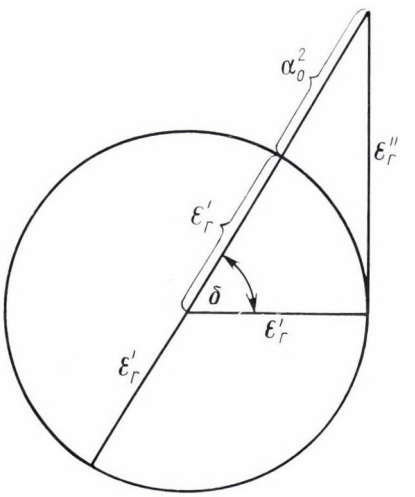

Fig. 6. Interpretation of the attenuation coefficient

On the basis of the drawing, equation (6) can be simplified in the following way: ε_r'' is the tangent drawn to the circle with radius ε_r', which according to the elementary geometry is the geometric mean value between the two segments of the secant drawn from the same point. According to this:

$$\varepsilon_r''^2 = \alpha_0^2 (2\varepsilon_r' + \alpha_0^2)$$

from which

$$\alpha_0 = \frac{\varepsilon_r''}{\sqrt{\varepsilon_r'}} \sqrt{\frac{1}{1 + \sqrt{\operatorname{tg}^2\delta + 1}}}$$

or, according to equations (9) and (6):

$$\alpha = \frac{\pi}{\lambda_0} \frac{\varepsilon_r''}{\sqrt{\varepsilon_r'}} \sqrt{\frac{2}{1 + \sqrt{\mathrm{tg}^2 \delta + 1}}} \tag{10}$$

The expression under the square root in the case of a small tg δ can be taken as a unit, its values are summarized in Table 2. Equation (10) can be written in the following simple form:

$$\alpha = C \frac{\pi}{\lambda_0} \frac{\varepsilon_r''}{\sqrt{\varepsilon_r'}} \tag{11}$$

Table 2

Correction factor values of "C"

tg δ	C
0.2	0.99
0.3	0.99
0.4	0.98
0.5	0.97
0.6	0.96

where the values of the correction coefficient C are summarized in Table 2. In the measurements tg $\delta < 0.5$, therefore C with an error less than 3 per cent can be taken as unit.

The following conditions make the evaluation of the results simple on the basis of equation (11):

1. The value of $\sqrt{\varepsilon'}$ in dilute solutions is practically equal to the value of $\sqrt{\varepsilon'}$ related to clean water (Hasted et al., 1948).

2. The change of ε' of water at 40 °C is negligible between 1.8 Gc/s and 4.0 Gc/s, it is less than 2 per cent (Grant et al., 1957; Hasted, 1961).

In the light of the above two conditions, on the basis of equation (11) the attenuation increase caused by the effect of the KCl content is

$$\Delta\alpha = \frac{\pi}{\lambda_0} \frac{\Delta\varepsilon_r''}{\sqrt{\varepsilon_r'}} \tag{12}$$

From equation (8) the value of $\Delta\varepsilon_r'' = \varepsilon_r'' - \varepsilon_{r\,H_2O}''$ can be expressed by $\Delta\sigma = \sigma - \sigma_{H_2O}$:

$$\Delta\alpha = \frac{1}{2\varepsilon_0 c \sqrt{\varepsilon'}} \Delta\sigma \tag{13}$$

c = speed of light in vacuo

According to equation (13) in the case of constant ε'_r the attenuation depends only on the conductivity of the sample, and therefore it is especially suitable for the study of the microwave conductivity–frequency dependence. For this purpose the attenuation coefficient on the basis of

$$\Lambda = \frac{10^3 \Delta\sigma}{c_{aeq}}$$

is expressed by the equivalent conductivity Λ (c_{aeq} is the concentration in equivalents).

$$\Delta\alpha = \frac{10^{-3}}{2\,\varepsilon_0\, c\,\sqrt{\varepsilon'}}\,\Lambda\, c_{aeq} \tag{14}$$

According to equation (14) the value of Λ can be determined from the angular coefficient of the straight line in Fig. 5. The angular coefficients of the regression straight line corresponding to the data measured on some frequencies and the Λ values calculated from them are shown in Table 3.

Table 3

Λ values determined between 1.8 Gc/s and 4.0 Gc/s

f GHz	tg β	Λ
1.8	0.181	176
2.0	0.174	170
3.0	0.179	174
4.0	0.175	171
		173 ± 3

The value of conductivity corresponding to the KCl content between 1.8 Gc/s and 4.0 Gc/s and at 40 °C, taken as a function of the frequency does not show perceptible changes, according to the data of the Table.

This conclusion is not in contradiction with the Debye–Falkenhagen theory (Falkenhagen, 1953) because according to the theory a conductivity increase of less than 1 per cent may be expected between 1.8 Gc/s and 4.0 Gc/s (the concentration used by us) which is smaller than the error caused by the relaxations used in the calculation. Nevertheless, we were unable to demonstrate a conductivity increase of 20–25 per cent, as it could be expected from the date of Little and Smith (1955). The microwave conductivity of the electrolytes is usually investigated in such a way that a value measured on a single microwave frequency is compared to values obtained from audio-frequency measurements (Little, Smith, 1955; Hasted, Roderick, 1958; Sevcsik, Vettyerj, 1965; Weiss et al., 1965). According to these data Little and Smith demonstrated a conductivity increase of 30 per cent in KCl solution of 0.05 mole at 10 cm, and Hasted and Roderick a conductivity increase

of 10 ± 9 per cent at 51.1 cm. Our investigations emphasize the significance of the comparative measurements in the microwave band. The calculation of Weiss et al. (1965) also supports this. These authors have namely shown that in the determination of the absolute value of the conductivity an error of 5 to 10 per cent can be expected in microwave measurements.

Our data indicate, at a temperature and at a frequency where the relaxation processes in the water do not play an important role yet, no significant change in the conductivity. On the basis of this conclusion one may divide the conductivity of biological substances, similarly to the radio-frequency investigation (Rajewsky, 1938; Cook, 1951; Schwan, 1963) in two parts: one which in the first place is characteristic of the structure and is frequency dependent, and another one which is characteristic of the ion content and is frequency independent.

Acknowledgement

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Effect of Ultrasound on the ^{24}Na-Exchange in Isolated Frog Muscle*

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^{24}Na-exchange in isolated frog muscle was investigated at different temperatures. Activation energy was calculated by the help of penetration time constants obtained from these experiments. The effect of ultrasound treatment decreased the activation energy compared to that of the control. Consequently it is supposed that ultrasound activates the partial metabolic process directing penetration into frog muscle, and influences active Na transport in this way.

The effect of chemicals influencing the partial metabolic process in the experiments also indicates that there is a relation between the delivery of Na and the partial metabolic process responsible for energy supply.

Introduction

In previous experiments the diffusion, and the penetration of Na-ions into frog muscle were studied. The constants characteristic of the moving of Na-ions were determined, and the extent to which these factors were influenced by ultrasound was investigated (Tamás, Rontó, 1961; Tamás, Rontó, 1963). The present paper deals with experiments on the ^{24}Na-exchange in isolated frog muscle performed at different temperatures, with and without ultrasound treatments, and with the effect of chemicals on the active Na transport influencing the partial metabolic processes.

Methods

Sartorius preparations were made of about 100 frogs. Before starting the experiment the muscles were kept for 3 hours in a Ringer solution containing 20 ml 50 μC ^{24}Na. According to the literature (Mc Lennan, 1957; Mc Lennan, 1958; Trosin, 1957; Harris, Steinbach, 1956) the major part of Na will be exchanged in the muscle during this time. Part of the muscles containing ^{24}Na was placed in 20 ml inactive Ringer solution; the incubation was interrupted at different time

* Lecture held at the IVth Biophysical Congress Budapest 1966, and at the IInd International Biophysical Congress Vienna, 1966.

intervals, and the decrease in radioactivity of the muscle was measured with a GM tube. A similar procedure was applied to the other part of the muscles, with the exception that the muscles while kept in inactive Ringer solution were treated with ultrasonic vibration of a frequency of 3000 kHz and an intensity of 2 w/cm^2.

The excitability of the muscles was continuously under control and the data of those muscles which were destroyed in the course of incubation were not evaluated.

The time constant was obtained by solving the differential equation $\frac{dc}{dt} = -kc$ (Harris, Burn, 1949)

$$k = \frac{2.3 \cdot \log \frac{c_1}{c_2}}{t_2 - t_1}$$

where c_1 and c_2 are the concentrations of ^{24}Na in the muscle at times t_1 and t_2, respectively, that is to say, the number of counts equal to them.

The experiments at different temperatures were performed in a constant temperature chamber of the desired temperature. The activation energy was calculated from the following equation (Wartiovaara, Collander, 1960)

$$E_2 = \frac{2.3 \cdot R \cdot T_1 \cdot T_2}{T_2 - T_1} \cdot \log \frac{k_2}{k_1}$$

where k_2 and k_1 are the time constants measured at the absolute temperatures T_2 and T_1, and R is the gas constant.

The following equation was used to calculate the temperature coefficient:

$$\log Q_{10} = \frac{10}{T_2 - T_1} \cdot \log \frac{k_2}{k_1}.$$

Results

The time curve of ^{24}Na release by the muscle was followed both with and without sonication. By plotting the results in a semi-logarithmic coordinate system Fig. 1 was obtained. The specific activity of ^{24}Na per 1 g muscle is plotted against the time in minutes of soaking in inactive Ringer solution. The permeability time constant determined by the help of the curve is:

$$k_K = (1.31 \pm 0.0215)/\text{hour}.$$

The release of ^{24}Na became five times faster, owing to the effect of ultrasound; the calculated time constant is:

$$k_U = (6.5 \pm 0.04)/\text{hour}.$$

The ^{24}Na release by the muscle with and without sonication at different temperatures was investigated next. In Fig. 2 the penetration time constant obtained at different temperatures is plotted against the reciprocal of the absolute temperature in a semi-logarithmic system. The straight line runs higher in the case of sonication, according to the fivefold increase in the Na-flux, but at the same time the angle of inflexion with the abscissa is greater. The activation energy was also calculated. Without sonication the value obtained was 9250 cal/mole, in case of sonication, this value became 6980 cal/mole. It can be seen that the actívation energy decreases as compared to that of the control in the case of subjecting

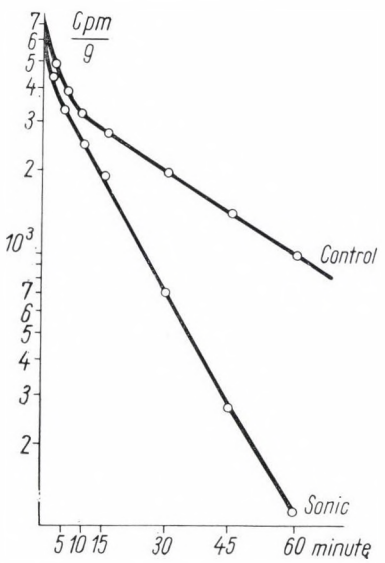

Fig. 1. The time curve of ^{24}Na release with and without sonication

the frog sartorius to ultrasonic vibration. The temperature coefficient was calculated, and so was Q_{10}. In the case of the control $Q_{10} = 1.72$, upon sonication $Q_{10} = 1.51$.

The next step was to study the effect of some chemicals on the partial metabolic processes, which are well known from the literature (Harris, 1960). Strophanthine-K at a concentration of 10^{-3} M decreased the ^{24}Na flux by 39 per cent. There was no change in the case of iodoacetate at a concentration of 10^{-3} M but the effect of dinitrophenol at the same concentration caused a 33 per cent increase in the flux. Finally the effect of potassium was investigated, when the potassium concentration of the outer medium was changed, the changes shown in Fig. 3 were obtained, when the concentration of potassium was lower than 2.5 mM, the time constant decreased, when the potassium concentration was increased, the

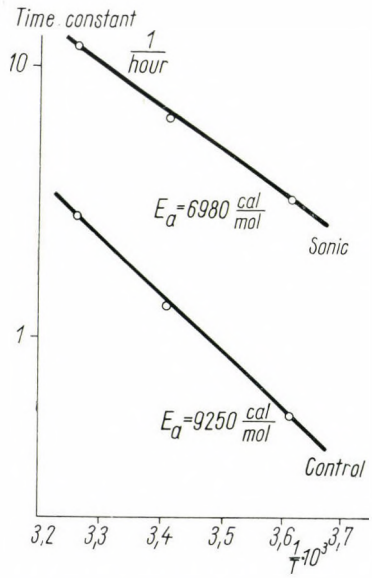

Fig. 2. The time constants obtained at different temperatures plotted against the reciprocal of absolute temperature

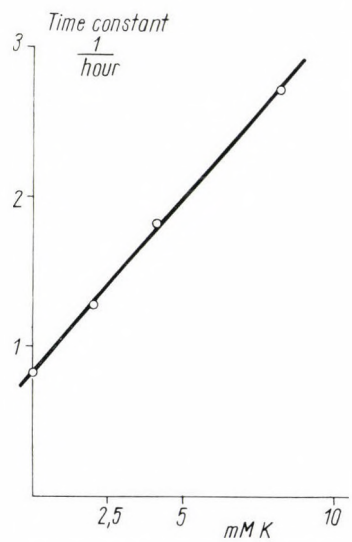

Fig. 3. Correlation between the potassium concentration of the outer medium and the time constants

time constant also increased. The measurements were statistically evaluated. The mean value of the measurements significantly differs from zero, $P < 0.01$.

Discussion

The rather high values of the activation energy and of Q_{10} suggest that a relation exists between the release of Na and the partial metabolic process supplying the energy. Na is being released against a very high concentration gradient, because of the known distribution of the Na-ions. Consequently it cannot be a spontaneous reaction, it must be connected with energy supply. This is proved by the effects of the chemicals used for influencing the partial metabolic processes, too. It is not the aim of this paper to discuss the nature and the mechanism of the system supplying the energy. We only refer to the fact that the ATPase activity of the cell is specifically inhibited by strophanthine-K, is activated by 2,4 dinitrophenol, and is influenced by changing the potassium concentration of the surrounding medium, as it is known from the literature (Harris, 1960). At the same time, iodoacetate effects neither this enzym, nor the release of Na.

As an effect of sonication the release of ^{24}Na shows a fivefold increase. In a previous paper (Tamás, Rontó, 1957) it has been established that ultrasound increases the breakdown of ATP and the development of lactic acid in the muscle. The increased Na-flux definitely must have some correlation with these metabolic processes which are enhanced upon sonication. The finding that in the case of sonication the activation energy decreases compared to that of the control also supports this assumption. The decrease of the activation energy is known to indicate the increase of the reaction velocity. From this the conclusion can be drawn that sonication activates the partial metabolic system directing the penetration into the isolated frog sartorius, and in this way exerts its effect on the active Na transport. According to what has been said above it is presumable that the energy source of ^{24}Na transport is the chemical energy of the intermediates produced in the course of ATP breakdown. The intense activation energy indicates that the penetrating ions come upon some resistance somewhere on their way (Wartiovaara, Collander, 1960). This resistance acts as the threshold of potential and only the ions supplied with a high energy are able to overcome this potential threshold. Those particles which have a kinetic energy below the value of this threshold are retained. It follows that only ions which are in an activated state are able to penetrate. According to the Maxwell–Boltzmann theory of velocity distribution only one out of 10^7 ions is activated under these experimental conditions.

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The Role of Inorganic Ions in the Automaticity of Frog Hearts

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The role of inorganic ions in the automaticity of frog heart was investigated. The hearts were treated with Ringer solution lacking either Ca^{2+} or Na^+ or K^+ until the hearts stopped beating. After this the hearts were stimulated by short d. c. impulses. Electric stimulation did not result in the continual spontaneous activity of the hearts. Only the hearts which were treated with K-less Ringer solution performed a single contraction. But the automaticity was re-established when the solution lacking one ion was exchanged for normal Ringer solution.

Introduction

Heart automaticity is one of the fundamental problems of biophysics and muscle physiology. The role of inorganic ions in excitation and heart automaticity was emphasized by many authors (Ernst, 1963, 1966); thus, it seemed to be of considerable interest to investigate the effects of Ca^{2+}, Na^+ and K^+ ions on the automaticity of frog hearts.

Methods

Hearts of Rana esculenta were prepared and tied to Straub cannules which contained the physiological solution. The hearts prepared on the same day were divided into three groups. The first group was treated during and after the preparation with Ringer solution lacking K (6.8 g of NaCl, 0.2 g of $CaCl_2$ sicc., 0.2 g of $NaHCO_3$ in 1000 ml of bidistilled water). With the two other groups normal Ringer solution (nR) was used during the preparation. Later the treatment of the second group was continued with Ringer solution lacking Ca (6.8 g of NaCl, 0.2 g of KCl, 0.2 g of $NaHCO_3$ in 1000 ml of bidistilled water), and the third group was treated with Na-less Ringer solution (72.3 g of sucrose, 0.2 g of KCl, 0.2 g of $CaCl_2$, 0.2 g of $NaHCO_3$ in 1000 ml of bidistilled water). The cannules were emptied and refilled with a new portion of the same solution about every half hour, altogether 15 to 20 times, until the hearts stopped beating.

These hearts were used for further experiments. To record the heart activity, a capacity transducer was designed and constructed. Details about the capacity

transducer used in our experiments are reported by Mórocz-Juhász and Örkényi (1967). Fig. 1 shows the schematic diagram of the experimental arrangement. One end of the light lever constituted the moving plate of the capacity, to the other end of the lever the hearts were attached. The voltage change caused by the heart activity was directly connected to a Kipp Micrograph BD 2 and continuously recorded. The hearts could be stimulated by short d. c. pulses of different amplitudes as can be seen in the diagram. The hearts having stopped beating, were attached to the lever of the capacity transducer and stimulated by short d. c. impulses. The time duration of the d. c. pulses was about 0.1 sec. The amplitude of the stimulus was varied gradually from 2.5 to 50 V. During the experiment the heart

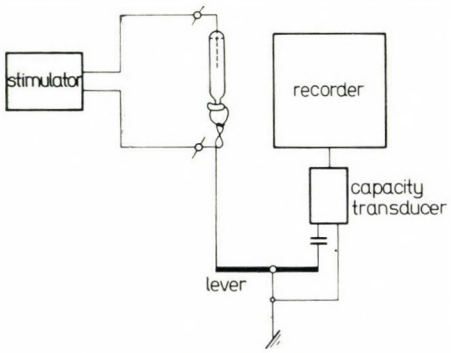

Fig. 1. Schematic diagram of the experimental arrangement

activity and the electric stimuli were continuously recorded on the micrograph with a chart speed of 1800 mm/h.

Results

The hearts treated with Ringer solution lacking Na or Ca showed no activity upon electric stimulus. Figs 2 and 3 show the records of our experiments. When the Na- or Ca-less Ringer solution was exchanged for normal Ringer solution and this exchange was renewed two or three times, the hearts restarted to beat.

In contrast, the electric shock caused a single contraction of the hearts treated with Ringer solution lacking potassium, but no sign of automaticity could be observed. Fig. 4 shows one of our records. After the exchange of the solution for normal Ringer the hearts restarted to beat normally in a few minutes.

Altogether 191 experiments were performed (64 experiments with Na-less Ringer solution, 62 experiments with Ca-less solution and 65 experiments with K-less Ringer solution) with the same result described above. The phenomenon was found to be reversible.

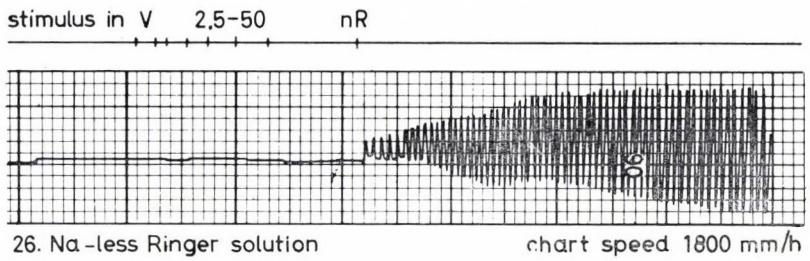

Fig. 2. Record of the activity of a frog heart treated with Na-less Ringer solution. On the upper line the electric stimuli and the exchange of the solution are represented

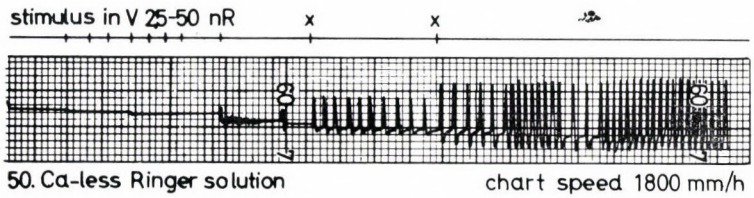

Fig. 3. Record of the activity of a frog heart treated with Ca-less Ringer solution. On the upper line the electric stimuli and the exchange of the solution are represented

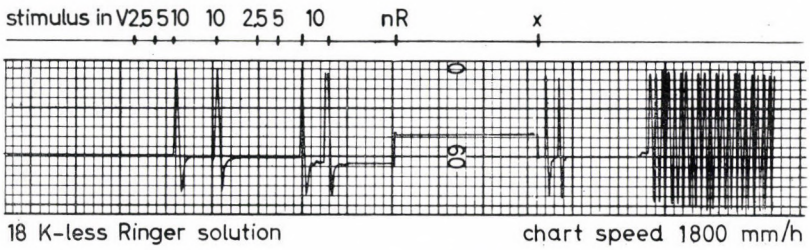

Fig. 4. Record of the activity of a frog heart treated with K-less Ringer solution. On the upper line the electric stimuli and the exchange of solution are represented

Discussion

The experiments described above show unambiguously the role of inorganic ions, Ca^{2+}, Na^{+}, K^{+} in the automaticity of frog hearts. The activity of the hearts stopped after having been treated with Ringer solution lacking one of the cations. Moreover, the hearts lost their irritability as well after they were treated with Ringer solution lacking Ca or Na, since in that case the hearts did not begin to beat even upon electric stimulus with direct current.

The hearts treated with K-less Ringer solution performed a single contraction after the stimulus. This phenomenon could be repeated. It has been pointed out (Ernst, 1966) that the K-content of the hearts treated with K-free Ringer solution is still high enough (they contained 30 to 50 per cent of their original

K-content), and therefore, the treatment with Ringer solution lacking K does not hinder the process resulting in the excitation caused by the electric stimulus and in the contraction.

The stimulation did not injure the frog hearts, because the automaticity was re-established in a short time, when the solution lacking a certain ion was exchanged for normal Ringer solution.

The experiments carried out earlier and published from our Institute (Niedetzky, 1966; Lakatos, Kollár-Mórocz, 1966) have referred to the role of inorganic ions in the process of automaticity. Our results on hearts treated with K-less Ringer solution are in a good agreement with the experiments carried out by Lakatos and Niedetzky. According to their experiments the presence of K-ions can be substituted by gamma-irradiation, or by light irradiation with hearts photostimulated by eosin dye, at least as far as the re-establishment of the automaticity is concerned. In many respects similar results were obtained after illumination, in relation to the contraction of the striated muscle photostimulated by Na-eosin (Van Lin-Fan et al., 1962; Sazonenko, 1965). Taking into account the results of all three experiments, it can be concluded that potassium plays an important role in a charge-transfer process due to stimulus.

In contrast, the experiments carried out on hearts treated with Ca- or Na-less Ringer solution showed no sign of heart's activity neither in our nor in Lakatos' experiments. But it has been pointed out (Niedetzky, 1966) that the hearts which had stopped beating upon treatment with Ca-free Ringer solution restarted to beat after gamma-irradiation. It was reported earlier by many authors (see e. g. Pulatova, et al., 1961; Belágyi et al., 1965) that free radicals are induced during gamma-irradiation, to which free radical reactions are attributed.

Inorganic ions – it may be supposed – are taking part in specific processes, similar to those mentioned above, which are associated with electron migration.

Acknowledgement

The authors are indebted to Professor E. Ernst for calling attention to this problem and for his advices.

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Differentiation between Shortening and Force

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1. A method suitable for the simultaneous study of shortening without force and of force without shortening was developed.

2. The method was employed to investigate the contractions with stretch and without stretch caused by single successive stimulations of the m. gastrocnemius of the Läwen–Trendelenburg frog perfused with hypotonic (1/4 normal) Ringer solution.

3. It was established that these two types of contraction processes differ from each other. By increasing the relative water content of the muscle, stretching and shortening appear as two different functions.

Introduction

The nature of the two types of muscle contractions, shortening and stretching (effort), respectively, has long been a much debated problem in the literature. On the basis of the assumption that two different processes were acting here (Ernst, 1963), an attempt was made to differentiate between shortening and force. Upon perfusion with hypotonic solutions a tetanoid reaction for a single stimulation can be observed (Mányi, 1959). This phenomenon was used for studying the two types of contractions in the experiments. Similar attempts were made earlier in our Institute, but shortening and stretch were investigated with different methods (Belágyi, Biró, 1962, 1963). In the present work a method has been designed which makes the simultaneous registration of both shortening without force and force without shortening possible. Only after solving this problem was it possible to start the actual experiments.

Methods

Läwen–Trendelenburg frog preparation perfused with hypotonic Ringer solution (1/4 normal) was used for the experiments. [The composition of the solution was the following: 3.3 g NaCl, 0.1 g KCl, 0.1 g $CaCl_2$ (anhydrous) + 1000 ml twice distilled H_2O, and 0.1 g $NaHCO_3$ + 1000 ml twice distilled H_2O.]

The right side m. gastrocnemius of the frog was stimulated directly by single stimulations and the contractions accompanying both shortening and stretching

* Our dear and excellent colleague died on the 9th of June, 1966.

were transformed into capacity changes with the help of a mechano-electric transducer, and recorded by a cathode-oscillograph.

The experimental equipment was arranged in the following way (Fig. 1): A hypodermic needle (2) moves in a glass tube 40 mm long and 1.6 mm in internal diameter (1). A rubber band 1 mm in diameter (3) was pulled through the hypo-

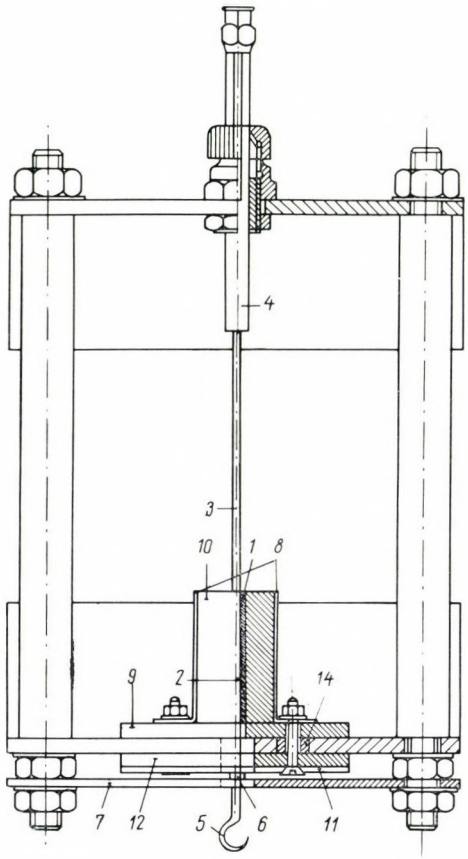

Fig. 1. The experimental equipment applied for the investigation of force and shortening. For explanation see text

dermic needle, and through a brass tube 80 mm long and 6 mm in outer diameter (4). It was attached to the end of the tube by the socket of the hypodermic needle. The brass tube was split 1 mm wide for two-thirds of its length. A steel hook (5) was spot-welded by a welding rod to the end of the hypodermic needle (2) (this hook was inserted into the Achilles tendon of the m. gastrocnemius of the Läwen–Trendelenburg frog preparation), and an iron slip (6) was welded on

it 15 mm from its fore-end, which exactly fitted into the center bore of the steel sheet (7). By turning the hook, the iron slip pressed against the steel sheet and prevented any further longitudinal movement. When the slip of iron adjusted itself into the bore, the rubber band represented 50 pond stretching force at a length of 160 mm (preliminary calibration), and this ensured the resting length of the m. gastrocnemius used in the experiment. Two condensers had to be applied for the measurements of the capacity changes. 1. The moving electrode of a displacement detector was represented by the hypodermic needle (2), the other armatures were two brass sheets bent rectangularly (8), which were stuck to the side of the trolitul prism (10). This trolitul prism contained the glass tube (1) and was attached to a trolitul sheet (9). 2. The condenser sheet of the dynamometer was a brass sheet (11) and this was fitted to a trolitul sheet (12) exactly equal to

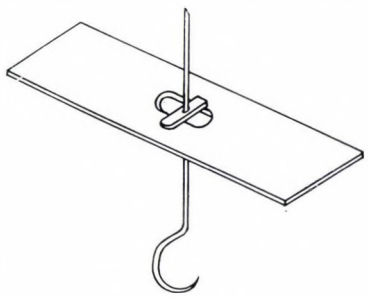

Fig. 2. The position of the iron slip when recording force

trolitul sheet (9), and the two sheets were screwed together by two brass countersunk screws. The spring sheet (7) made of stainless steel was the other sheet of the condenser used for the measurement of force.

In the investigation of the shortening without force ("isotonic contraction") during muscle contraction, the hypodermic needle passes unhindered through the steel sheet (7) and the capacity caused by (2) and (8) will change. When expenditure of force without shortening (isometric twitch) was studied, the hook inserted into the Achilles tendon was turned to such an extent that the iron slip (6) by pressing against the sheet (7) prevented the movement of the needle (Fig. 2). In this case the spring sheet (7) stretches, and the capacity change appears on the condenser of the dynamometer.

The operation principle of the apparatus is the following: The transistor marked by T generates high-frequency oscillations at the frequency determinated by L_2 and $C_3 + C_x$. The circuit L_3C_5 is tuned to the same frequency (Fig. 3). As the muscle shortens or exerts a force the capacitance of the capacitor C_x decreases, the frequency of the circuit $(C_3 + C_x)L_2$ is shifted, and therefore a d.c. voltage change arises on the output of the discriminator circuit, which can be recorded on the oscilloscope after adequate amplification. In our measurements the frequency of the circuit $(C_3 + C_x)L_2$ in the resting state was equal to 980 kc/s,

the detuning was 1.8 kc/s at 1 cm displacement, or 2 kc/s at a force development of 1 kp. The d.c. voltage changes were on the output −0.125 V, or 0.14 V.

The input circuit of the oscilloscope is illustrated in Fig. 4. The voltage change from the displacement transducer, which has a negative sign and a relatively slow time course, and the voltage pulses from the stimulator, which are of a

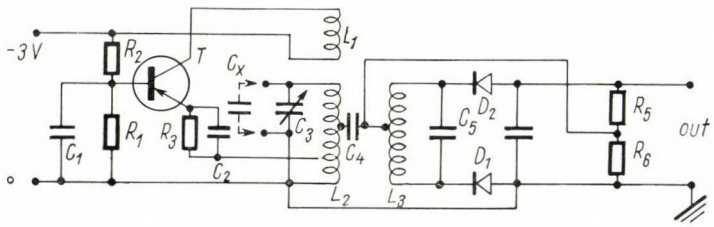

Fig. 3. Connection diagram of the mechanoelectric transducer

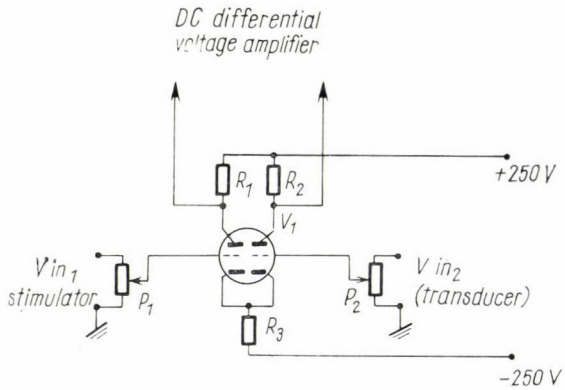

Fig. 4. Connection diagram of the input of the oscillograph

positive sign and of rapid time course, were connected to the grids of the first stage of the differential amplifier. In such a way the stimulus and the contraction appear on the same beam without the disturbance of one instrument by the other. To control the time durations an a.c. voltage of 50 c/s was connected to the input of the second channel of the oscilloscope. Stimulation was done by single stimulations, and by square impulse of a frequency of 100 c/s and a duration of 0.5 msec, respectively. The time of delay of the stimulus was 25, 100 and 250 msec. The potential of the stimulus varied between 1 and 10 V. In one part of the experiment the stimulation was done by constant voltage within the given value, in the other part, increasing stimulation voltage was used. The change appearing on the screen of the oscilloscope caused by the effect of stimulation was registered by a camera.

Experimental results

Before starting the perfusion with hypotonic solution, two photographs were taken to record the contraction connected with shortening without force, and that associated with force without shortening. They can be seen in Fig. 5 and

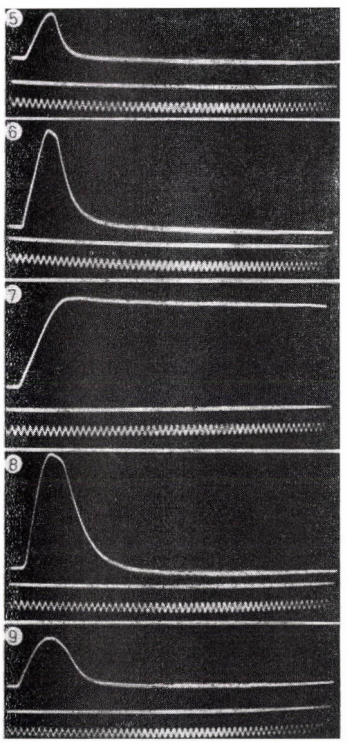

Fig. 5. The shortening of the m. gastrocnemius of the frog upon single stimulus. Upper line: contraction curve. Lower line: indication of time (district a.c. current of a frequency of 50 c/s). The dot preceding the shortening curve represents the stimulus

Fig. 6. Force without shortening

Fig. 7. Shortening ("tetanoid twitch") induced by single stimulus during perfusion with hypotonic solution (1/4 n Ringer)

Fig. 8. Force induced by single stimulus during perfusion with hypotonic solution (1/4 n Ringer)

Fig. 9. Shortening induced by the second stimulus applied after the first tetanoid shortening

Fig. 6. There is a marked difference between the two. The amplitude of the isometric contraction is greater, the ascending slope of the curve is steeper, and the attainment of the acme occurs sooner than in the case of isotopic twitch. These data indicate the process of the tension to proceed faster. After taking these two photographs, perfusion with hypotonic 1/4 n Ringer solution was started, and

the muscle was stimulated at given intervals, and both the contraction without tension and the contraction with force exertion were investigated.

A change in the time course of the shortening without force was experienced 2 to 15 minutes after the beginning of the perfusion, and tetanoid twitch (Fig. 7) was induced by the applied single stimulus. (This phenomenon is already known from earlier literary data.) The contraction with tension did not change, or only slightly changed in those pictures which were recorded immediately before or after contraction. Fig. 8 shows the picture of the "isometric" contraction. The ascending slope of the tension curve is steeper here than that of the curve representing shortening without force. These tetanoid reactions to single stimulations were observed for 10 to 12 minutes after their first appearance in most of the experiments. (In some cases it was observable for 20 to 24 minutes.) If the muscle was stimulated again immediately after a tetanoid contraction without tension, the tetanoid reaction was not so convincing (Fig. 9) and very often did not appear at all.

Discussion

There are several theories concerning the initiation of muscle contraction. One of the most recent theories attributes a very important role to the change in the water content of the muscle. The relative water content increases in the muscle when it is perfused with hypotonic solution and this leads to a decreased ion concentration. This phenomenon may be responsible for the fact that instead of the usual contraction curve, exhibiting a sine function of time, a tetanoid twitch appears to the single stimulus. This is not a real tetanus as proved by the fact that almost normal contractions appear to the second or to the third stimulus applied immediately after the first one, and that the unreal tetanus collapses. By increasing the number of stimuli the capability of this "great contraction" disappears in the muscle; more tetani cannot be produced 10 to 12 minutes after the appearance of the first one. These phenomena do not exist in the case of force without shortening, the time course of the contraction cannot be influenced by perfusion with hypotonic solution. Consequently by increasing the relative water content of the muscle, which is accompanied by a decrease in the ion concentration, experimental conditions can be produced, which make it possible to differentiate the two types of contraction. The difference between the time courses indicates that such process takes place in the force exertion of the muscle and that this process differs from the basic process of shortening.

Acknowledgement

Authors express their thanks to Professor E. Ernst for encouragement and help in this work.

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On the Rôle of Thermoosmosis in Root Pressure

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The concentration of the bleeding sap of sunflower plants was found to be five or six times lower than that of the sap of the root cells. The quantity and the concentration of the bleeding sap varied as a function of time, the temperature and the concentration of the solution in which the roots were kept. The possibility of the operation of a thermoosmotic mechanism is raised to explain the production of root pressure.

Introduction

The basically unsolved problem of root pressure is practically as old as plant physiology. Many investigators have been looking for its explanation. Since 1937 (Ernst, 1937) several papers from this Institute (e.g. Vető, 1963; Ernst, 1967) pointed to thermoosmosis as a possible mechanism by which a small temperature difference between two solutions of the same concentration is able to produce a hypotonic solution from the warmer side to the colder one across a semipermeable membrane against a significant pressure. In order to test the possible role of such a mechanism, first some basic experiments were carried out to reveal the regularities of bleeding of sap excretion. The following questions were examined: 1. Is there any relation between the concentrations of the bleeding sap and of the sap of the root cells? 2. Do the concentration and the quantity of the bleeding sap vary as a function of time, the temperature, and the concentration of the solution in which the roots were kept?

Methods

3—7 weeks old sunflower seedlings grown either on moist filter paper, or in soil in little flower pots, were used. The bleeding sap was collected as usual with a Pasteur pipette from the cut surfaces of the roots of plants, which were kept at the desired temperature (refrigerator, thermostat) in a vapour saturated atmosphere (Szalai, Frenyó, 1962). The volume of the fluid was measured in small graded glass tubes, and the refractive index and dry matter content, respectively, were deter-

mined by the Abbe refractometer. Although this procedure does not give exact informations about the osmolarity, it does permit, however, to get rough estimates about the concentration. (Methods for measuring vapour pressure of fluids of small volume are in preparation.) The concentration of the sap pressed out of the roots was measured in a similar way.

The effect of the external medium, i.e. the solution in which the roots were kept, was determined as follows: The water on the roots and on the filter paper was replaced by a solution of the desired concentration one and a half hour before the measurement. This solution was replaced with a fresh one again immediately before the cutting, to make sure that the effect was due to the solution and not to the water previously present.

When the role of the temperature was to be investigated, the plants were placed at the desired temperature (3, 13, 23, or 33 °C) one and a half hour before their "decapitation", and the temperature of the soil was measured. (The above temperatures might have fluctuated by 1 or 2 degrees.)

The approximate empiric formula of the functions was established by plotting the data of the measurements in a coordinate system. The dots were connected by a curve which graphically represented the function in question. The curve was "straightened" by applying an adequate transformation, if it was necessary. The constants of the formula were determined by calculating the regression coefficient (and its deviation), and on the basis of the diagrams obtained.

Results

1. The dry matter content of the bleeding sap of the 3 – 7 weeks old sunflower seedlings was 0.4 ± 0.1 per cent according to the data of 36 measurements. For each measurement about 15 plants were used.

The dry matter content of the sap pressed out of the roots of similar plants was 2.2 ± 0.3 per cent according to the data of 10 measurements. For each measurement about 10 plants were used.

2. The *concentration* change of the sap; *a)* The concentration of the bleeding sap decreased as a function of time. The measurements were made up to 4 hours after cutting. The correlation coefficient was found to be -0.69 ± 0.14. The probability of this value being derived from a series of pairs of values showing no relation is $P < 0.01$. Consequently, the sap, excreted in the first hour after cutting is more concentrated (about 0.5 per cent) than that excreted later, e.g. in the 4th hour (about 0.2 per cent). *b)* On the basis of measurements made in temperature range of 3 to 33 °C it can be established that the concentration of the sap decreases with increasing temperature. The correlation coefficient is -0.60 ± 0.13, and $P < 0.01$. Thus, while the concentration of the sap excreted at 3 °C is about 0.6 per cent, the concentration of the sap excreted at 30 °C is only about 0.3 per cent. *c)* If the concentration of the solution surrounding the root increases (beginning with distil-

led water and raising the concentration to a 0.5 per cent sucrose solution) that of the sap excreted also increases (from about 0.2 per cent to 0.9 per cent). The correlation coefficient in this case is 0.85 ± 0.09 and $P < 0.01$.

Changes in the *quantity* of the sap: *a)* The quantity of the bleeding sap is directly proportional to the time, if the medium is soil, water, or a 0.25 per cent sucrose solution (measurements were carried out for 4 hours). Thus, the quantity of the bleeding sap (μl/plant) under these circumstances generally is $v = bt$; where b is the regression coefficient, and t is the time in hours. The value of b will be $b_0 = 4.41 \pm 1.13$ μl/plant × hour, in the case of distilled water. Using 0.25 per cent sucrose solution as a medium, this value becomes $b_{0.25} = 2.97 \pm 0.59$ μl/plant × hour. The amount of sap excreted per unit time gradually decreased (Fig. 1) and after a certain time it dropped to zero, when the roots were kept in a

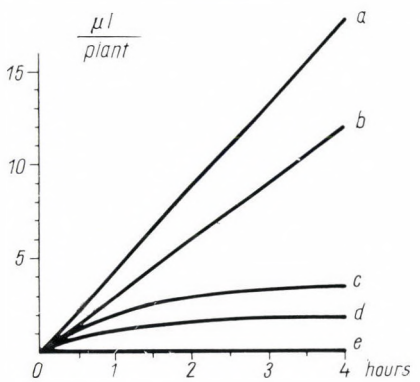

Fig. 1. Changes in the quantity of the bleeding sap plotted as a function of time. *a)* When the roots were kept in water, the regression coefficient was $b_0 = 4.41 \pm 1.13$ μl/plant × hour. *b)* When the roots were kept in a 0.25 per cent sucrose solution the regression coefficient was $b_{0.25} = 2.97 \pm 0.59$. *c)* When the roots were kept in a 0.5 per cent sucrose solution the approximate relation was $v = \frac{t}{bt + 0.28}$, where v is the bled sap per plant, t the time in hours and $b = 0.21 \pm 0.09$. *d)* When the roots were kept in a 1.0 per cent sucrose solution the relation was $v = \frac{t}{bt + 0.43}$, similar to the former one. Here the value of b was $b = 0.41 \pm 0.13$. *e)* When the roots were kept in a 2.0 per cent sucrose solution there was no bleeding at all.

more concentrated solution (0.5 to 1.0 per cent). This can be expressed by the formula $y = \frac{x}{bx + a}$. Thus in the case of a 0.5 per cent solution the formula is: $v = \frac{t}{0.21 \cdot t + 0.28}$ and for a 1.0 per cent solution it is: $v = \frac{t}{0.41 \cdot t + 0.43}$. When solutions of higher concentrations (2 to 4 per cent sucrose solutions) were used, there was no bleeding at all. *b)* The quantity of sap excreted per unit time depends on the temperature of the environment, too. For example Fig. 2 shows an experi-

mental result, where the quantities of the sap excreted by the plants at 3, 23, and 33 °C are plotted against time. The regression coefficients of the straight lines are the following:

$$b_3 = 0.76 \pm 0.14 \ \mu l/\text{plant} \times \text{hour}$$
$$b_{23} = 3.80 \pm 0.33 \ \mu l/\text{plant} \times \text{hour}$$
$$b_{33} = 7.40 \pm 0.63 \ \mu l/\text{plant} \times \text{hour}$$

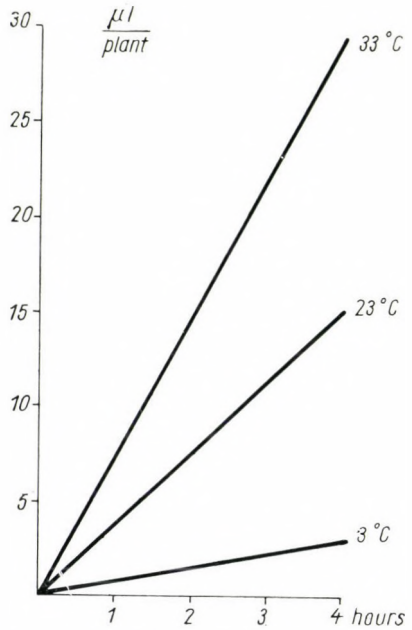

Fig. 2. The quantity of the sap excreted by the plants at different temperatures plotted as a function of time

Another straight line can be obtained if the logarithms of these coefficients are plotted against temperature, and the direction tangent of this is: $b' = 0.332 \pm 0.067$. This is actually the logarithm of the van't Hoff coefficient (Q_{10}) which will be $Q_{10} = 2.15$, and denotes how many times the quantity of the bleeding sap increases with a temperature change of 10 °C. The mean value of the van't Hoff coefficients, determined in the above mentioned way, was in all measurements: $Q_{10} = 2.4 \pm 0.3$. (The quantity of the bleeding sap can be expressed as

$v = 0.65 \cdot 2.4^x \cdot t$, where $x = \frac{\tau - 3}{10}$, τ is the temperature in °C, and t is the time in hours.)

It must be mentioned that a relative slowing down of the process was experienced in a few cases when measurements were performed around 33 °C. Most probably the optimum temperature was exceeded in these cases. *c)* By plotting the concentration of the solution, surrounding the roots, against the quantity of the sap excreted per unit time Fig. 3 is obtained. This can be approached by the following equation: $v = \frac{6.45}{x + 1} - 2.29$; where x is the concentration of the solution in per cent and the numerator is 6.45 ± 0.93.

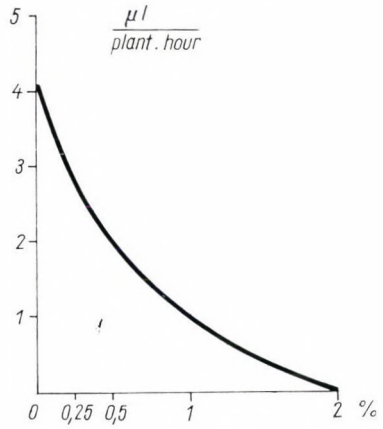

Fig. 3. The concentration of the solution surrounding the roots plotted against the quantity of the sap bled per unit time. $v = \frac{b}{x + 1} - 2.29$, where x is the concentration of the solution in per cent and $b = 6.45 \pm 0.93$

Discussion

The most important facts revealed by this experiment were that the concentration of the bleeding sap is much lower than that of the cell sap, and that the larger the quantity of the bleeding sap, the lower is its concentration. These data are in favour of a thermoosmotic interpretation of root pressure. The well-known schematic equation for the suction force of the cells (osmotic equivalent or diffusion pressure deficit) is: $S = P + A - T$; where S is the suction force, P the osmotic pressure, A the "active pressure of the plasm", and T the turgescence (hydrostatic pressure) in atmosphere. By replacing the general and undefined idea of "active pressure of the plasm" by the thermoosmotic pressure caused by ΔT (thermomolecular pressure effect; Prigogine, 1961), the formula will be more definite, and becomes suitable for experimental work. (See Appendix.) Consequently the investigation (e.g. by micro-couples) of that crucial spot (probably the endodermis) where the presumable ΔT can be demonstrated is very important. It seems possible that with the help of the LASER technology, developed in

the latest decades, artificial intracellular ΔT can be brought about, and the effect of this selective heating on water mobilization can be investigated.

If the activation energy (E) of the process, or according to Spanner (1954) the parallel heat of transfer (Q^*) are calculated from the measured $Q_{10} = 2.4$, the equation $E \approx Q^* \approx \frac{\ln (1.034 \cdot Q_{10}) \cdot RT^2}{10} = 15530 \pm 1940$ cal/mole at 20 °C is obtained. According to the theory of irreversible thermodynamics (De Groot, 1952; Gyarmati, 1960) significant differences in concentration and hydrostatic pressure, respectively, can be caused even by small ΔT values. This temperature difference is approximately of about 0.01 °C in order of magnitude, which may easily be supposed to occur in biological objects too.

Appendix

For an ideal semipermeable membrane a working hypotesis expressed by the following equation can be used:

$$\Delta P = \frac{1}{v}\left(RT \ln \frac{c_2}{c_1} + H \frac{\Delta T}{T}\right) \tag{1}$$

where hydrostatic pressure difference ΔP (expressed in atmosphere) keeps equilibrium with the sum of the osmotic pressure $\Delta \pi = \frac{1}{v} RT \ln \frac{c_2}{c_1}$ and the thermoosmotic pressure $\Delta \tau = \frac{H \Delta T}{vT}$. $R = 82\,\text{cm}^3\,\text{atm/degree} \cdot \text{mole}$, $v = 18$ ml/mole, $c_2 = \frac{n_0}{n_0 + n_2}$ is the mole fraction of water in a less concentrated solution and $c_1 = \frac{n_0}{n_0 + n_1}$ is the mole fraction of water in a more concentrated solution, ΔT is the small temperature difference which is positive if the less concentrated (c_2) solution is warmer and negative if the more concentrated one (c_1) is warmer; T is the average temperature, and H is the molecular heat of water vaporization (about 10000 cal/mole $\approx 4.13 \cdot 10^5\,\text{cm}^3\,\text{atm/mole}$). If there is no equilibrium, i.e. $\Delta \pi + \Delta \tau - \Delta P \neq \neq 0$, the so-called "*suction force*" S arises and thus $S = \Delta \pi + \Delta \tau - \Delta P$, or

$$S = \frac{RT}{v} \ln \frac{c_2}{c_1} + \frac{H \cdot \Delta T}{vT} - \Delta P \tag{2}$$

Equation (1) can be derived by combining the formula well known for osmotic pressure in physical chemistry with the Clausius–Clapeyron equation. The initial conditions which are valid for the starting formula also, limit the applicability of equations (1) and (2). The system is taken as a quasi isotherm one because of the small ΔT. The application of equation (1) and equation (2) offers a simple possibility for rough calculations. The theory of irreversible thermodynamics (De Groot, 1952; Gyarmati, 1960) gives an explicit but more complicated description of this problem.

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Amino Acid Sequence of One Pair of Analogous Peptides of D-glyceraldehyde-3-phosphate Dehydrogenase from Beef Muscle and Pig Muscle

Short Communication

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Molecular genetics has raised some clearly defined problems which can be studied by comparing the structure of functionally identical proteins from different species. A reconstruction of the past evolution of the structure of individual enzymes can be achieved through such studies. Moreover, by determining the invariant regions within the complete amino acid sequence of an enzyme, useful information can be obtained on the relationship between enzyme structure and enzyme function.

In the case of D-glyceraldehyde-3-phosphate dehydrogenase (GAPD) the sequence of amino acids in the immediate vicinity of the reactive cysteinyl residue is known (Harris et al. 1963). Perham and Harris (1963) have shown that in the enzymes isolated from rabbit muscle, pig muscle and yeast cells, respectively the reactive cysteinyl residue is found in an identical peptide consisting of 18 amino acid residues. The analogous peptides of the enzyme from halibut and lobster and also of the human enzyme were slightly different (Allison, Harris, 1965).

Our studies were concerned with differences in the amino acid sequence of GAPD from pig, beef and Rhesus muscle. Such differences are apparent in such parts of the sequence which lay outside the known vicinity of the reactive SH groups. Pig and beef enzymes were prepared by the method of Előd i and Szörényi (1956), the enzyme from Rhesus muscle was obtained by Szörényi's method (Szörényi, 1960). The enzyme preparations were four times recrystallized and then acetone powders were prepared. The dry powder was oxidized with performic acid. 200 mg samples of the oxidized protein were dissolved in 0.1 M ammonium bicarbonate, pH 7.8, and hydrolyzed for 40 hours with two times 1/30 weight of trypsin. Hydrolysis was stopped by boiling and this was followed by freeze-drying of the samples. No precipitate formed on boiling. The dry hydrolysate was dissolved in 3 ml 0.05 M $(NH_4)OH$ and filtered through a column, 1×150 cm, of Sephadex G-25 fine pore gel.

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Fig. 1 shows that upon elution with 0.005 M $(NH_4)OH$ and recording the absorbance at 280 mμ four peaks were obtained. No appreciable difference was found in the elution profile of the three enzymes. The fractions were pooled. No tubes were discarded when pooling, in order to avoid the eventual loss of any component of the hydrolysate. The pooled fractions were freeze-dryed and fingerprints were prepared from each.

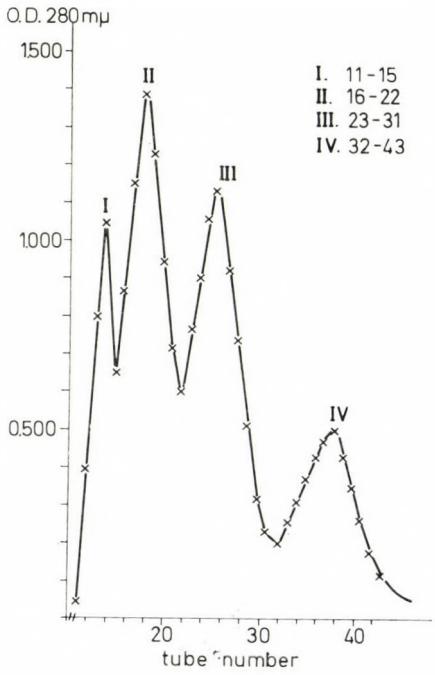


Fig. 1. Fractionation by Sephadex filtration of the tryptic hydrolysate of pig muscle GAPD. 200 mg dry hydrolysate was dissolved in 3 ml 0.05 N $(NH_4)OH$ and this was loaded onto a column, 1 × 150 cm, of Sephadex G-25, fine pore. Elution was carried out with 0.005 N $(NH_4)OH$ at a flow-rate of about 30 ml/h. The volume of the fractions was 3 ml. Identical diagrams were obtained with the tryptic hydrolysates of beef muscle and Rhesus muscle GAPD

Fractions I, II and IV of the beef and pig enzymes gave fully identical fingerprints, while there was some difference in fraction III. Fraction III of the Rhesus muscle enzyme gave a fingerprint which was markedly different (Fig. 2).

It is seen from the fingerprints (Fig. 2) that the hydrolysate of pig muscle GAPD contains two peptides (P 1 and P 4), one strongly basic and one neutral, which are absent from the hydrolysate of the beef enzyme. Peptide P 1 was not always clearly shown on the fingerprints, it was usually found as a more or less blurred spot. It turn, two slightly basic peptides are seen in the hydrolysate of beef muscle GAPD (B 5 and B 6) which are absent from the pig muscle enzyme.

Peptides P 4 and B 6 were isolated by fingerprinting on a preparative scale. Aliquots containing 5 mg dry matter were spotted. Following separation by electrophoresis and chromatography, the spots were developed on the paper by spraying with a 0.005 per cent ninhydrin solution. Homogeneity of the eluted peptides was checked by high voltage electrophoresis at pH 1.9.

The amino acid composition of the peptides was determined with a Grossman–Hannig type amino acid analyzer (Bender and Hobein), following 16-hour hydrolysis of the dried peptide with 6 N HCl. Peptide P 4 proved to be a hexapeptide composed of His(1), Thr(1), Gly(1), Val(1), Phe(1) and Lys(1), while the composition of the hexapeptide B 6 was found to be Asn(1), Thr(1), Gly(1), Val(1), Phe(1) and Lys(1). The presence of Asn instead of Asp in peptide B 6 is indicated by the electrophoretic mobility of the peptide in pH 5 buffer, since the net charge of the peptide is slightly basic at this pH, whereas it should be neutral in case it contained Asp.

The amino acid sequence of the peptides was determined by stepwise degradation with phenyl isothiocyanate followed by quantitative amino acid analysis. Edman's procedure as modified by Konigsberg and Hill (1962) was applied. The results shown in Table I were obtained by determining the amino acid composition of the residual peptides resulting from the stepwise removal of the N-terminal residues.

Table I

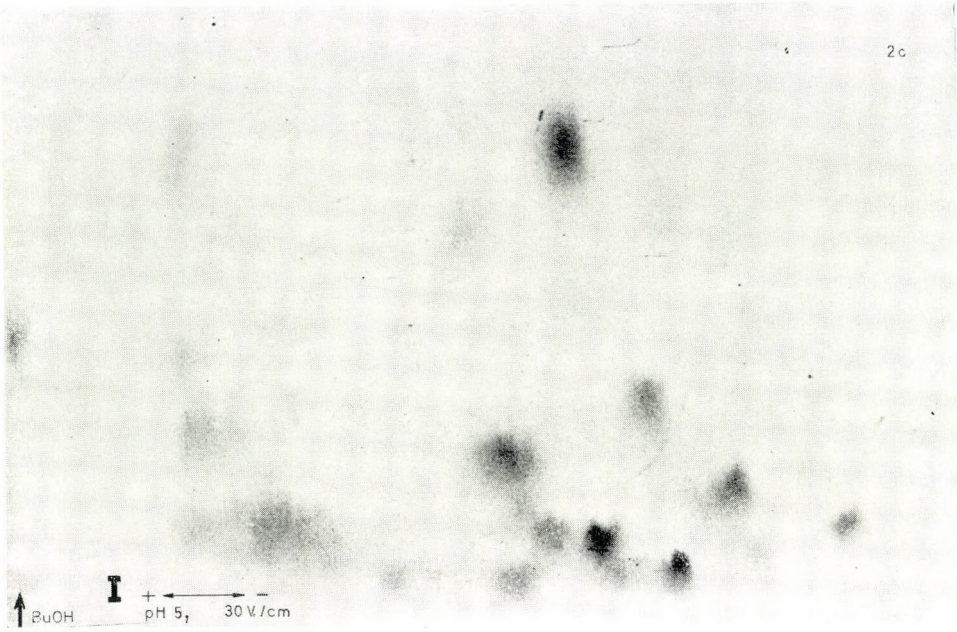
The N-terminal sequence of peptides B 6 and P 4

Edman's procedure as modified by Konigsberg and Hill (1963) was used for the stepwise degradation of peptides. Aliquots of the residual peptides were hydrolyzed with 6 N HCl and the amino acid composition of the hydrolysates was determined with the automatic analyzer

Peptide	B 6				P 4			
Number of degradations	0	1	2	3	0	1	2	3
Lys	1.2	1.1	1.0	0.9	1.2	1.1	0.9	1.0
His	—	—	—	—	0.8	0.9	0.9	1.0
Asn	0.9	1.1	1.1	0.9	—	—	—	—
Thr	1.0	1.0	0.9	*0.3*	1.0	1.1	1.1	*0.1*
Gly	1.0	1.1	1.2	1.0	1.1	0.9	1.2	1.3
Val	1.1	0.9	*0.2*	0.3	1.0	1.1	*0.3*	0.2
Phe	0.9	*0.3*	0.1	0.2	0.7	*0.2*	0.2	0.1

Sequence Phe. Val. Thr./Gly, Asn/Lys Phe. Val. Thr./Gly, His/Lys

Since the complete amino acid sequence could not be determined by Edman's procedure, the partial acid hydrolysate (conc. HCl, 72 h, 37 °C) of peptides P 4 and B 6 was also studied. Evaporation to dryness was followed by double-buffered electrophoresis according to Dévényi (1963), and then by paper chromatography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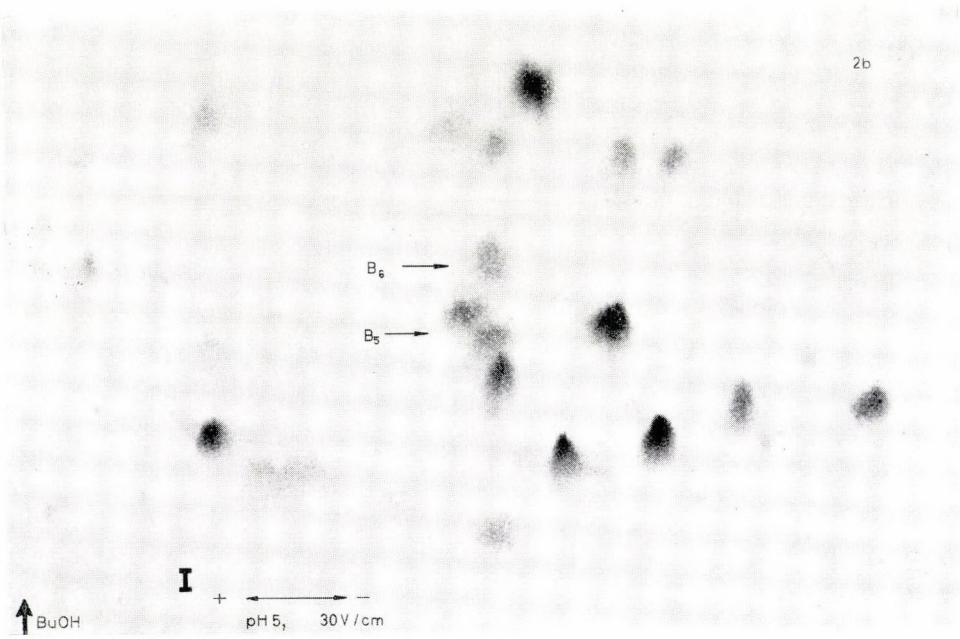
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B5
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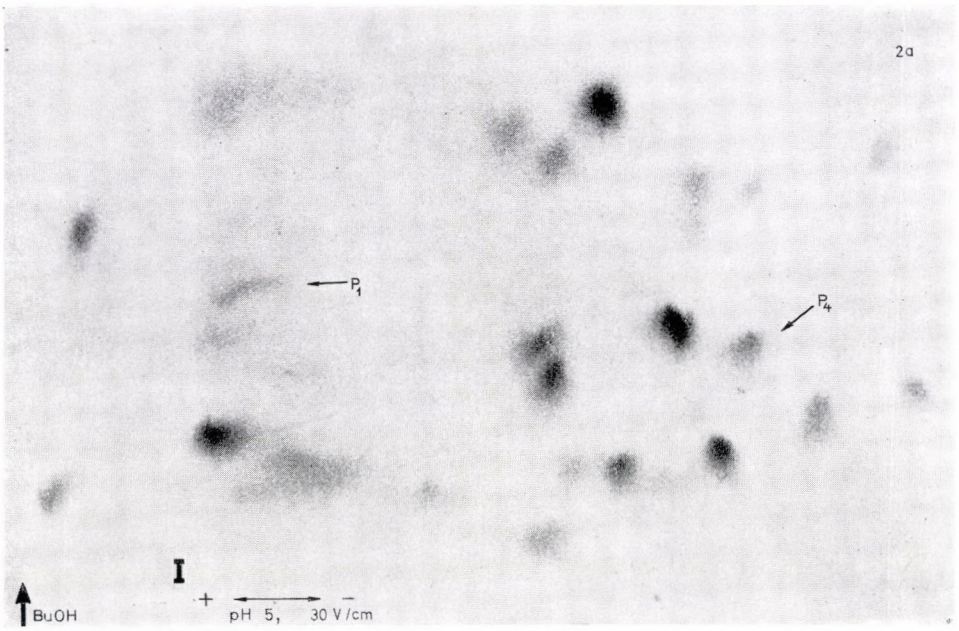

Fig. 2. Fingerprint of fraction III of the tryptic hydrolysate of GAPD isolated from beef, pig and Rhesus muscle. Electrophoresis was carried out in pyridine acetic acid buffer (30 V/cm, 2 hours) pH 5, and descending chromatograms were run with butanol: acetic acid: water = = 120 : 30 : 50. a) Pig muscle GAPD; b) Beef muscle GAPD; c) Rhesus muscle GAPD;

with the amylalcohol : pyridine : water = 35 : 35 : 30 solvent of Wittmann and Braunitzer (1959). The chromatograms were developed with a 0.005 per cent ninhydrin solution, the spots were eluted and their amino acid composition was determined. The following peptides were isolated: Val(1), Thr(1), Gly(1); Gly(1), His(1); and His(1), Lys(1) from the hydrolysate of peptide P 4, and Thr(1), Gly(1); and Thr(1), Gly(1), Asn(1) from the hydrolysate of peptide B 6.

In conclusion, the following amino acid sequences were established:

P 4 Phe. Val. Thr. Gly. *His* Lys
B 6 Phe. Val. Thr. Gly. *Asn.* Lys

We did not attempt to determine the composition of the second pair of peptides (P 1 and B 6, respectively) which were different in the pig and beef enzymes, because considerable difficulties were encountered in their isolation.

Nierenberg et al. (1965) have shown that the triplets coding for Asn are GAU and GAC, while CAU and CAC code for His. Thus the observed Asn/His substitution corresponds to a G/C substitution in the code triplet.

Our results indicate that there is only a slight difference between the primary structure of GAPD isolated from beef muscle, and pig muscle, respectively. Apparently, the primary structure of the Rhesus muscle enzyme is so much different that a determination of the specific sites or sequences in the Rhesus enzyme would require the establishment of a greater part of the complete sequence.

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The Hydrodynamic Properties and the Molecular Weight of Aldolase and Aldolase-T

Short Communication

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(Received December 8, 1966)

It has been shown by Biszku and Szabolcsi (1964) that digestion of enzymically active aldolase containing 10 mercaptide bonds with small amounts of trypsin results in the formation of a high molecular weight product which exhibits about 50 per cent of the original enzymic activity. This product has been called aldolase-T. Aldolase-T was found to be a homogeneous protein by ion exchange chromatography (Biszku, Szabolcsi, 1964) and by acrylamide gel electrophoresis (Biszku et al. 1967).

In the present work we compare the hydrodynamic properties of native aldolase and aldolase-T. Three times recrystallized aldolase prepared according to Taylor et al. (1948) was used. The preparations were characterized by a E_{250}/E_{280} ratio of 1 : 2.8. E^{1cm}_{280} divided by 0.74 corresponded to 1 mg of aldolase per ml solution (Biszku, Szabolcsi, 1964). The specific activity of the preparations as measured by the dinitrophenylhydrazine test (Swenson, Boyer, 1957) was between 80 and 100. Aldolase-T was prepared as described previously (Biszku, Szabolcsi, 1964). All samples were dialysed against several changes of 0.05 M phosphate buffer pH 7.5, and their homogeneity was controlled with acrylamide gel electrophoresis. The final dialysis media were used as reference solvents for subsequent dilutions and measurements.

The partial specific volume was determined from 10 parallel measurements by the picnometric method at 20 °C. The values obtained were 0.740 ± 0.001 and 0.738 ± 0.001 for native aldolase and aldolase-T, respectively.

Sedimentation velocities were measured in a MOM Model G-120 analytical ultracentrifuge in synthetic boundary cells using schlieren optics. The rotor speed was 40,000 to 60,000 r. p. m. with a stability of ± 20 r. p. m. The temperature stability was better than ± 0.05 °C. Measurements were made at several concentrations of protein and the values obtained were corrected for the viscosity and density of water at 20 °C. Both native aldolase and aldolase-T sedimented as single peaks. The values of $s^{\circ}_{20,w}$ obtained by extrapolation to zero protein concentration were found to be 8.0 ± 0.02 and 6.8 ± 0.02 for native aldolase and aldolase-T, respectively.

Diffusion coefficients were measured in a Zeiss diffusion-meter equipped with interference optics. All experiments were carried out at $20\,°C \pm 0.01$ °C. The values

of $D^{\circ}_{20,w}$ were obtained by extrapolation to zero protein concentration and were found to be $4.47 \pm 0.02 \times 10^{-7}$ and $4.69 \pm 0.02 \times 10^{-7}$ cm^2/sec for native aldolase and aldolase-T, respectively.

The molecular weight calculated from the sedimentation and diffusion coefficients, using the Svedberg equation was 158 000 $\pm$ 3000 for native aldolase and 127 000 $\pm$ 3000 for aldolase-T.

Viscosity measurements were carried out at 20 °C in an Ostwald viscosimeter (outflow time 100 sec). From the experimental data intrinsic viscosity was calculated. The values obtained were 0.038 $\pm$ 0.003 dl/g and 0.036 $\pm$ 0.005 dl/g for native aldolase and aldolase-T, respectively. The molecular weights were also calculated from the intrinsic viscosity and sedimentation constant according to the equation described by Scheraga and Mandelkern (1953) as follows:

$$M = \frac{N^{3/2} (s^{\circ}_{20,w})^{3/2} [\eta]^{1/2}}{\beta^{3/2} (1 - \bar{V}\varrho)^{3/2}}$$

where β has the value of 2.16×10^6 for spheres and compact ellipsoidal molecules of moderate axial ration. We used this value since the data on intrinsic viscosity (cf. Table I) preclude elongated rod or random coil conformations (Tanford, 1961). The molecular weight obtained in this way was 157 000 $\pm$ 4000 for native aldolase and 122 000 $\pm$ 4000 for aldolase-T.

Table I

Sample	$s^{\circ}_{20,w}$ Svedberg	$D^{\circ}_{20,w} \times 10^7$ cm^2/sec	$[\eta]$ dl/g	$\bar{V}$ ml/g	$M_{\overline{S,[\eta]}} \times 10^{-3}$	$M_{\overline{S,D}} \times 10^{-3}$	$M_w \times 10^{-3}$
Native aldolase	8.00 $\pm$ 0.01	4.47 $\pm$ 0.02	0.038 $\pm$ 0.003	0.740 $\pm$ 0.001	157 $\pm$ 4	158 $\pm$ 3	162 $\pm$ 8*
							159 $\pm$ 3**
Aldolase-T	6.80 $\pm$ 0.02	4.69 $\pm$ 0.02	0.036 $\pm$ 0.005	0.738 $\pm$ 0.02	122 $\pm$ 4	127 $\pm$ 3	129 $\pm$ 8*
							125 $\pm$ 3**

* obtained by Archibald's method
** determined in sedimentation equilibrium

Archibald's method: measurements were carried out with 0.1 – 0.4 per cent protein solutions at low speed in transient equilibrium states approaching sedimentation equilibrium. A speed of 4000 to 10 000 r. p. m. was found to be suitable. The temperature was 20 °C. The concentration gradient function was determined in a normal cell by means of the schlieren optical system. The initial concentration was measured in synthetic boundary cell. Photographs were taken 80 minutes after

reaching the final speed. In order to assure the proper formation of the lower meniscus CCl_4 was layered under the solution at a height of a few millimeters.

Calculations were based on the procedure of Klainer and Kegeles (1956).

$$M_w = \frac{M_i^2 c_i}{M_i c_i} = \frac{RT\,\partial c/\partial x}{(1 - \bar{V}\varrho)\,\omega^2 xc}$$

where $\bar{V}$ is the partial specific volume, ϱ the density of the solvent, ω the angular velocity, $\partial c/\partial x$ the concentration gradient at the upper or lower meniscus, x the distance from the axis of rotation. The c concentrations at the upper and lower meniscus were determined from the initial concentration c_0, and the concentration gradient:

$$c = c_0 \pm \frac{1}{x^2} \int_a^b x^2 \frac{\partial c}{\partial x}\, dx$$

Homogeneity of the proteins could be established by the measurements made in transient equilibrium states. The molecular weights calculated from the values obtained for the upper and lower meniscus, respectively, were equal and did not change during the course of the determinations. The molecular weights were found to be $162\,000 \pm 8000$ and $129\,000 \pm 8000$ for native aldolase and aldolase-T, respectively.

Sedimentation equilibrium experiments were carried out as proposed by Van Holde and Baldwin (1958), using schlieren and Rayleigh interference optics, simple double-sector cells with 0.1–0.5 per cent protein solutions and a column height of 1 to 2 millimeters. Equilibrium was attained in 6 hours. The initial concentration was measured in a synthetic boundary cell.

The weight and z average molecular weights were calculated from the equilibrium patterns. The data suggest that the protein samples were homogeneous since the plots of log c vs. x^2 were linear. Thus the molecular weights calculated are the unique molecular weights: $159\,000 \pm 3000$ and $125\,000 \pm 3000$ for native aldolase and aldolase-T, respectively. Fig. 1 shows a typical sedimentation equilibrium plot.

To sum up, native aldolase was found to have a molecular weight of $159\,000 \pm 3000$. This result falls within the range of values reported in previous studies (Taylor, Lowry, 1956; Stellwagen, Schachman, 1962; Deal et al. 1963; Tanford, 1966) when the appropriate corrections are made (Kawahara, Tanford, 1966).

In Table I we compared the hydrodynamic parameters and the molecular weight of native aldolase and aldolase-T. The results indicate that aldolase-T is a homogeneous protein. The intrinsic viscosity, sedimentation velocity and diffusion coefficient show that the decrease in molecular weight is not accompanied by the loosening of the molecule.

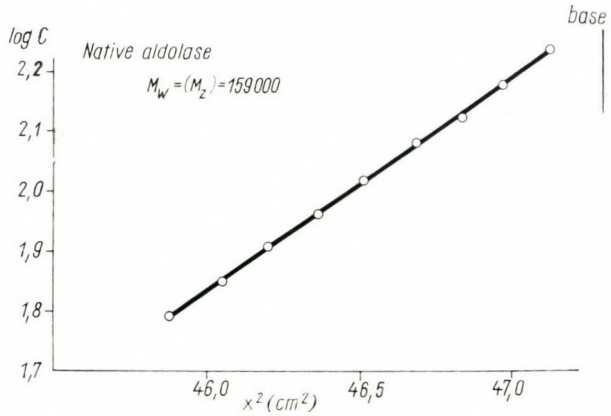

Fig. 1. Molecular weight determination of native aldolase at low speed in a sedimentation equilibrium experiment. The ordinate gives the logarithm of the protein concentration and the abscissa represents the square of the distance (cm^2) from the axis of rotation. Initial concentration of native aldolase was 0.2 per cent in 0.05 M phosphate buffer, pH 7.5. The rotor speed was 6000 r. p. m. and the length of sedimentation experiment was 8 hours at a temperature of 20 °C. Column height 1.2 mm

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Ultrastructure of the Ergastoplasm of Pancreas Acinar Cells as Revealed by Topooptical Staining Reactions

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(Received January 28, 1967)

The ergastoplasm of pancreas acinar cells was found to show intensive anisotropic staining reaction with toluidine blue and rivanol if post-staining stabilization with ferricyanide was applied. Analysis of the birefringence induced by the anisotropic staining reaction indicated a regular alignment of the dye molecules on the ultrastructural framework of the EP, oriented perpendicularly with their light absorbing bonds to the membranes of the EP. The high values of dichroic ratio of toluidine blue stained EP were indicative of a high degree of orientation of the dye molecules and also of a high ultrastructural order in the EP. Analysis of the optical findings suggested that the RNA of EP was membrane-oriented and was in a close structural relation with the membrane lipids.

The ultrastructural implications of the optical findings are discussed in their relation to recent electron microscopic findings on the ultrastructural organization of the EP and to the functional significance of the ribosome-membrane association.

Introduction

The few polarization optical studies of the submicroscopic structure of the ergastoplasm (EP) which have been published so far were made on unstained pancreatic acinar cells and on plasma cells (Kautz et al., 1957; Munger, 1958; Nolte, 1947; Olivecrona, Hillarp, 1949; Ries, 1940; Sjöstrand, 1953). They showed that the EP had a weak intrinsic birefringence: negative with respect to the length of its striation. This indicated the presence of a lipoprotein membrane structure in the EP. These investigations, however, provided no conclusive evidence of the ultrastructural organization of the RNA in the EP, which in the electron micrographs of chemically fixed cells appeared as a particulate RNP component arranged in rows along the EP membranes (Ekholm, Edlund, 1959; Palade, 1956; Weiss, 1953).

It was because of the weak anisotropy of the unstained EP that polarization optical studies could not contribute much to the question of the ultrastructural organization of the EP.

This paper is a report on our studies based on polarization optical analysis of the anisotropic staining reactions of the EP of the pancreatic acinar cells. The aim of the experiments was to shed some more light on the ultrastructural organi-

zation of the EP. These studies were made possible by the finding that staining with some cationic dyes (such as toluidine blue and rivanol), if followed by a post-staining treatment with ferricyanide, produced a strong anisotropic staining reaction of the EP, due to an oriented binding of the dye molecules to the ultrastructural framework of the EP.

Analysis of this topooptical staining reaction under various conditions and after elimination of the RNA and /or the lipid component from the EP supported the conclusion that the RNA of the EP was membrane oriented as well as membrane bound. This suggested a close microstructural relation between the ribosomal RNA and the membrane element of the EP. This seems to be in good agreement with recent electron microscopic findings obtained on ultrathin sections of frozen-dried material (Sjöstrand, Baker, 1958; Hanzon et al. 1959; Hanzon, Hermodsson, 1960; Sjöstrand, Elfvin, 1964) or with electron microscopic findings on negatively stained emulsions of microsomal material (Sabatini et al., 1966) and also with more recent biochemical data supporting the view that in the process of protein synthesis a close functional relation exists between membrane material and ribosomes and that a labile membrane-ribosome structural unit may be considered of basic importance in the initial stage of protein synthesis of the cell (Hendler, 1965; Székely, 1965; Henshaw et al., 1963).

Material and Methods

Pieces of rat, dog and cat pancreas were freshly fixed in 10% formalin and in Bouin fixative for 24 hours, and embedded in gelatine. Unstained and stained frozen sections 8 – 10 μ thick were used for the light and polarization microscopical studies.

Staining procedures

The slices were stained according to a procedure described in a previous paper (Romhányi, 1963), using a post-staining stabilization by treatment with ferricyanide solution (2%). This treatment was originally introduced on the assumption that ferricyanide used as a precipitant in the quantitative determination of amino-acridine dyes (Albert, 1951) might be able to stabilize the dye molecules in the pattern as they had been bound to the cytological structures. This proved to be indeed the case and therefore this type of anisotropic staining reaction was a useful means for the study of biological ultrastructures by polarization microscopy (Romhányi, 1963, 1967).

In the present experiments the following staining methods were used: 1.staining with rivanol (0.1%) for 10 minutes. The dye solution was blotted off the slice and, after short rinsing with water, ferricyanide solution (2%) was dropped on the slice. Thereafter the slice was mounted in gum arabic containing ferricyanide (0.2%). The gum arabic layer was allowed to dry. In such preparations the anisotropic

staining effect of the different structural elements proved to remain unchanged for more than 10 years. 2. Staining with pH 4.5 toluidine blue (0.1%), buffered with sodium-veronal-acetate, to which increasing amounts of calcium chloride or sodium chloride were added to give ionic strengths from 0.1 to 1.5. Apart from omission of the rinsing with water, staining and post-staining treatment were similar to those in rivanol staining.

For polarization optical analysis a Leitz Ortholux polarization microscope equipped with rotating compensators of 18 and 57 mμ and a Berek compensator was used. Microddensitometry measurements were made with a Zeiss Schnellphotometer. The examinations in monochromatic light were carried out with a Leitz monochromator and a Zeiss interference filter.

Results

The data in Table 1 showing the optical characteristics of the EP of unstained pancreas acinar cells indicate the presence of a weak intrinsic negative birefringence with respect to the striation of the EP. After lipid extraction only a weak positive form — birefringence is seen in water, which may be attributed to the protein component of the EP membranes and to the RNP of the EP.

Table 1

Optical characteristics of unstained EP of formol-fixed pancreas acinar cells

	In water	In gum arabic
Control	∅	6—8 mμ (—)
Lipid-extracted+	6—8 mμ (+)	∅

∅: isotropic.
+ Treatment of the slices: with alcohol or methanolchloroform from 1 to 24 hours

Anisotropic staining reactions of EP

We have found that the birefringence of the EP is greatly increased by staining with toluidine blue or rivanol, but only if post-staining treatment with ferricyanide is applied. This treatment stabilizes the dye molecules in the pattern in which they have been bound to the ultrastructure of the EP, and in addition, greatly increases the birefringence of the EP by forming in situ an anisotropic complex with the dye molecules bound to the structure.

As shown in Fig. 1, Fig. 8/a and Table 2, the increase in birefringence of the EP by this type of staining reaction is very considerable. The retardation of the unstained EP is raised from 6–8 mμ to 60–65 mμ with rivanol, and to 30–35 mμ with toluidine blue staining.

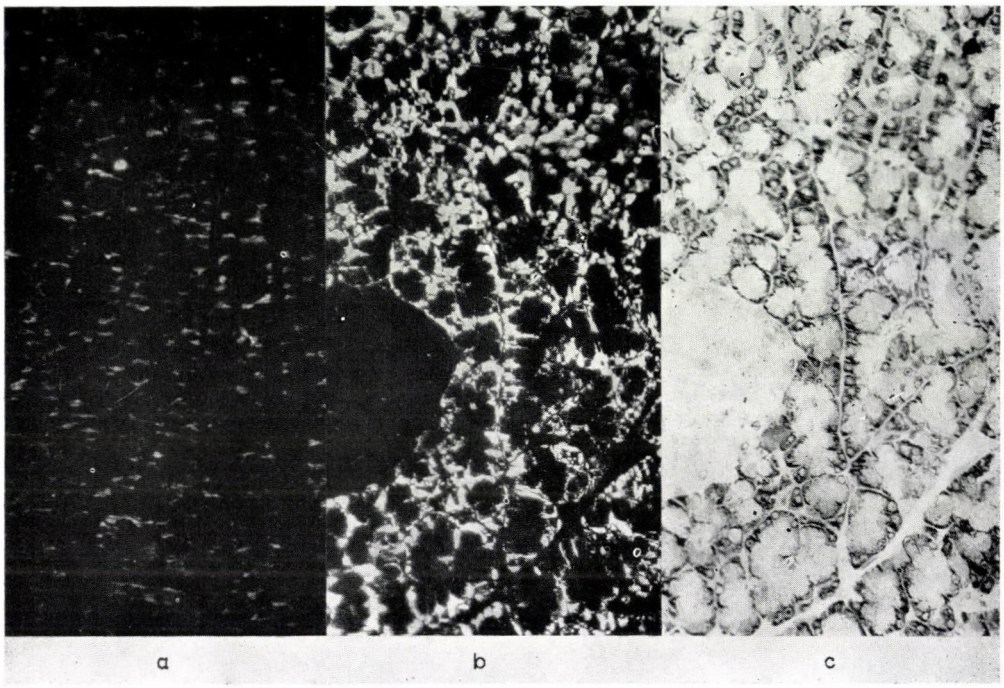

Fig. 1. Frozen section of rat pancreas in gum arabic. *a)* Unstained section at crossed polaroids, showing the EP of the acinar cells with weak intrinsic birefringence. *b)* Section stained with toluidine-blue (at 1.0 ionic strength of sodium chloride) at crossed polaroids. The strongly increased birefringence of EP due to the anisotropic staining reaction is apparent. The isotropic cell nuclei are clearly delineated from the birefringent EP. On the left the isotropic field of a Langerhans islet is seen. *c)* The same as in *b)* in bright field microscope taken with light of λ 540 mμ. In many cells the striations of the basophil EP on both sides of the cell nuclei are clearly seen. × 250

Optical characteristics of the topooptical staining reaction of EP

Fig. 2 shows the dispersion of birefringence of the toluidine blue and rivanol stained EPs as a function of wave-length. The toluidine blue-stained EP exhibits anomalous dispersion with an inversion in the region of λ 540 mμ; the birefringence being negative for the long wave-side and positive for the short wave-side of the spectrum (with respect to the length of the striation of the EP). The curve

intersects the line of isotropy in the spectral region of the absorption band of the metachromatic dye complex of toluidine blue (λ 530 – 540 mμ).

Toluidine blue-stained EP was found strongly dichroic, negative with respect to its striation, i.e. with a maximum absorption for light polarized perpendicularly to the striation and being maximum for wave-lengths in the spectral region of λ 540 mμ (Fig. 3). In cells with EP, well oriented with respect to the plane of the section, considerable values of dichroic ratio (E⊥/E ||) of EP up to 4 – 5 were found in densitometric measurements of photographic films. Such values are not far from those ($\approx$ 8) of mechanically oriented toluidine blue films (Land,West 1946), indicating a high degree of orientation of the dye molecules on the structure of the EP.

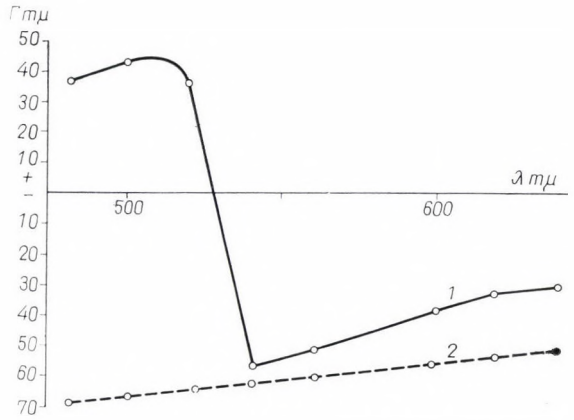

Fig. 2. Dispersion curves of birefringence of the toluidine-blue (1) and rivanol (2) stained EPs. 1. Shows inversion in the region of λ 530 mμ corresponding to the metachromatic absorption band of toluidine-blue, 2. rivanol-stained EP is negatively birefringent for all wave-lengths of the visible spectrum

Rivanol-stained EP is negatively birefringent for all wave-lengths of the visible spectrum. No inversion of the curve of birefringence occurs; theoretically, this should occur in the spectral region of the absorption maximum of rivanol (λ 350 mμ), however, in our studies in which optic instruments of glass were used it could not be traced. For a similar reason (lack of absorbancy in the visible light) the rivanol-stained EP did not show dichroism in the visible spectrum.

Thus the optical characteristics of the toluidine blue and rivanol stained EPs are indicative of an association on the EP of dye molecules with their light absorbing or retarding bonds oriented perpendicularly to the membranes of the EP, i. e., parallel to the lipid molecules in the membrane.

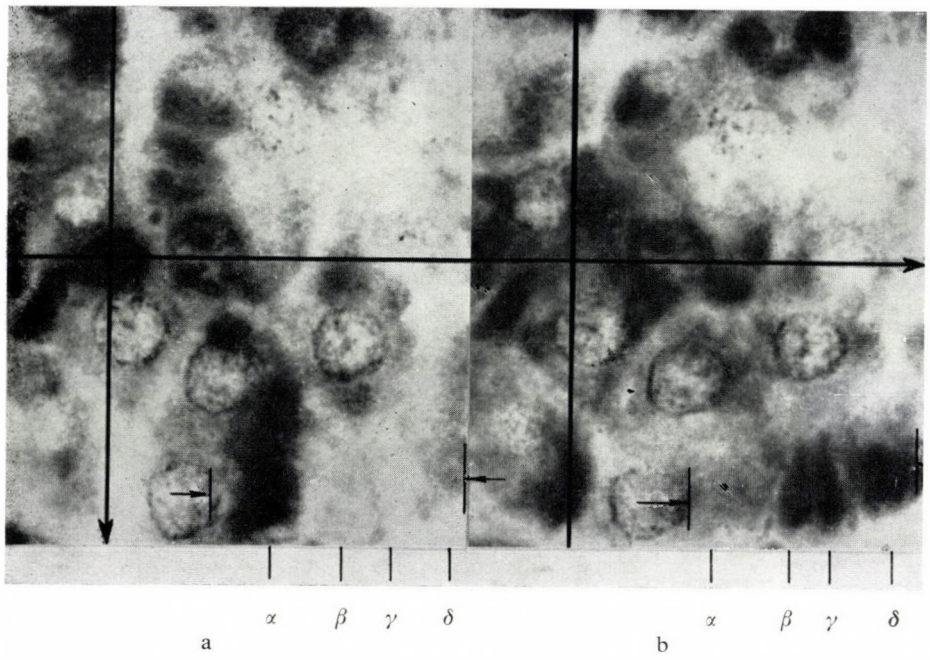

Fig. 3. Dichroism of toluidine-blue stained EP. *a)* and *b)* show the same field taken with light of λ 540 mμ and polarized in *a)* vertically, in *b)* horizontally ($\times$: 1500). The strong dichroism of the EP in well oriented cells is apparent. EP shows maximum absorption for light polarized perpendicularly to its striation (negative dichroism). *c)* Densitometric tracings of the two photographic negatives of *a)* and *b)* between the zones marked by arrows. The values of the dichroic ratios ($E_{\perp}/E\,||$) of the lettered EP striations are: α: 1.6, β: 4.3, γ: 4.8, δ: 3.7

Effect of the ionic strength of the medium on the topooptical staining reaction of EP

While the anisotropic staining reaction of the EP with rivanol was not dependent on the ionic strength of the dye solution a definite dependence was observed with toluidine blue. Fig. 4 shows that an optimium anisotropic staining reaction with toluidine blue could be obtained at an ionic strength of 1.0 – 1.3. At lower ionic strengths the anisotropic staining reaction gradually decreased, in spite of the increase in dye-binding, which is shown by the extinction curve of the EP as measured by cytophotometry at λ 540 mμ.

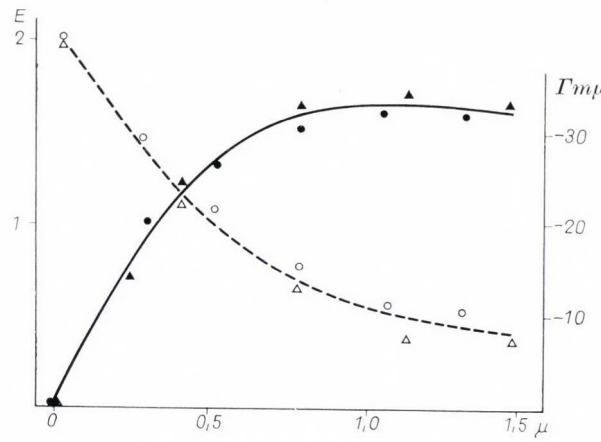

Fig. 4. Dependence of anisotropic staining of EP with toluidine blue on the ionic strength of the dye solution: ———————— retardation of EP stained with toluidine blue at different ionic strengths of NaCl (●) and $CaCl_2$ (▲); - - - - - - - - Extinction of EP after staining with toluidine blue at different ionic strengths of NaCl (○) and $CaCl_2$ (△). The increase in birefringence and decrease in extinction of EP with increasing ionic strengths of the dye solution are apparent, maximum birefringence being obtained at about μ: 1.0

Thus at low ionic strengths an isotropic (unoriented) overstaining of the EP occurs, therefore at crossed polaroids the EP appears quite isotropic and in red colours, whereas after staining with toluidine blue at optimum ionic strength it appears in green birefringence-colour and with increased birefringence and marked dichroism indicating a maximum degree of dye molecule-orientation on the framework of the EP. From the curves in Fig. 4 it can be seen that more than half of the available negatively charged groups on the EP must be neutralized by cation adsorption in order to provide a possibility for the toluidine blue molecules to be bound in a highly oriented pattern on the EP structure.

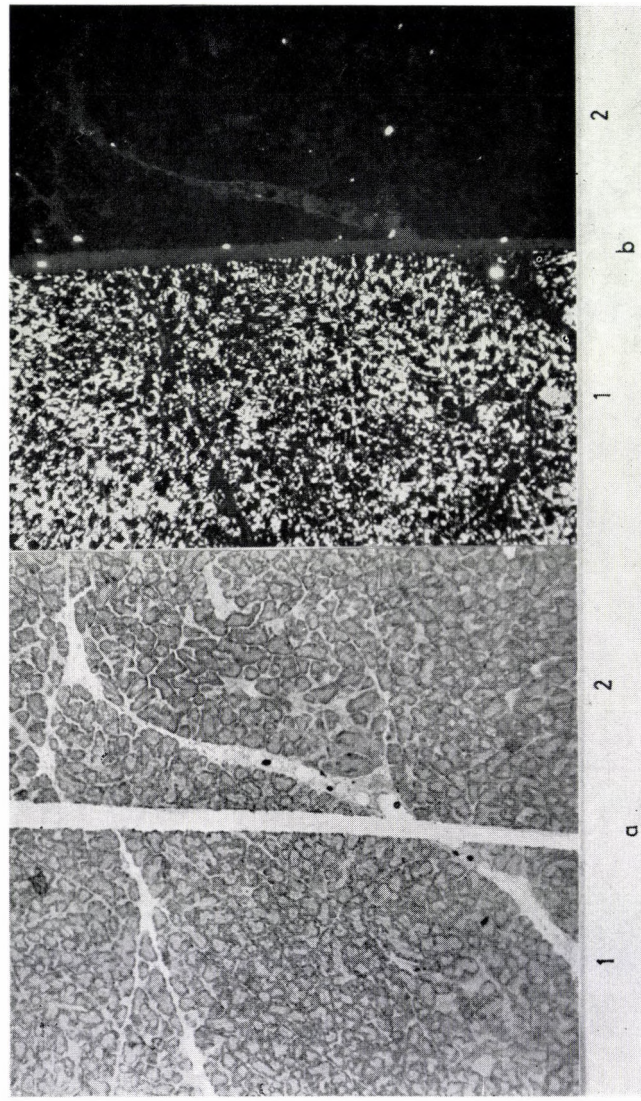

Fig. 5. Two halves of the same frozen section of rat pancreas, from one of which (2) the structural lipids have been extracted before staining with toluidine blue; (1) is the control half. *a)* In the bright field microscope, *b)* the same at crossed polaroids. It can be seen that the lipid extracted half of the section stains with toluidine blue with the same intensity as the control however, at crossed polaroids its staining is completely isotropic, indicating an unoriented binding of the dye molecules on the RNA of the lipid extracted EP. ×: 100

Effect of elimination of RNA and/or the structural lipids on the topooptical staining reaction of EP

The data in Table 2 show the effect of the elimination of RNA, and/or the structural lipids, on the anisotropic staining reaction of the EP with rivanol and toluidine blue. A striking finding was the disappearance of the anisotropic staining reaction of the EP with toluidine blue following the elimination of the struc-

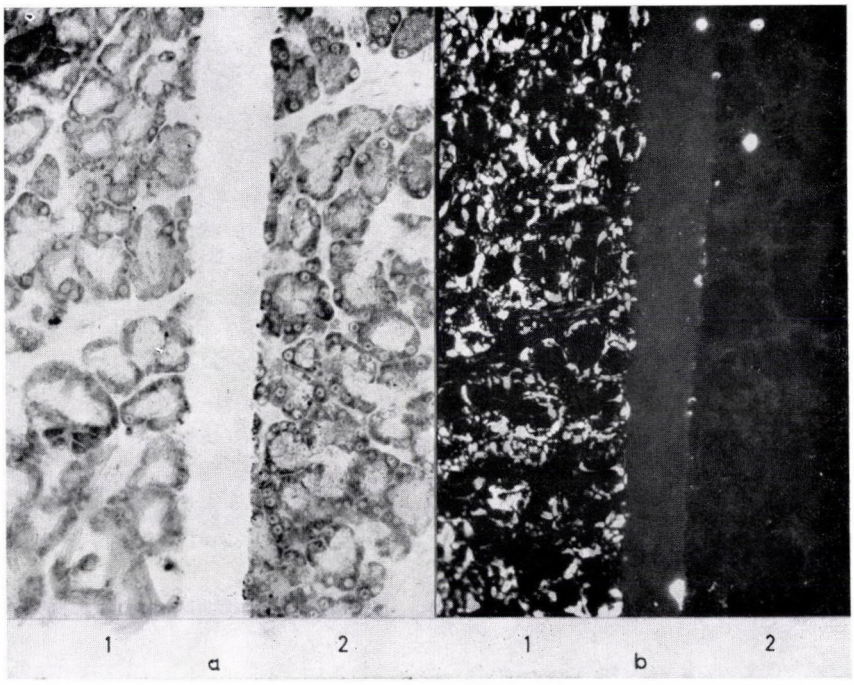

Fig. 6. Similar as in Fig. 5 at higher magnification showing more clearly the cellular details and the complete loss of the anisotropic staining ability of the RNA in the lipid-extracted EP. × : 250

tural lipids. In Figs 5, 6 and 7 it can be seen that RNA in lipid-extracted EP was unable to bind the toluidine blue molecules in an oriented pattern, but no quantitative change occurred in its dye-binding capacity. In contrast, lipid extraction did not abolish the anisotropic staining reaction of the EP with rivanol (Fig. 8), and only a relatively small decrease in the retardation of the EP was observed (Table 2).

Lipid extraction followed by RNAse treatment completely abolished the anisotropic staining reaction of the EP both with rivanol and toluidine blue (Table 2). All these findings indicate that RNA itself has an oriented polarized

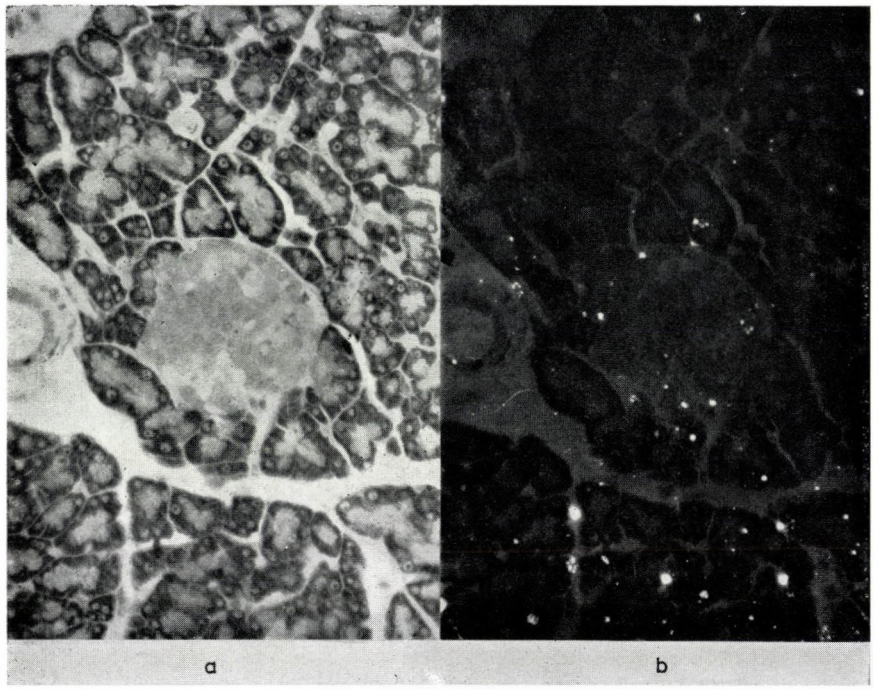

Fig. 7. Frozen section of rat pancreas, stained with toluidine blue (at 1.0 ionic strength of sodium chloride). The slice was treated with crude phospholipase for 18 hrs (Rezek, Sir, 1954). An intensive basophil staining of the EP is seen in *a)*, however, the staining is isotropic as shown at crossed polaroids *b)*, indicating an unoriented binding of the dye molecules on RNA. × : 250

Table 2

Optical characteristics of EP of pancreas acinar cells stained with toluidine blue and rivanol

	Control	Lipid-extracted+	RNAse++	Lipidextr. and RNAse
Toluidine-blue pH 4.5 ferricyanide	30—34 mμ (—)	∅	14 mμ (—)	∅
Rivanol-ferricyanide	60—65 mμ (—)	45 mμ (—)	20 mμ (—)	∅

+ Treatment of the slices 1) with absolute alcohol, or methanol chloroform for 24 hours; 2) with 1 per cent sodium deoxycholate for 18 hours; 3) with crude phospholipase (Rezek, Sir, 1954) for 18 hours.

++ RNAse (Reanal, Budapest) 2 mg/ml, 4 hours; decrease of the extinction of toluidine blue-stained EP (at low ionic strength) from 1.7 to 0.40.

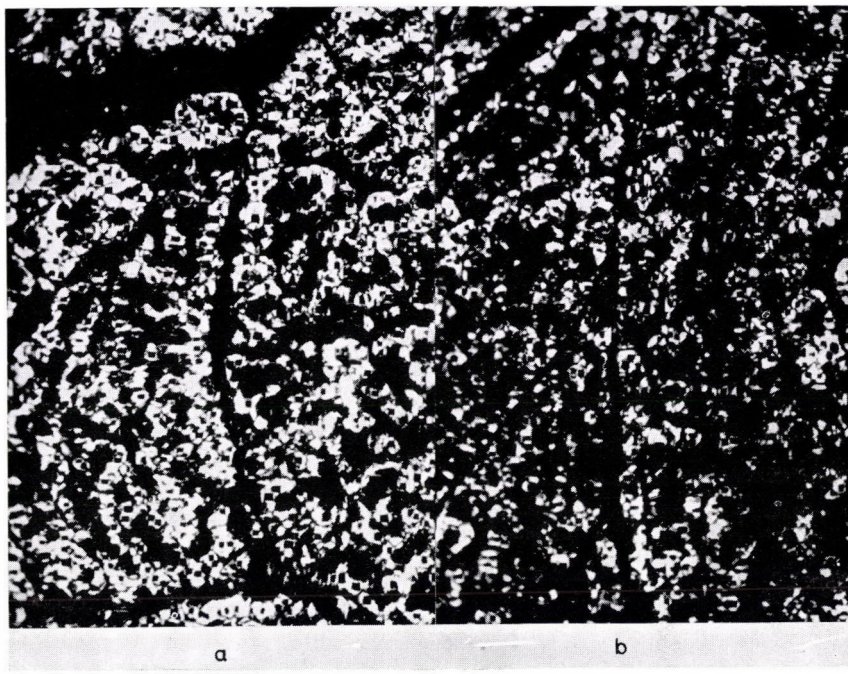

Fig. 8. Frozen section of rat pancreas. Stained with rivanol. *a)* Control, *b)* after extraction of the lipids with methanol chloroform. It can be seen that lipid extraction did not abolish the anisotropic staining reaction of RNA of the EP; only a slight decrease in the anisotropic staining reaction is seen in *b* (see Table 2). ×: 250

ultrastructure; however, this is not detectable in the lipid-extracted EP with toluidine blue but only with rivanol.

Discussion

Our findings show that the weak negative intrinsic birefringence of the unstained EP of pancreas acinar cells is greatly increased by the anisotropic staining reaction with toluidine blue or rivanol if post-staining treatment with ferricyanide is applied. However, without this treatment only an isotropic staining effect is achieved. This is the reason why Ries (1940), who was the first to study the EP with polarization optics, could not obtain an anisotropic staining effect with toluidine blue. The optical analysis of the anisotropic staining reaction with toluidine blue and rivanol indicated that the dye molecules were arranged with their light absorbing bonds (corresponding to the planes of the dye molecules) perpendicular to the membranes of the EP, i.e., parallel to the chains of fatty acid residues of the lipid layers in the membrane.

In the ultrastructural interpretation of our findings a comparison between the anisotropic staining reaction of the RNA in the EP and that of DNA in the cell nuclei seems to be of value. The comparison is made possible by our earlier studies on the anisotropic staining reaction of interphase nuclei (Romhányi, 1967).

The electronegatively charged surface of RNA in the EP is more freely available for oriented dye binding than that of DNA in formol-fixed cell nuclei since it is able to give an anisotropic staining reaction even in the presence of its protein component, while DNP of formol-fixed cell nuclei shows the anisotropic staining reaction only after trypsin pretreatment which is known to liberate DNA from the histone component (Romhányi, 1967).

A very striking finding in our studies was the dependence of the anisotropic staining reaction of RNA of the EP with toluidine blue on the presence of the membrane lipids. Although extraction of the structural lipids did not cause a decrease in the quantitative dye-binding capacity of the EP it turned the anisotropic staining reaction into an isotropic one indicating that the dye molecules were bound unoriented to the RNA. This could not be attributed, however, to a derangement of the ultrastructural surface-pattern of RNA, possibly caused by the lipid extraction procedures, since, after the same procedures RNA was still able to give an intensive anisotropic staining reaction with rivanol. It seemed therefore reasonable to assume that RNA of the EP had some sort of linear anisotropic polarized ultrastructure with periodically distributed negatively charged surface groups oriented in a definite direction relative to the EP membrane. Thus, we reach the conclusion that RNA of the EP is membrane-oriented.

If in our chemically fixed material the RNA of the EP were present in separate particles arranged in rows along the EP membranes as seen in electron micrograps of chemically fixed pancreas acinar cells, then it must be assumed that the individual ribosomes have a polarized anisotropic ultrastructure and are oriented in a definite relative direction to the membranes. Otherwise ribosomes, even with an inherent anisotropic ultrastructure, but unoriented with respect to each other or to the membranes, could not provide the postulated structural basis for the anisotropic staining reaction of RNA as observed in our studies. In earlier electron microscopic studies ribosomes were not infrequently seen attached in linear order to the membranes (Ekholm, Hyden, 1965; Pfisler, Lundgren, 1964; Röhlich et al., 1965; Schjeide et al., 1966), but no definite information was obtained concerning the presence of a polarized ultrastructure in the individual ribosomes or their definite orientation with respect to the membranes. However, in more recent studies Sabatini, Tashiro and Palade (1966) have found biochemical and electron microscopical evidence that ribosomes were attached by their larger subunits to the microsome membranes in a definite order. In high resolution electron micrographs of negatively stained glutaraldehyde-fixed suspensions of liver and pancreatic microsomes they found that the groove separating the large and small subunits of ribosomes was parallel to the membrane. These authors also pointed out that the ribosomes in their preparations were found very close to each other and

formed an almost continuous layer of particles on the membranes not seen in the osmium-fixed, plastic-embedded materials, in which the individual ribosomes are seen more separated.

In this respect the electron microscopic findings of Sjöstrand and Elfvin (1964) are of interest. In frozen-dried mouse pancreas they have seen no particulate ribosomes and the ribosomal material was spread out in the form of a continuous carpet-like layer on the EP membranes. The authors, assuming that the method of freeze-drying is the more likely to preserve the structure of the living cell, have suggested that the ribosomes may be artefacts formed during the chemical fixation or the dehydration of the cytoplasm, or because of mechanical disintegration.

The topooptical staining reactions of pancreatic EP as found in our studies indicate the presence of a membrane-oriented anisotropic RNA component on the EP. This seems to be in agreement either with the view of a continual ribosomal film as suggested by Sjöstrand and Elfvin (1964) or with an orientated attachment of anisotropically structured ribosomes on microsomal membranes as indicated by the recent findings of Sabatini et al. (1966).

Another characteristic feature of the electronegative surface pattern of the RNA of the EP is the dependence on the ionic strength of the anisotropic staining reaction with toluidine blue. An optimum anisotropic staining effect with high dichroic ratio could be obtained at relatively high ionic strength, the high dichroic ratio of the EP being quite comparable to that of artificially oriented dye films (Land, West, 1946). This indicated a very high degree of orientation of the dye molecules on the framework of EP. However, at low ionic strengths an isotropic basophil overstaining occurred with toluidine blue. Histophotometric measurements showed that the amount of the dye bound at low (0.1) ionic strength had to be decreased to about 1/3 if maximum orientation of the bound dye molecules was to be achieved. This finding may reflect a high density or an irregularity in the distribution of the negatively charged groups on the molecular surface of RNA, partly due to its more irregular molecular shape (Spirin, 1960) as compared with the regular form of DNA which does not show isotropic basophil overstaining at low ionic strengths.

It is of further interest that rivanol does not induce isotropic overstaining of RNA in the EP. This may partly be due to spectral characteristics or conformation of the dye molecules. The rivanol molecule lacks absorbancy in the visible light and therefore it seems possible that its molecules, even when bound unorientedly to RNA, do not overshadow the anisotropic effect caused by the dye molecules attached in an oriented pattern. Another point to be considered is that the rivanol (2–5 diaminoacridine) molecule has two positively charged side groups. Each rivanol molecule can therefore probably combine with two negatively charged groups on the RNA surface and be held in an oriented pattern. In this way the surface does not become overcrowded with dye molecules even at low ionic strengths. However, toluidine blue, possessing only one positively charged side group, can combine only with one negatively charged group on the surface of

RNA, which thus can be overcrowded with dye molecules at low ionic strengths. The dye molecules mutually inhibit their regular orientation on the surface, and isotropic overstaining results.

It remains to be clarified in further studies whether the quantitative differences in the anisotropic staining effect of the two dyes (Table 2) as well as the striking difference in the anisotropic staining capacity of the EP, dependent on the presence or absence of the structural lipids, can be related to the molecular shape of these dyes. From a molecular morphological point of view it is remarkable that while rivanol is bound with regular orientation on the surface of RNA even in the absence of the structural lipids, toluidine blue is unable to be bound in an oriented pattern on the RNA of lipid-extracted EP.

The finding that toluidine blue molecules are bound in an oriented pattern by the EP only when the structural membrane lipids are present, can be interpreted by assuming that the dye molecules are bound by the negative side groups of the RNA, and oriented by the structural lipids of the EP: the hydrophobic part of the dye molecules possibly being intercalated between, and oriented by, the lipid molecules. These findings suggest a very close structural relation on a molecular level between ribosomal RNA and the lipid film of the EP membrane. This ultrastructural interpretation deserves attention in the light of recent biochemical data indicating the functional significance of association of ribosomes and membranes. Several investigators have shown (Campbell et al., 1964; Hendler, Tani, 1964; Hendler et al., 1964; Henshaw et al., 1963; Sabatini et al,. 1966; Schlesinger, 1963; Székely, 1965; Tani, Hendler, 1964) that the activity of ribosomes in amino acid incorporation is greater when they are attached to the membranes than when they are present as free ribosomes. Hendler (1965) reviewed the evidence in the literature indicating the importance of the functional association of membranes and ribosomes in protein synthesis and concluded that the major synthetic activity was associated with membrane-attached ribosomes. Also Sabatini et al. (1966) pointed out the strong attachment of the in vivo active ribosomes by their large subunits to the microsome membranes.

In agreement with the biochemical and functional data and recent electron microscopical findings, our polarization optical findings suggest a close correlation between an oriented, polarized RNA ultrastructure and a lipid film of the membrane in the EP. The presence of such an oriented interphase between ribosomal RNA and membrane lipid was infered in our studies from the optical evidence of a combined action of RNA and membrane lipids on the binding and orientation of toluidine blue molecules. The dependence of this effect upon the electrolyte ionic strength and upon the conformation of the dye molecules seems to be of special microstructural interest.

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Microwave Investigation of Biological Substances. II

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From the investigation of the audio-frequency and microwave conductivity of ash-free and KCl containing gelatine solutions in a temperature range from 10 to 50 °C the following conclusions can be drawn:

1. In the microwave conductivity of ash-free gelatine hydrated water and proton migration in correlation with the structure hydrated water apparently play an important role.
2. The electric field of the polyelectrolyte changes upon sol–gel transition.
3. At a frequency corresponding to the dielectric relaxation of water an increase in the conductivity of the electrolyte solutions is to be expected.
4. At the sol–gel transition the microwave conductivity of KCl dissolved in gelatine changes. This indicates a decrease of hydrated water according to the Fricke theory.

Introduction

According to the general conception the sol–gel transition of polyelectrolyte solutions is not accompanied by a characteristic change in the electric parameters (e. g. Kuhn, 1963). The radio-frequency measurements of Ernst (1935), and Koczkás (1938), however, called the attention to the role played by the structure of the gel, and their microwave measurements indicate that the structural changes connected with the sol–gel transition have an influence on the values of the measurements (Masszi, Örkényi, 1966). Earlier publications generally report investigations performed at the temperature of the sol–gel transition.

In the present paper structural characteristics of gelatine solutions and the structural changes connected with gelatination will be reported. The investigations were performed by audio-frequency measurements and microwave measurements at a temperature range from 10 to 50 °C.

Materials and Methods

The measurements were carried out on purified gelatine solutions according to Loeb's (1924) method. The ash content of the solutions was less than 0.1 mg/g and their pH was 4.7. The dry matter content of the solutions was determined after drying the samples for 15 hours at 110 °C.

In one group of the experiments the measurements were performed on gelatine solutions containing 0.08, 0.06, 0.04 and 0.02 per cent KCl, respectively. The solution series was prepared from a 0.08 per cent solution by proportional dilution.

The microwave measurements were performed at 3 GHz ($\lambda = 10$ cm) according to the method described in detail in our previous article (Masszi, Örkényi, 1967). One part of the coaxial transmission line is filled up with sample and the voltage U is determined. U is proportional to the amplitude of the microwave running through the sample. From the ratio of the voltage measured with different samples the difference between the attenuation coefficients can be simply calculated.

The increase ($\Delta\alpha$) of the attenuation coefficient of ash-free gelatine solutions related to distilled water was determined, and on the basis of the equation

$$\Delta\alpha \approx \frac{\sigma}{2\,\varepsilon_0 C \sqrt{\varepsilon_r'}} \tag{1}$$

the conductivity (σ) corresponding to the gelatine content was calculated. In the equation $\Delta\alpha$ is the difference of the attenuation coefficient between the gelatine solution and distilled water ($\Delta\alpha = \alpha_{gel} - \alpha_{H_2O}$); ε_0 is the dielectric constant of the vacuum; C is the velocity of light; ε_r' is the relative dielectric constant of the gelatine solution. Equation (1) is only approximative because there is a 6–8 per cent divergence between the square roots of the dielectric constants of the water and those of the gelatine solution, respectively. The error originating from approximation is 10 to 15 per cent at most.

With solutions containing KCl from the increase of the attenuation coefficient corresponding to the KCl content the equivalent conductivity Λ of KCl was determined (Masszi, Örkényi, 1967) according to the equation valid for low concentrations

$$\Delta\alpha = \frac{10^{-3}}{2\,\varepsilon_0\, C\sqrt{\varepsilon_r'}}\,\Lambda\, c_{aeq} \tag{2}$$

Here $\Delta\alpha = \alpha_{KCL\text{ in gel}} - \alpha_{gel}$ is the difference between the attenuation coefficients of the gelatine containing KCl and the ash-free gelatine, c_{aeq} is the KCl concentration in equivalents. Fig. 1 summarizes the data concerning the aqueous solution of KCl. The $\log \frac{U_0}{U}$ value on the ordinate is proportional to $\Delta\alpha = (\alpha_{KCL\text{ in }H_2O} - -\alpha_{H_2O})$ (Masszi, Örkényi, 1967) thus the individual curves show the concentration dependence of the microwave attenuation and a comparison of the different curves indicates the temperature dependence of the attenuation. The same data for a 10 per cent gelatine solution can be seen in Fig. 2. The value of Λ can be determined

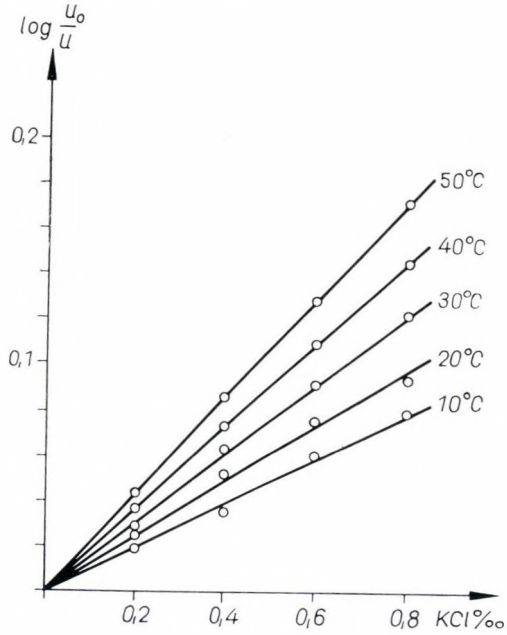

Fig. 1. Values obtained by microwave measurements on aqueous solution of KCl

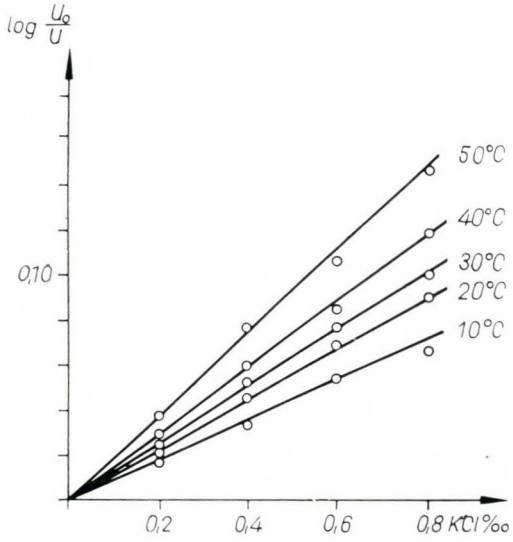

Fig. 2. Values obtained by microwave measurements on KCl dissolved in 10 per cent gelatine

from the slope of the regression lines shown in the figures on the basis of equation (2). According to this method a single A value is determined by five measured points.

The value of ε_r' for water was calculated on the basis of equation

$$\varepsilon_r' = 78.5\,(1 - 4.6 \times 10^{-3}\,(t - 25) + 8.8 \times 10^{-6}\,(t - 25)^2) \qquad (3)$$

and the 1 to 2 per cent decrease starting at 3 GHz and corresponding to the relaxation process was taken into account (Hasted, 1961).

The value ε_r' relating to gelatine solutions was determined from the equation

$$\varepsilon_r' = \varepsilon_{rH_2O} - \delta c_p \qquad (4)$$

where c_p = the concentration of gelatine g/100 ml solution

δ = the dielectric decrement, whose value is 1.00 (Buchanan et al., 1952).

The audio-frequency (2 kHz) conductivity was determined by the RC bridge. To the Wheatstone bridge the coaxial cell as used for measuring microwaves was attached. The cell was previously calibrated with a measuring vessel containing platinum electrodes. The electrodes were 4 cm^2 in surface and were 4 cm far from each other.

Results

1. Ash-free gelatine

Table 1 summarizes the data concerning an 8.5 per cent ash-free gelatine solution. The fourth column of the Table shows that the value of the microwave conductivity is 4 to 5 times higher than that of audio frequency.

Table 1

Low frenquency conductivity (σ_l) and microwave conductivity (σ_m) of 8.5 per cent ash-free gelatine solutions

T °C	σ mMho cm^{-1}		$\frac{\sigma_m}{\sigma_l}$
	σ_l	σ_m	
10	0.49	2.70	5.5
20	0.66	3.19	4.8
30	0.83	3.87	4.6
40	1.04	4.18	4.0
50	1.19	4.90	4.1

To investigate the temperature dependence of the conductivity measured at microwave and at audio frequency, on the basis of the following equation (Szkanavi, 1953)

$$\sigma = \sigma_0 \, e^{-\frac{\Delta H^x}{RT}} \tag{5}$$

the following correlation may be written for the data of Fig. 3

$$\log \sigma = -\frac{\Delta H^x}{10^3 \, R}\left(\frac{1}{T}\, 10^3\right) \log e + \log \sigma_0. \tag{5a}$$

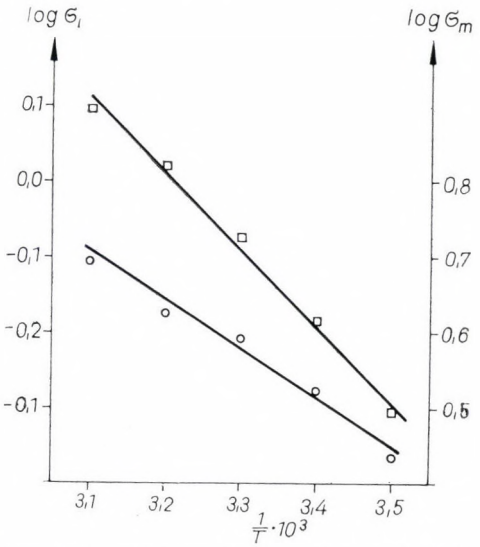

Fig. 3. Determination of the activation energy of ash-free gelatine (□—□ values of audio-frequency conductivity, ○—○ microwave values)

(Here ΔH^x = activation energy, R = 2 cal/grad. mol, T = absolute temperature, σ_0 = constant.)

From the slopes of the straight lines of Fig. 3 the following values (ΔH^x) for the activation energy were determined on the basis of Equation (5a):

At audio frequency: 4.5 ± 0.4 Kcal/mole
At microwave: 2.8 ± 0.3 Kcal/mole

According to the Eyring theory (Glasston et al., 1941) λ_{sx} ("Sprungwellen-") may be estimated from the activation energy value of the microwave conductivity and this is the wavelength at which the intensity of the processes

partaking in the microwave conductivity is at its maximum. According to the theory (Müller, Schmelzer, 1950)

$$\frac{1}{\tau} \approx \frac{kT}{h} e^{-\frac{\Delta H^x}{RT}} \tag{6}$$

and

$$\lambda_s = 2\pi c\tau \tag{7}$$

where k is the Boltzmann constant, h is the Planck constant and τ is the relaxation time.

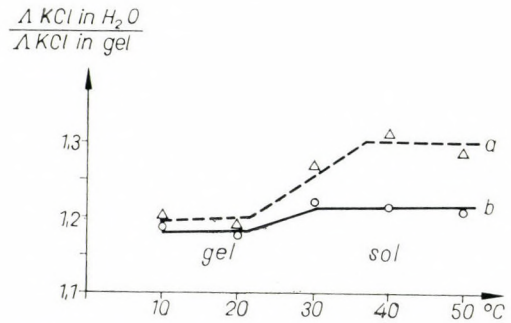

Fig. 4. The ratio of the microwave conductivity of KCl dissolved in water and in gelatine plotted against temperature

λ_s is about 3—4 cm in accordance the above equations. The relaxation processes are situated in the area of the decimeter waves if it is considered that the calculation is approximative and Fricke's (1939) data are considered, according to which the conductivity of gelatine gradually increases from the audio-frequency value, and at 65.6 MHz reaches a 4—5 fold increase which has been demonstrated by us as well.

2. Gelatine containing KCl

In the first group of the experiments measurements were performed on 10 per cent gelatine solution and the concentration of KCl was related to the combined amount of gelatine+water. Table 2 summarizing the data shows that in gelatine the equivalent conductivity corresponding to KCl is about 20 per cent smaller than in an electrolyte solution. The ratio of these two conductivities changes between 20 to 30 °C, in the range of sol–gel transition, as shown in the fourth column of Table 2 (Fig. 4, curve *b*).

In the second group of the experiments the KCl concentration and the quantity of water in the gelatine solution were compared. The quantity of water was determined from the dry weight of the gelatine. According to the data of Table 3

Table 2

KCl equivalent conductivity in water ($\Lambda_{KCL\ in\ H_2O}$) *and in a 10 per cent gelatine solution* ($\Lambda_{KCL\ in\ gel}$)

T °C	Λ Mho cm²		$\frac{\Lambda_{KCl\ in\ H_2O}}{\Lambda_{KCl\ in\ gel}}$
	$\Lambda_{KCl\ in\ H_2O}$	$\Lambda_{KCl\ in\ gel}$	
10	97	82	1.19
20	116	99	1.17
30	145	119	1.22
40	174	144	1.21
50	204	168	1.21

in this case the change of the $\frac{\Lambda_{KCL\ in\ H_2O}}{\Lambda_{KCL\ in\ gel}}$ ratio (Fig. 4, curve *a*) shows even more definitely that with the gel → sol transition the conductivity of KCl dissolved in gelatine increases to a lesser extent than that of a pure KCl solution at the same temperature.

The audio-frequency conductivity of KCl dissolved in water is practically identical with the microwave conductivity, but in the case of KCL dissolved in gelatine the microwave values show a 20 to 30 per cent increase in conductivity.

According to Fig. 5 the low frequency conductivity of KCl measured in gelatine cannot be characterized by a single activation energy value, the activation energy values calculated on the basis of the other two curves are the following:

KCl in water: 3.8 ± 0.2 Kcal/mole

KCl in gelatine at microwave: 3.7 ± 0.2 Kcal/mole

According to the microwave measurements of Sandus and Lubitz (1961) the value of the activation energy necessary for the rotation of the water molecules

Table 3

KCl equivalent conductivity in water ($\Lambda_{KCl\ in\ H_2O}$) *at audio-frequency* (Λ_1) *and at microwave* (Λ_m), *as well as in 8.5 per cent gelatine* ($\Lambda_{in\ gel}$) *at audio-frequency and microwave, respectively*

T °C	$\Lambda_{in\ H_2O}$		$\Lambda_{in\ gel}$		$\frac{\Lambda_{m\ in\ H_2O}}{\Lambda_{m\ in\ gel}}$
	Λ_1	Λ_m	Λ_1	Λ_m	
10	96	92	69	76	1.20
20	115	113	79	96	1.18
30	142	144	104	113	1.27
40	170	173	115	131	1.32
50	179	208	120	161	1.29

is 3.95 Kcal/mol, the activation energy of KCl dissolved in water and gelatine is very close to this value. This indicates that at microwave frequency the energy necessary for breaking the hydrogen bonds supplies the energy barrier of the ion movement even in the case of polyelectrolyte surroundings.

This conclusion presents a basis for regarding the presence of protein in microwave measurements simply as the inhomogeneity of the well conducting medium, in which medium the "microwave mobility" of the ion is not influenced

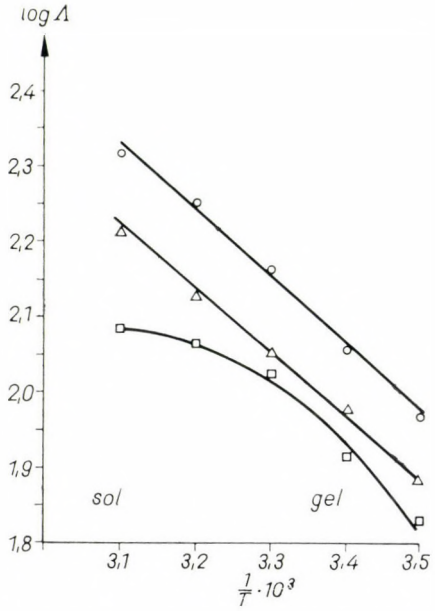

Fig. 5. Determination of the activation energy of KCl
(○—○ KCl in water at microwave, △—△ KCl in 8.5 per cent gelatine at microwave, □—□ KCl in 8.5 per cent gelatine at audio-frequency)

by the presence of the protein. The conductivity decrease which is observable in the presence of protein depends on the shape and volume of the protein molecule according to the theory of inhomogeneous dielectrics. In accordance with Fig. 4 the change in ratio $\frac{\Lambda_{\mathrm{KCL\ in\ HO}}}{\Lambda_{\mathrm{KCL\ in\ gel}}}$ indicates a change in protein structure upon the sol–gel transition. To analyse the conductivity for our data the following equation may be written on the basis of Fricke's equations (1924)

$$\Lambda_{\mathrm{KCL\ in\ H_2O}} - \Lambda_{\mathrm{KCL\ in\ gel}} \frac{\beta p}{1-p} \Lambda_{\mathrm{KCL\ in\ gel}} \tag{8}$$

where β is a constant depending on the shape of the protein, p is the volume of the protein. By measuring dielectric constants Buchanan et al. (1952) have shown that the value of β does not change during the sol–gel transition. $\beta = 1.7$ with thread-like gelatine molecules. The above equation makes it possible to estimate the total volume of protein. As the specific volume of gelatine (Stuart, 1953) is 0.7, the value of

$$v = \frac{p}{k} - 0.7 \tag{9}$$

gives the volume of hydrated water in the protein (k = protein concentration in

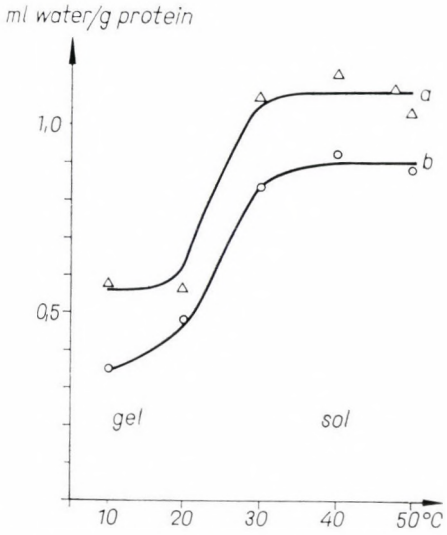

Fig. 6. Changes in the amount of hydrated water upon sol–gel transition

per cent). The values of v are shown by curve a in Fig. 6. A closer estimation was made by supposing that the hydrated water did not play a role in the dissolution of the ions, and thus instead of $\Lambda_{\text{KCL in gel}}$ the value

$$\Lambda^{x}_{\text{KCL in gel}} = \Lambda_{\text{KCL in gel}^{x}} \frac{1-k}{1-p} \tag{10}$$

was substituted into Equation (8).

In this case Equation (8) is a cubic equation. The values for v which are obtained by solving Equation (8) are shown by curve b in Fig. 6. The calculation which is indicated by Fig. 6 shows that upon gelatination the quantity of hydrated water in the gelatine decreases.

To explain Fig. 4 it is obvious to suppose that part of KCl is bound by the gelatine upon the gel → sol transition and as a consequence the ratio $\frac{\Lambda_{\text{KCL in } H_2O}}{\Lambda_{\text{KCL in gel}}}$ increases.

From this point of view the microwave ΔH^x does not show such a change during the gel → sol transition which would indicate an increased rate of ion binding, but the scattering of the value ΔH^x is great enough to conceal a change of this character. We will continue our experiments to elucidate this problem.

Discussion

1. According to the measurements on ash-free gelatine, the anomalous dispersion originating from the rotation of the protein molecule cannot be taken as explanation for the relaxation processes expected with the decimeter waves (Schwan, 1957). Rather the conductivity increase is brought about by the relaxation processes of the hydrated water on the surface of the protein. Investigation concerning the relaxation of bound water have demonstrated that the frequency of the relaxation processes of bound water shifted from the frequency (λ_s = 1.5 cm) of the relaxation processes of clear water towards the longer waves (Schwan, 1957; Hasted, 1961). For a biological interpretation of the demonstrated conductivity increase the surface conductivity of proton is also important as suggested by Kirkwood and Shumaker (1952, 1952a). It is very probable that the rotation of hydrated water of the surface of the protein and the surface proton migration are closely related processes. This is pointed out also by the direct current investigations of Riehl (1957) on gelatine of small water content.

2. Fig 5 shows that the audio-frequency conductivity of KCl in gelatine cannot be characterized by only one activation value of ΔH^x, the slope of the curve expressing the temperature dependence of the conductivity changes strikingly, especially in the surroundings of the sol–gel transition. Taft and Malm (1939) have performed measurements directly at the temperature of the sol–gel transition, and from these measurements Katchalsky (1954) concludes the following: ". . . the sol gel transition of the gelatine hardly affects the conductivity indicating that the polyelectrolyte field acting on the ions is the same in the sol and gel phases." On the basis of the data obtained at a higher temperature interval it can be supposed that in those biological processes which are accompanied by sol–gel transition, a change in the electrostatic field influencing ion migration must also be taken into account.

3. Our observation that the activation energy of the rotation of the water molecules nearly equals the activation energy of the microwave conductivity of KCl dissolved in water and gelatine indicates according to the Eyring theory, mentioned above, that at a frequency which is nearly equal to the relaxation frequency of water an increase in electrolytic conductivity can be expected. This

conclusion is in line with the calculation of Little and Smith (1955) according to which at a wavelength of 1.5 cm a conductivity increase of about 300 per cent may be expected. At longer wavelengths we were unable, however, to demonstrate any conductivity increase fitting the Little and Smith theory (see the second and third columns of Table 3; Masszi, Örkényi, 1967).

4. Investigations on the estimation of the quantity of bound water yielded very different results: Jacobson (1954) supposed the existence of an "ice-like" hydratation of great extension which according to O'Konski's (1955) calculations would be of the order of 40 ml water/g protein; according to Fricke (1939) 1.5 g water/g protein; according to Dumanskii and Kurilenko (1949) 0.42 g water/g protein; according to Zhukov and Stepin (1965) 0.3 to 0.4 g water/g protein; according to Buchanan et al. (1952) and Haggis et al. (1951) 0.2 to 0.4 g water/g protein. Pócsik (1966, 1967) found that the density of muscle water started to increase at a value of 0.3 g water/g protein.

The different data of the above list and the vapour pressure measurements performed by Ernst et al. (1950) show that no pronounced dividing line can be drawn between "bound" water and "free" water. The estimations represent a mean value the magnitude of which greatly depends on which properties of water are taken to indicate the bound state. Our investigation gives an estimation of the quantity of water bound to the surface of the protein. This water together with the protein molecule can be regarded as the inhomogeneity of the conducting medium. Curve *a* of Fig. 6 was taken on the basis of the supposition that the hydrated water contributed to the dissolution of ions exactly in the same way as "free" water with curve *b* on the other hand, it was supposed that the hydrated water did not act as a solvent. The curves corresponding to both extreme cases indicate that the examined amount of bound water decreases with the sol → gel transition. According to the investigations of Meyer and A van der Wyk (1937, 1940) gel formation is a crystallization process. It can be supposed that the decrease of water binding, which conclusion is drawn from the Fricke theory, is in connection with the formation of crystalline range. From this point of view our investigations yield data to Ernst's conception according to which the decrease of water binding due to the stretching of muscle (Ernst, 1925; Ernst et al., 1954) is a partial phenomenon of crystallization of the protein (Ernst, 1963).

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Magnetochemical Measurements on Gelatine

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A Theorell-type magnetic microbalance was built to investigate the magnetic properties of biological objects. The sensitivity of the apparatus is 0.0003×10^{-6} emu.

In this paper the measurements made on gelatine solutions of different concentrations are reported. It has been established that the mass susceptibility of gelatine is -0.571×10^{-6} emu., Wiedemann's law is valid, but the susceptibility of ferric salt-gelatine solution is less than that of ferric salt dissolved in water. This is due to the ferric-gelatine compound.

Introduction

The magnetic properties of muscle were investigated in our Institute many decades ago (Koczkás, 1935) and in the last few years many articles have been published to get informations about the role of trace elements in the excitation process and heart automaticity (Lakatos, 1962; Ernst, 1963; 1967; Niedetzky, Hajnal-Papp, 1963).

It seems to be of considerable interest to carry out magnetochemical measurements, because such studies may shed some light on the mechanism of the trace elements.

The ions of the trace elements are usually paramagnetic, and therefore, the interaction between the paramagnetic ion and the diamagnetic medium can be followed by susceptibility measurements (Hutchinson, 1955).

As the biological processes take usually place in water milieu and because of the great diamagnetic screening of water, it is necessary to construct a magnetic balance with a sensitivity of at least 10^{-10} emu. This value is about 10^4 times smaller than the susceptibility of water.

For our purpose the improvement of the Gouy's balance proposed by Theorell seemed to be suitable. Here the magnetic forces acting on the sample and the gravitation are perpendicular to each other, the magnetic susceptibility is proportional to the force compensating the magnetic force, and the sensitivity required for the measurements can be achieved.

As biological model gelatine was used, because its physico-chemical characteristics, amino-acid composition are well known (Loeb, 1924; Meyer, Mark, 1953).

Materials and Method

The construction of our balance is similar in many respects to that described by Theorell and Ehrenberg (1950).

The experimental arrangement is shown schematically in Fig. 1. The most important part of the apparatus is the light measuring tube (*t*), which is suspended by two fine wires (*L*). The tube experiences an attractive or a repulsive force from the inhomogeneous magnetic field and moves in a horizontal direction. The change in the position of the tube can be compensated by means of a micrometer screw

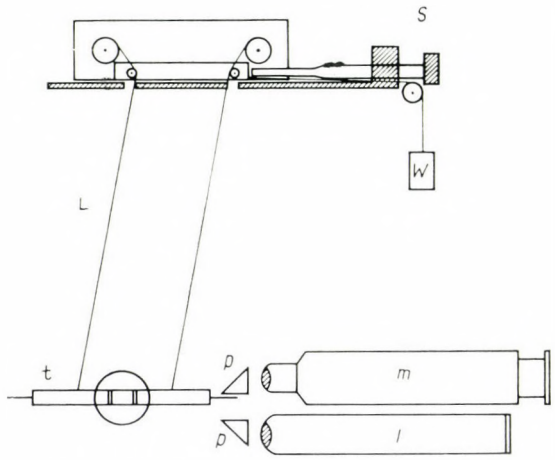

Fig.1. Schematic diagram of the experimental arrangement

(*s*) removing the suspending wires at the points where they are fixed. The change at the micrometer screw required to keep the tube in the original position is a measure of the magnetic susceptibility.

The wheels holding the suspending wires can be displaced together or also independently of each other in two perpendicular directions, and therefore, the measuring tube can be set rapidly between the pole gaps. The wheel holder is propped against the micrometer screw by the counterbalance (*w*).

The 70 mm long tube containing three chambers was made from plexi-glass, it weighs about 500 mg. The two greater outer cells contain the solutions to be measured and calibrated; the little chamber in the middle of the tube ensures a better damping of the tube. The volume of each cell is about 0.35 ml. A light aluminium ring is mounted in the middle of the surface of the tube, which can be removed in both directions. If the sample containers are filled with identical solutions, by removing the ring it can be achieved that the position of the tube does not change either with or without the excitement of the magnetic field. The measuring tube is suspended on tungsten wires 10μ in diameter. The position of the

tube can be controlled at any time by means of a microscope (m) by observing a 0.5 mm platinum wire mounted to the cap closing the tube. The magnification is 100 fold. The illumination required for the microscope is provided by a little light source (l) through two rectangular prisms (p). It was provided for the elimination of the air-caused movements as well.

The electromagnet applied was a Weiss-type one with 110° top angle. For excitation direct current of 15 A was supplied by a standard-type power unit (Konverta OT 110/30 type). Since the iron core was magnetized almost up to saturation, the excitation current was not stabilized. The magnetic field strength between the pole gaps was measured with a Metra type magnetometer, and the measurement of the temperature was accomplished by means of a thermistor and a Wheatstone bridge.

The temperature during the measurement was constant to $\pm$ 1 °C.

To calculate the mass susceptibility, it was necessary to determine the density of the solutions. This was carried out by pycnometric method.

As measuring objects, gelatine solutions of different concentrations were used. Their dry weight content was 0.5 to 7 per cent. The following procedure was used. The commercial gelatine was purified with manifold decantation to an ash content of less than 1 per mille. The water used was redistilled in an all-glass distillator. Preliminary measurements in the ultracentrifuge showed that the purified gelatine had a molecular weight of about 10^5 and a pH of 4.8. This is in good agreement with the data given by Loeb (1924) for the iso-ionic point of gelatine.

Other materials used were of the purest grade available (mostly analytical grade).

Results

Taking into account the forces acting on the tube in a magnetic field, we obtain

$$\kappa = \kappa_0 + k\,(Ms - M_0 s_0)$$

where κ is the susceptibility of the gelatine solution, κ_0 is the susceptibility of the calibrating solution, M and M_0 are the total masses of the tube filled with gelatine solution and with the calibrating solution, respectively, s and s_0 are the readings on the micrometer, and k is the constant of the apparatus.

The value of k can be determined after having measured two solutions with known susceptibility. In our case water and $2 \cdot 10^{-3}$ M $MnCl_2$ solution were used. The volume susceptibility of water according to the data reported by Selwood (1943) can be accepted as -0.7200×10^{-6} emu. For the Mn ion it can be supposed a spin only paramagnetism as it is usually done at the most salts of the iron group (see e. g. Vonsovskij, 1956), and therefore, the effective magnetic moment is $\mu_{eff} = 5.92\,\beta$ where β is the Bohr magneton. Since the Wiedemann- and Curie-

laws are valid for most solutions, the difference in magnetic susceptibility between water and a 2×10^{-3} M $MnCl_2$ solution is 0.0276×10^{-6} emu at 20 °C.

The sensitivity of the apparatus can be estimated on the basis of the following equation:

$$\Delta\kappa = \frac{Mg}{q\,H_0^2}\,\frac{\Delta s}{L}$$

where M is the mass of the tube filled with the solution, H_0 is the maximum field strength, L is the length of the suspending wire, q is the area of the cross-section of the tube, g is the gravitational constant, Δs is the displacement read on the micro-

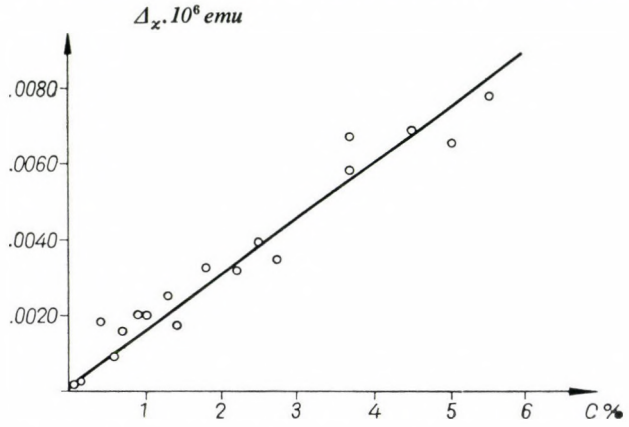

Fig. 2. Susceptibility measurements made on gelatine solution

meter screw. The theoretically calculated sensitivity is about 0.0001×10^{-6} emu, but during the measurement we experienced a sensitivity of 0.0003×10^{-6} emu.

The results obtained with 0.5 to 6 per cent gelatine solutions are plotted in Fig. 2, where $\Delta\chi$ means the difference in susceptibility between the gelatine solution and water. It may be seen from Fig. 2 that the results gave a straight line with close approximation in the interval measured by us. The line in Fig. 2 is the calculated regression line.

In further experiments ferric chloride ($FeCl_3$) and 1×10^{-3} to $6\times10^{-3}M$ ammonium ferric sulphate ($NH_4Fe(SO_4)_2$ dissolved in 2 to 6 per cent gelatine-gel were investigated.

The results obtained with ferric chloride are illustrated in Fig. 3. All values are the means of several determinations. In the figure the susceptibility difference $\Delta\chi$ is represented by taking the ferric salt concentration as the abscissa. The upper line refers to ferric chloride dissolved in water, and $\Delta\chi$ means the difference in susceptibility between the ferric chloride solution and water, and the lower line represents the measurements made on ferric salt gelatine solutions. In this case $\Delta\chi$

means the net difference in susceptibility between the ferric chloride solution and water (without the diamagnetic contribution of gelatine).

The susceptibility is proportional to the concentration of Fe^{3+} ions in both cases, but the susceptibility referring to ferric salt gelatine solution is less than that of ferric salt dissolved in water.

The results are closely similar to those obtained for ammonium ferric sulphate dissolved in water and gelatine.

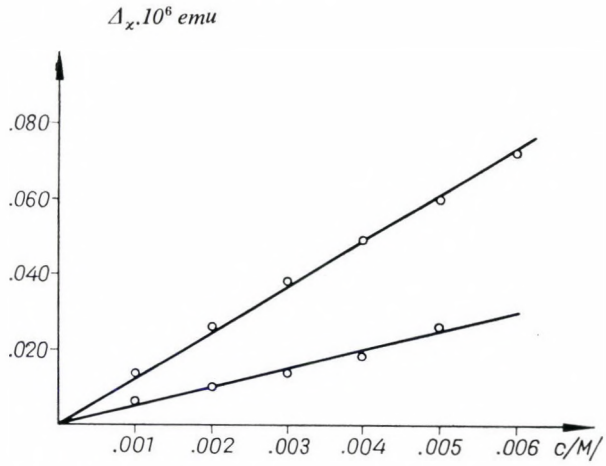

Fig. 3. Susceptibility measurements made on ferric chloride.
Upper line: ferric chloride dissolved in water
Lower line: ferric chloride dissolved in gelatine

Discussion

According to the measurements made on gelatine solutions there is a linear correlation between susceptibility and concentration. This result is in agreement with the generally experienced relation at diamagnetic substances. Therefore, the Wiedemann's formula is valid. In this case we could easily calculate the mass susceptibility of gelatine. According to the measurements

$$\chi = -0.571 \times 10^{-6} \text{ emu/g.}$$

Our result is similar to those obtained for amino acids, which compose the gelatine macromolecule.

The susceptibility difference obtained in the case of ferric chloride dissolved in gelatine solution can be interpreted as follows: The ferric ion starts to precipitate in hydroxide form at a pH value lower than that of the iso-ionic point for gelatine. This precipitation does not occur if the gelatine concentration is high

enough, – according to our measurements higher than 1.5 per cent – which fact refers according to Charley et al. (1963) to complex formation. It can be supposed that the ferric-complex has a dimer structure $[Fe_2(OH)_2.(H_2O)_4]^{4+}$ (Aasa et al., 1964) where the ferric ions of the dimer structure are bound to the carboxylic groups of gelatine. The amino acids, which might be considered to bind metal ions such as iron are aspartic and glutamic acids (Kirby, 1957).

Since the dimer structure possesses a diamagnetic property (Schoffa, 1964), the susceptibility must decrease as it was observed in our experiments. But the susceptibility does not drop to zero, as it could be expected, because of the dimer formation. It remains to be explained whether the paramagnetic excess is due to colloidal aggregates, or perhaps to magnetic interaction between the neighbouring ferric ions.

Acknowledgement

The author is indebted to Professor E. Ernst for calling his attention to this topic.

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Bound Water in Muscle

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Experiments on the density of muscle water were continued. To investigate the gradually increasing "boundedness" of muscle water the behaviour of the "*own*" *water content of the muscle* was investigated rather than that of the content of water which had been added to the muscle. The mass and the volume of the gradually dried muscle were measured: the values measured after drying at 105 °C were taken as the dry matter content of the muscle. Supposing, that there was only water loss in the muscle, the mass and volume of the dry substance remained constant during drying. Thus, the data have shown a gradual increase of about 30 per cent in the density of muscle water. The data of the measurements were evaluated also by a statistical method (120 pairs of data). In our opinion, the gradual increase in the density of muscle water indicates the gradually increasing boundedness of muscle water.

Introduction

As pointed out in an earlier paper (Pócsik, 1966) the problem of the "boundedness" of the water content of muscle was re-investigated because of contradictory literary data (Dydynska, Wilkie, 1963; Ernst, 1963). Investigations of muscle plus added water were carried out by one group of research workers (Dydynska, Wilkie and others), and experiments on the gradual decrease of muscle water were performed by the other group (Ernst et al., 1950). The present work was also based on the assumption that adequate results can be obtained by studying the *own water content* of the muscle. In the present paper some experimental data concerning the density of the own water of muscle will be presented. If the muscle is a swollen system the relative water content gradually decreases and the value of the swelling pressure gradually increases during gradual drying. According to Katz's investigations (1924) the swelling pressure of the swollen system gradually increases with decreasing relative water content. In the case of increasing swelling pressure a gradual increase in the density of muscle water can be expected. This assumption has been proved by the experiments.

Experimental

The density (d_i) of muscle water can be calculated by the following equation (Pócsik, 1966)

$$d_i = \frac{M_i - m}{V_i - v} \tag{1}$$

where M_i and V_i are the mass and volume, respectively, of the muscle in phase *i* after a certain degree of drying; m and v are the mass and volume, respectively, of the dry matter of muscle. It was supposed that the decrease of both the mass and the volume of the muscle were caused *exclusively* by loss of water and m and v remained constant during drying. Therefore to determine the density of muscle water, mass and volume measurements are to be performed.

a) *Mass measurements.* One of the musculus gastrocnemius of the frog was removed in such a way that a piece of tendon of about 1 mm was left on the muscle. A fine platinum wire 36 mm long weighing about 6 mg threaded in it. The wire was shaped into a bridle. By the help of this wire the muscle was suspended on an analytical balance and the total mass of the fresh muscle and the platinum wire was weighed. (The balance was counterbalanced on both arms by two metal wires about 60 mm long, with hooks at the ends serving for the suspension of the muscle.)

b) *Determination of volume.* Weighing the mass in air was followed by the determination of the volume of the muscle by a hydrostatic method which, as it is known, is based on the following equation

$$V_i = \frac{M_i - M_{bi}}{d_b} \tag{2}$$

where M_{bi} is the mass of muscle in a liquid (in our case benzene) of known density, (the mass which virtually corresponds to the smaller weight) and d_b, the density of benzene at a given temperature. After weighing the mass in air the muscle was placed within about 15 to 20 seconds in benzene of 23 °C. Greater water losses can be avoided in this way. The loss with fresh muscle is about 1 mg in one minute at room temperature and at 60 per cent relative air humidity.* The measurement was carried out according to the sketch shown in Fig. 1. (The platinum bridle was also submerged about 6 mm deep in benzene).The volume can be calculated from the data.

To observe possible benzene binding or fat release, the muscle was weighed again in air after taking it out from the benzene. The above-mentioned measure-

* The speed of the loss of water decreases after this to a few tenths of mg/min; it is only 0.01—0.02 mg/min after a 75 per cent mass decrease. Muscles take up vapour at a rate of 0.01—0.02 mg/min from the air in the last phases of drying.

ments were completed in a few minutes following the preparation. The muscle was then placed in a small glass vessel in a desiccator, above $CaCl_2$ for drying. The desiccator was placed in a refrigerator. When necessary the little glass vessel could be closed with a glass stopper. Thereafter, the other muscle of the frog was also removed and the measurements were performed in a similar way as described above. In this series the experiments were carried out on 10 muscles. (Altogether 70 muscles were used in the series of experiments performed so far.)

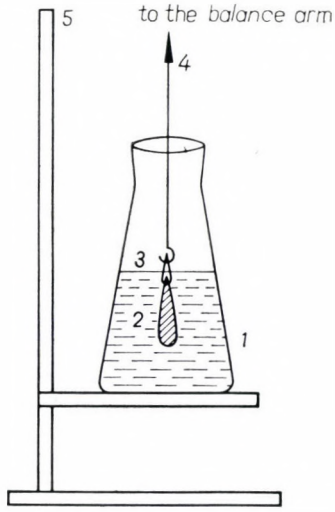

Fig. 1. Schematic drawing of mass measurement performed in fluid. 1) Glass vessel. 2) Muscle. 3) Platinum bridle. 4) Metal wire. 5) Frame

The second phase of the experiment followed after drying the muscle for about four hours (Table 1). The desiccator was taken out from the refrigerator and was allowed to stand until it warmed up to room temperature. The procedure was similar to that of the first phase of the experiment after taking out the muscle from the desiccator. The rate of water loss decreased when the muscle was placed right in the refrigerator without a desiccator (as after the 3rd phase) or when only a slight suction was applied (as after the 5th phase). Thus the dots of the phases get nearer to each other without changing the slope of the curve. From a mathematical point of view a single dot $P_i(V_iM_i)$ belongs to each phase in the "phase plane" determined by the VM axes.

After an about 77 per cent loss of water (7th phase) the muscle was weighed in air and placed in an empty glass vessel (Fig. 2). The glass vessel was connected to a flask (2 in Fig. 2) provided with a ground joint and with a stopcock with a *T*-bore (Fig. 2). The vessel was evacuated at an appropriate position of the stopcock. Benzene was poured into the flask and by turning the plug of the stopcock

to a suitable position it was poured on the muscle. The next step was to remove the flask (the temperature was controlled at the same time). The measurement was performed in the same way as described previously.

The dry matter content of the muscle was obtained by drying the muscle in a vacuum desiccator above $CaCl_2$ for at least 5 hours. Care was taken that the muscle be in a closed space, it was in air only for 15 to 20 seconds.

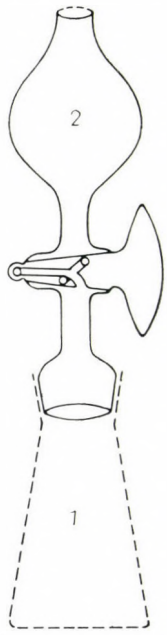

Fig. 2. Glass flask used to fill benzene into the muscle in vacuum

It has to be noted that an additional force of 1.88 mpounds acts downwards on the platinum bridle which weighs about 6 mpounds and submerges into benzene to a depth of about 0.5 cm. This force is the resultant of the surface tension and the buoyant force affecting the bridle.

Results

The data of measurements on one muscle are shown in Table 1. The phases of the experiment are indicated in the first column, and the combined masses of the muscle and bridle ($\overline{M}_i$) measured in air are represented in the second column. The data of the third column give the combined masses of the muscle and the platinum bridle measured in benzene ($\overline{M}_{bi}$). The time elapsed since the first mass meas-

urement (time of drying) is indicated in the fourth column t and the temperature of drying in °C (T) in the fifth one. The last column shows the way in which the muscle was dried before mass measurement i in a desiccator (*de*) or in a refrigerator (*r*), at room temperature (*t'*) or in a thermostat *(therm)* etc. Designations like *de* + *r* mean that the muscle was in a desiccator and the desiccator was placed in a refrigerator.

Table 1

Experimental data of muscle No. 5

The combined mass of the muscle and the platinum bridle in air ($\overline{M}_i$) and in benzene ($\overline{M}_{bi}$). The time calculated from the first mass weighing is (t). The temperature of drying is (T). The ways of drying are: *de* = desiccator, *r* = refrigerator, *t'* = room temperature, *therm* = thermostat. The mass of the bridle is 5.84 mg

Phase	$\overline{M}_i$ (mg)	$\overline{M}_{bi}$ (mg)	t		T (C°)	
			hours	days		
1	1025	184.70	0		—	—
2	816	157.40	4		2—4	*de* + *r*
3	553	124.80	8		2—4	*r*
4	494	117.58	21		2—4	*de* + *r*
5	326	96.18	28		2—4	*de* + *r*
6	269.0	88.27	44		2—4	*de* + *r*
7	247.78	84.63		3	2—4	*de* + *r*
8	237.62	82.60		5	2—4	*de* + *r*
9	233.74	81.77		8	2—4	*de* + *r*
10	228.87	80.63		16	2—4	*de* + *r*
11	225.30	79.65		21	23	*de* + *t'*
12	218.65	77.56		23	23—70	*de* + *therm*
13	209.09	74.25		30	70—105	*de* + *therm*

Equation (1) given for the calculation of the average density of muscle water may be transformed in the following way:

$$d_i = \frac{M_i - m}{\frac{M_i - M_b}{d_b} - \frac{m - m_b}{d_c}} = \frac{d_b (M - m)}{(M_i - m) - (M_b - m_b)} = \frac{d_b}{1 - \frac{M_{bi} - m_b}{M_i - m}} \qquad (3)$$

where m and m_b are the mass values in air and in benzene, respectively, in phase 13. Benzene has a density (d_b) of 0.8747 ± 0.0001 g/cm³ at 23 °C.

The density of muscle water in the 1st phase is

$$d_1 = 1.012 \text{ g/cm}^3$$

by using the data of Table 1. The calculated values for the following phases can be seen in the 3rd column of Table 2. The errors of the densities of muscle water from the 1st to the 8th phase can be estimated as ± 0.001 g/cm^3 and those from the

Table 2

Values of the relative water content (i), *the average density of muscle water* (d_i) *and the density of muscle* (D_i) *at different phases of drying in muscle No.* 5

Phase	i	d_i (g/cm^3)	D_i (g/cm^3)
1	4.01	1.012	1.059
2	2.99	1.014	1.073
3	1.69	1.025	1.113
4	1.40	1.032	1.129
5	0.58	1.076	1.209
6	0.30	1.141	1.261
7	0.19	1.192	1.282
8	0.14	1.234	1.292
9	0.12	1.26	1.296
10	0.10	1.28	1.299
11	0.08	1.31	1.301
12	0.05	1.33	1.302
13	—	—	1.300

9th to the 12th stage as $\pm 0.01 - 0.02$ g/cm^3. The relative water content (i) is indicated in the 2nd column of the Table 2 and can be calculated on the basis of the following equation:

$$i = \frac{M_i - m}{m} \tag{4}$$

It is worth-while to determine the density of muscle (D_i) in the different phases of drying. According to equation (2)

$$\frac{V_t}{M_i} = \frac{1 - \frac{M_{bi}}{M_i}}{d_b} = \frac{1}{D_i} \tag{5}$$

from which

$$D_i = \frac{d_b}{1 - \frac{M_{bi}}{M_i}}$$

For example the density of the muscle in the 13th phase, or rather the density of the dry matter can be calculated. Table 1 shows the combined mass of the muscle and the platinum bridle weighing 5.8 mg. Thus, taking into consideration the buoyant force of the air weighing 0.2 mg as well, we get

$$M_{13} = m = 209.1 - 5.8 + 0.2 = 203.5 \text{ mg}$$

The mass weighed in benzene is

$$M_{b13} = m_b = 74.3 - (5.8 + 1.9) = 66.6 \text{ mg}$$

Here 1.9 mg is due to the effect of the surface tension, etc. Thus, the density of the muscle is

$$D_{13} = 1.300 \text{ g/cm}^3$$

The 4th column of Table 2 contains the values of the density of the whole muscle in all phases of the experiment. The experimental error can be estimated to be about ± 0.001 g/cm^3. Similar values were obtained with every muscle of the experimental series whether referring to the density of the muscle or to the density of the muscle water.

Discussion

The results show that the average density of the water of the gradually drying muscle increases, and this indicates in our opinion the increasing boundedness of muscle water.

The problem may be investigated more generally by statistical methods. Let us plot M_b against M by using the data of Table 1. The point of origin of the coordinate system is represented by the coordinates of the dry substance (Fig. 3). It can be seen that at greater mass values in the starting phases of the drying process the dots representing the values of the measurements are linearly distributed. The linear regression was calculated from 65 pairs of values by using the data of measurements of the other muscles (Appendix). The correlation coefficient indicating the extent of the correlation is

$$c_1 = 1.000$$

The curve of the second order fits well in with the last phases of the drying process. Its equation was determined from further 55 pairs of values. The index indicating the degree of the correlation is

$$c_2 = 0.923$$

Taking into account equation (2) the above function correlations may be transformed into a function correlation between the mass and the volume of muscle

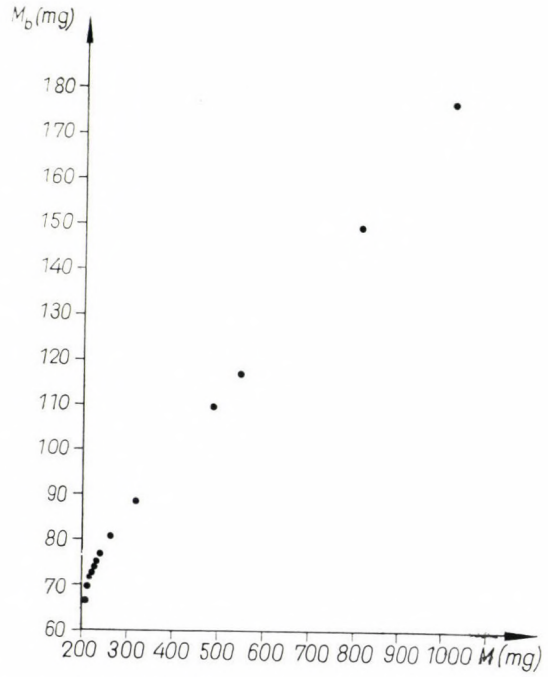

Fig. 3. Diagrammatic representation of the mass values of muscle No. 5 measured in fluid (M_b) and in air (M)

water ($v_I = f(m)$; $v_{II} = \phi(m)$), where m is the water content in the case of 200 mg dry weight. The two curves intersect at two points. The mass value is 72.97 and 51.55, respectively. The arithmetic mean value 62.26 of the two values is taken as an approximation. If the "local" density in the phase point is expressed as the derivative of v $\left(\frac{dm}{dv}\right)$ the following correlations are obtained:

$$d_I = 1.002 \qquad (i = 4 - 0.3) \qquad (6$$

$$d_{II} = \frac{1}{3.94 \times 10^{-3} m + 0{,}7523} \quad (i = 0.3 - 0) \tag{7}$$

The local density calculated on the basis of equations (6) and (7) plotted against $\left(\frac{1}{i}\right)$ is characteristic of the swelling of the curve (Fig. 4) similarly to the curve representing the decrease of the relative vapour pressure as a function of the relative water content (Ernst et al., 1950). Its value in the case of 200 mg dry matter content is constant till 262 mg (i = 0.31) whereafter it increases according to equation (7). This can be interpreted by assuming, that those water molecules get free from the muscle which have the greatest tendency to escape. Their effect can be measured by the vapour

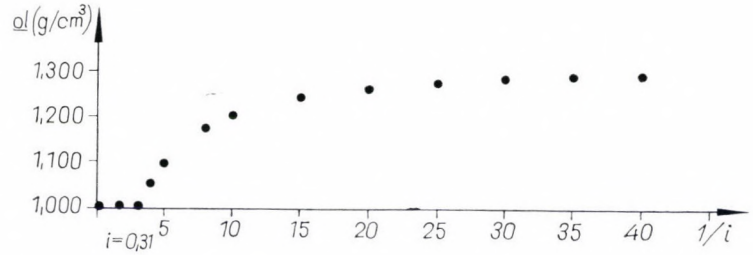

Fig. 4. Local density (d) of muscle water plotted against the reciprocal value $\left(\frac{1}{i}\right)$ of the relative water content

pressure established in a closed space above the muscle, on the one hand, and by the derivative $\frac{\Delta m}{\Delta v}$ if only water loss is supposed, on the other $\left(\text{at a limiting value by the derivative } \frac{dm}{dv}\right)$. This gives the mass of water being in a volume unit of the muscle at a given phase point.

The mass of the muscle weighed immediately after its treatment in benzene yields essential informations about the fat release and the binding of benzene (the muscle stood in benzene for one to two minutes). According to the measurements, the binding of benzene is negligible. In the experiments performed during the summer a few small fat drops were observed in the benzene in the first few phases, because the frogs were well fed, and this caused some fat release. In the present experiments performed during the winter this effect is even smaller and is negligible. It must be emphasized that the present experiments were concerned with the *own water content* of the muscle, and not with water added to the muscle. In the case of 200 mg dry matter content the effect of boundedness of the water begins to increase at 262 mg (i = 0.31). Similar results are obtained if the relative vapour pressure of the gradually drying muscle is investigated as a function of the

relative water content (Ernst et al., 1950). According to the results obtained by another method (Ritland et al., 1950) the water bound by 1 g protein is about 0.3 g.

To sum up the fact that the density of the water of the dried muscle increases shows that part of muscle water is bound. This contradicts Hill's (1930) opinion as well as Dydynska and Wilkie's recent suggestion (1963).

Appendix

For the statistical evaluation of the data 200 mg dry matter content is taken for each muscle. The mass of the muscle weighed in air and in benzene for example in the 13th phase is

202.00 mg and 66.00 mg, respectively.

The same in the 12th phase

210.00 mg and 69.00 mg etc.

By the conversion of the masses we get

$$M_{13} = 200 \qquad M_{b13} = \frac{66}{202} \times 200 = 65.32$$

$$M_{12} = \frac{210}{202} \times 200 = 207.92 \qquad M_{b12} = \frac{69}{202} \times 200 = 68.32$$

For the water content it is valid in the 12th phase (m_{12} and m_{b12}, respectively)

$$\begin{array}{r} 207.92 \\ -\ 200.00 \\ \hline m_{12} = \quad 7.92 \end{array} \qquad \text{or rather} \qquad \begin{array}{r} 68.32 \\ -\ 65.35 \\ \hline m_{b12} = \quad 2.97 \end{array}$$

In this way, out of the 10 muscles of the series 120 pairs of data can be obtained. Arranging the data according to their order of magnitude 65 pairs of value gave a straight line and 55 pairs of value a curve of second order. The equation of the straight line is

$$m_{bI} = 0.1274\, m + 6.61 \tag{8}$$

The correlation coefficient showing the extent of the correlation is

$$c_1 = 1.000$$

The equation of the curve of second order is

$$m_{bII} = -1.72 \times 10^{-3} m^2 + 0.3420 m + 0.13 \quad (9)$$

The correlation index showing the degree of the correlation is

$$c_2 = 0.923$$

By using equation (2) equation (8) may be written as

$$m - 0.874 v_I = 0.1274 m + 6.61$$

From here

$$v_I = 0.9976 m - 7.56$$

Similarly v_{II} can be obtained from equation (9)

$$v_{II} = 1.97 \times 10^{-3} m^2 + 0.7523 m - 0.15$$

By solving the two equations for m, two values are obtained

$$m_1 = 72.97$$

$$m_2 = 51.55$$

The mean value of these is $m = 62.26$.
It is known that the definition of density for inhomogeneous bodies is

$$d = \frac{\Delta m}{\Delta v}$$

The limit of this quantity is the derivative $\frac{\mathrm{d}m}{\mathrm{d}v}$ which may be called local density.
By differentiating v_I and v_{II} according to v

$$d_I = \frac{1}{0.9976} = 1.002 = \text{const.}$$

$$d_{II} = \frac{1}{3.94 \times 10^{-3} m + 0.7523}$$

At a mass value $m = 62.26$

$$d_{II} = \frac{1}{3.94 \times 10^{-3} \times 62.26 + 0.7523} = \frac{1}{0.9976} = 1.002 = \mathrm{d}_I$$

Acknowledgement

The author expresses his thanks to Professor E. Ernst who called his attention to this question.

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Analysis of Muscle Contraction

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An electronic equipment was constructed to study the shortening and force development of the striated muscle during contraction. To accomplish an exact analysis, the functions (shortening, force development) and their derivatives were simultaneously recorded during single contraction and under tetanic conditions. It has been concluded that 1. the analysis of the shortening confirms our previous results, according to which the time course of the shortening can be described in terms of a sine function, 2. the time course of the velocity lies close to the cosine function. Conclusions are drawn about the time course of force development as well.

The analysis of muscle contraction is one of the most extensively investigated problems of muscle biophysics and physiology. However, the study of this problem has not lost yet its actuality because there still exists no solution to this problem which would be free of all ambiguity. The conclusions drawn from the analysis may shed some light on the process of contraction. It does not seem to be unreasonable to redesign the experiments with more adequate mechanical parameters and with better electronics to obtain detailed informations about the behaviour of the muscle during contraction.

Numerous studies in the literature have been concerned with the time course of muscle contraction. On the basis of earlier data (Tigerstedt, 1910; Fenn, 1925) an analysis was performed by Ernst (1963). He concluded that the time course of the tension-free contraction can be described by a sine function. This conclusion was confirmed by Belágyi and Biró (1962) by using a photoelectric transducer. The investigations were extended to the analysis of the contraction of *in situ* muscle as well and the same results were obtained. In these experiments the descending branch of the contraction curve was not analysed because its features are depending mostly on the mechanical characteristic of the transducer, and not on the muscle. This fact was experienced in a work of Rosenblueth et al. (1958).

To carry out a more accurate analysis, it seemed necessary to record the first derivatives* of the contraction curves because the derivatives measured either by drawing the tangents or by the mirror method are not suitable for mathematical analysis.

* The second derivative, d^2r/dt^2 multiplied by the actual mass is the measure of the force exerted by the muscle during shortening. Experiments on muscle, during which the second derivative is recorded are in progress.

Methods

As regards the experimental requirements, the following must be fulfilled in any case:

1. The mechano-electrical transducer used for shortening is practically inertia-free, and has a well defined (mostly linear) characteristic.

2. The transducer used to study force development gives a fast response without overshooting, and

3. The electronic system amplifies linearly without distortion in a wide-frequency band.

The two first requirements could be fulfilled by using a special capacity transducer containing two variable condensers. The moving plate of both condensers can be attached to the muscle to be investigated. By regulating the position of the muscle both shortening and force development can be recorded successively one after the other. Details about the double capacity transducer were reported in a previous work (Mórocz-Juhász, Örkényi, 1967).

To fulfil the third requirement, a special four channel (two a. c. channels and two d. c. channels) oscilloscope was designed and constructed. Its important characteristics are the following: the width of the frequency band for all channels is 40 kc/s; the sensitivity for a. c. channels is 0.1 mV/cm, and for d. c. channels it is 5 mV/cm. A special electronic switch (Fig. 1, No. 8) is applied as well to get time markers of 5, 2, 1, 0.5, 0.2 and 0.1 ms.

The experimental arrangement used in our experiments is shown schematically in Fig. 1. The isolated muscle (m. gastrocnemius of frog *(Rana esculenta)* was attached to the capacity transducer (No. 4) by inserting a hook into the Achilles tendon. The other end of the muscle was fixed by its stub to a mobile slide. All the experiments were carried out with muscles at their resting length. This was ensured by a rubber spring about 160 mm long in case of shortening or by adjusting the mobile slide to measure the force development. The mechanical activity of the muscle was transformed into capacity change with the help of the transducer. The d.c. voltage change on the output of the capacity transducer was connected to the first channel of the scope and the sign was recorded after adequate amplification.

To yield the experiments more information, not only the function (shortening or force development), but also its derivative were simultaneously recorded, as can be seen from the diagram (No. 3 and 6). To study the behaviour of the muscle for a longer period of time, muscle activity was recorded on a high speed stripchart potentiometer (*ЭПП* 09 M 2 type) as well (No. 2). On the third and fourth channels the action potentials of the muscle and the nerve were recorded in other experiments. In these experiments only the third was used for time control (No. 7).

The stimulator (No. 1 and 5) and the sweep generator (No. 9) were synchronized to the camera with adjustable delay.

The muscle was stimulated with super maximum rectangular pulses. The time duration of the stimulating pulses was 1 ms. When the muscle activity was investigated under tetanic condition, an impulse-series of a time duration of 0.4 s was used with a frequency of 50 c/s.

Results

Fig. 2 shows one of our records of the shortening of an excised frog's gastrocnemius. The upper line represents the velocity curve, which attains its maximum value within a short time period. The oscillations on the derivation curve are

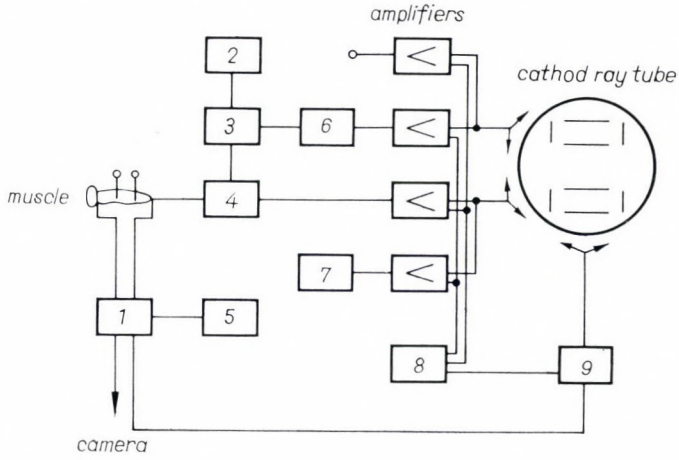

Fig. 1. Schematic diagram of the experimental arrangement. (1) stimulator, (2) recorder, (3) impedance transformer, (4) capacity transducer, (5) trigger oscillator, (6) differentiator circuit, (7) time marker, (8) electron switch, (9) sweep generator

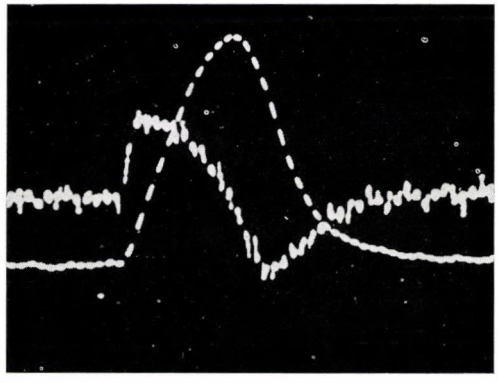

Fig. 2. Oscillogram of the shortening of the isolated frog's gastrocnemius. Upper line: velocity-curve. Lower line: time course of the shortening. Time mark: 5 msec.

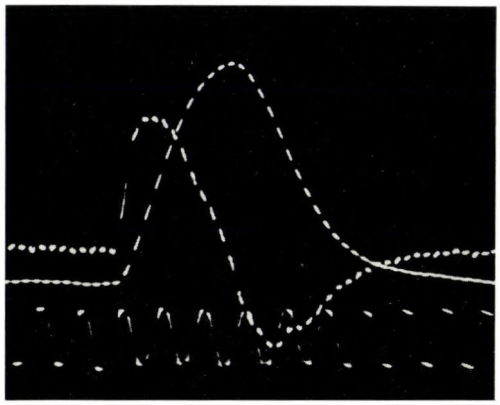

Fig. 3. Oscillogram of the force development of the isolated frog's gastrocnemius. Upper line: derivative of the force development, Lower line: time course the force development. Time mark: 5 msec, and 50 c/s

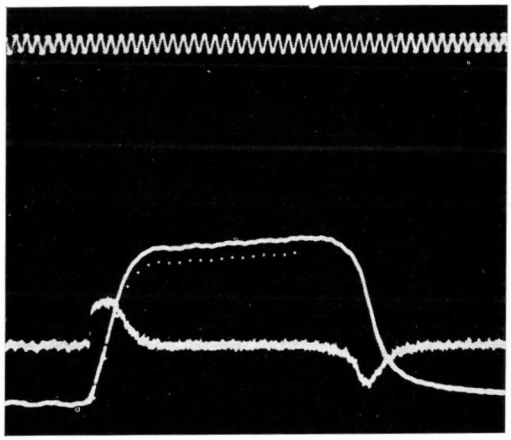

Fig. 4. Oscillogram of the shortening of the isolated frog's gastrocnemius under tetanic conditions. Upper line: velocity curve. Lower line: shortening. Time mark: 50 c/s

probably due to the irregular movement of the rubber spring keeping the muscle at resting length, or to other mechanical vibrations.

A record of the force development is represented in Fig. 3. The derivative dp/dt reaches its maximum value in the first third part of the contraction curve, which shows the presence of a reversal point in the rising phase. As can be seen

from the derivative curves in Fig. 2 and Fig. 3, the time courses of the shortening and the force development are different.

Fig. 4 and Fig. 5 show the muscle activity under tetanic conditions. The time duration of the stimulation in this case was 0.4 s; the stimulation frequency was 50 c/s.

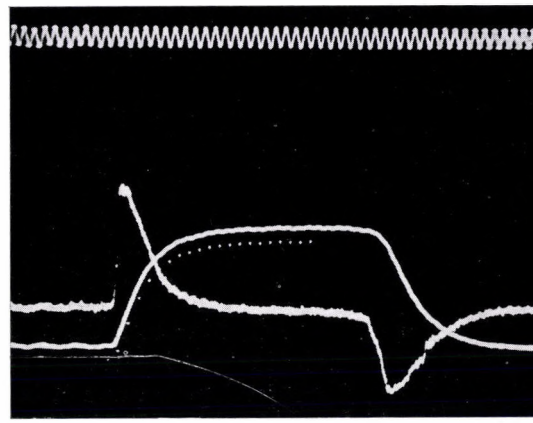

Fig. 5. Oscillogram of the force development of the isolated frog's gastrocnemius under tetanic conditions. Upper line: derivative of the force development, Lower line: curve of the force development. Time mark: 50 c/s

Discussion

The contraction curves obtained were enlarged and a detailed analysis was carried out from the magnified photographs. The contraction curves were measured and plotted as percentage of the maximum amplitude to eliminate the differences due to the different lengths of the muscles. The analysis showed that the shortening can be approached by the sine function:

$$\frac{r}{\mathrm{R}} = sin \frac{\pi}{2} \frac{t}{\mathrm{T}}$$

where r is the momentary shortening, t the time belonging to r; R the maximum shortening, T corresponds to the time during which the muscle reaches its maximum shortening. Its first derivative gives the time course of the velocity:

$$v = \frac{\mathrm{d}r}{\mathrm{d}t} = \frac{\pi \mathrm{R}}{2\mathrm{T}} \; cos \; \frac{\pi}{2} \frac{t}{\mathrm{T}}$$

From Fig. 2 it can be seen that the muscle at shortening attains its maximum velocity within a short time period – about 5 to 10 msec – which represents a small fraction of the rising phase of the contraction. If this part of the curve is neglected, a good agreement is found between the cosine function and the experimental data. This means that the velocity of the shortening is the highest in the first part of the contraction. Similar results were obtained by Abbott and Ritchie (1951).

On the other hand, Rosenblueth and Rubio (1959) have found by analyzing mammalian muscles' twitches under both isotonic and isometric conditions that a sudden reversal exists with the second derivatives. We have observed the same rapid change at the beginning of the shortening by taking into account the real form of the velocity curve in the oscillogram (Fig. 2).

A comparison of the time courses of the shortening and force development shows that the latter reaches its maximum earlier (Buchthal, Kaiser, 1951; Jewell, Wilkie, 1958). A similar result has been published by us earlier (Belágyi, Biró, 1963).

As regards the time course of force development, as it can be seen from Fig. 3 dp/dt reaches its maximum value relatively later than the derivative dr/dt. The maximum value shows the reversal point in the rising phase of force development. It means that the time course of force development cannot be described by the exponential formula proposed by Rosenblueth and Rubio (1959) for mammalian muscle in isometric twitches. (An accurate analytical description for force development is in preparation.)

Fig. 4 and Fig. 5 show the mechanical activity of muscle; shortening, force development respectively, under tetanic conditions. The electrical impulses stimulating the muscle are marked on the contraction curves as small points. As it can be seen from Fig. 4, the velocity reaches its maximum value after a rapid change and remains nearly constant in a time interval of 30 to 40 ms.

Ernst (1963) suggested that the rising phase of the tetanic shortening can be interpreted as the superimposing of single twitches. Since the superimposed first parts of the sine curves can be approached by a straight line, it can be expected that the velocity remains constant for a certain time. This is in good agreement with our experimental results.

On the other hand, the time course of force development, as shown in Fig. 5, is different from the shortening curve. This can be explained by various processes in the muscle during contraction, such as the crystallization of myosin during stretching.

Acknowledgement

The authors are indebted to Professor E. Ernst for his valuable advice and criticism.

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Relationship between the Resting Potential, Ion Content and Extracellular Space in Striated Muscle Treated with Hypertonic Solution

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The effects of 4 times hypertonic solution on resting potential, potassium content, inulin space, and chloride content of frog sartorii (Rana pipiens and Rana esculenta) were investigated. The hypertonic solution used was prepared with normal Ringer's by increasing the osmolality 4 times with sucrose. The muscles treated 60 minutes in hypertonic solution showed about a 50 per cent decrease of the resting potential, accompanied by a loss of weight of 30—35 per cent (water), no significant change in total muscle potassium, ca. 40 per cent relative increase of the inulin space and ca. 30 per cent decrease in the chloride content. These results appear inconsistent with the present formulations of the ionic theory of the membrane potential.

The problem of excitation — contraction coupling has in the past few years become one of the most prominent questions in muscle research (Sandow, 1952; Ernst, 1963; Fischman, Swan, 1964). In studying this problem (Tigyi, Ernst, 1959; Varga-Mányi, Tigyi, 1962) with the method of hypertonic solutions, we observed many details of the phenomenon. The dissociation of action potential and contraction comes into being regularly after 15—30 minutes of perfusion or incubation with hypertonic solution, and after changing back to normal Ringer's we can observe a relatively good reversibility of all functions (Ernst, 1959; Hodgkin, Horowicz, 1957). This cycle can be repeated about 3—5 times at room temperature. If we continue the perfusion or incubation of muscles longer than 30 minutes, the action potential also disappears, but it returns with a good reversibility after changing the hypertonic solution for normal Ringer's. Investigating the details of this latter phenomenon we measured the changes of the resting potential with internal microelectrodes (Tigyi, Fan Shih-fang, 1962). We have found that the resting potential begins to decrease after 30 minutes and drops to its half value by the first 60 minutes. This change of resting potential is also nearly reversible, but it lasts longer. 60 minutes after return to normal Ringer's the resting potential begins to increase; at 75 minutes the action potential also reappears, and at about 120 minutes the mechanical activity also returns.

In the present studies the details of changes of the resting potential in relation to changes in potassium content and in "extracellular" inulin space were investigated.

The main problem seems to be the following: according to the ionic theory the membrane potential is to be considered as the potassium equilibrium potential (Hodgkin, Katz, 1949; Hodgkin, 1958). On the other hand the data about the outward movement of potassium (Keynes, 1954; Harris, Burn, 1949) in the striated muscle suggested to us that such rapidly reversible changes of the potassium content as would be postulated by the membrane theory, are unlikely.

A significant number of publications emphasize the role of the chloride concentration in influencing the membrane potential, e. g. Hodgkin and Horowicz (1959) Kernan (1964). We have therefore also controlled the relative changes in the chloride content.

Methods

Isolated sartorius muscles of Rana pipiens and Rana esculenta were used. The frogs were kept a few days before the experiments in a cold room at 2 °C. Only the perfectly intact muscles were used for the experiments. The muscles used ranged between 40–200 mg in wet weight as in our earlier experiments. Solutions: The composition of Ringer's was: 112.5 mmoles of NaCl, 2.7 mmoles of KCl, 1.8 mmoles of $CaCl_2$, 2.4 mmoles of $NaHCO_3$. The hypertonic solution was prepared by adding 24.3 g (710 mmoles) sucrose to 100 ml of this Ringer's solution, so the ionic content of the hypertonic solution was exactly identical to that of normal Ringer's, but the osmotic concentration was elevated 4 times.

The determination of the resting potential was performed as described by Page (1962). Glass microelectrodes filled with 3 *M* KCl were used with a tip diameter of 0.5–1 μ. The water content of the muscles was measured by weighing the muscles to an accuracy of 1 mg. Before weighing the muscles were blotted by touching their surface slightly with ash-free filter paper.

For determination of potassium content, the muscles were dried and incinerated in an electric oven at 450 °C for 6 hours in platinum crucibles, then dissolved in 10 ml of distilled water, and measured with a flame photometer as previously described (Tigyi, 1962).

The inulin space was determined by using inulin carboxylic acid-C^{14}. The inulin-C^{14} was dissolved immediately before the experiments in solutions kept previously at 2 °C. Before the experiments the muscles were incubated for at least 12 hours in the labelled solution. At the end of the treatment the muscles were first carefully blotted with filter paper, and then extracted with 0.1 *N* HNO_3 solutions by shaking for 48 hours at room temperature (Page, Solomon, 1960).

The changes in the chloride content were measured using $NaCl^{36}$. The radioactive determinations were made with a Nuclear Chicago well type scintillation counter, using the Bray scintillation fluid (1962). The evaluation of the experimental results was generally carried out by a comparison of the two parallel sartorii from the same frog. The data are given in terms of mean $\pm$ standard error.

Results

1. Water loss in hypertonic solution

Fig. 1 shows the change of muscle weight during a 120 minute period after putting them into the hypertonic solution. The change of weight, which essentially parallels the water loss, has reached about 30 per cent in the first half hour, and by the end of the second hour has increased by only a few per cent. Weight losses greater than 45 per cent were found seldom in many hundreds of experiments

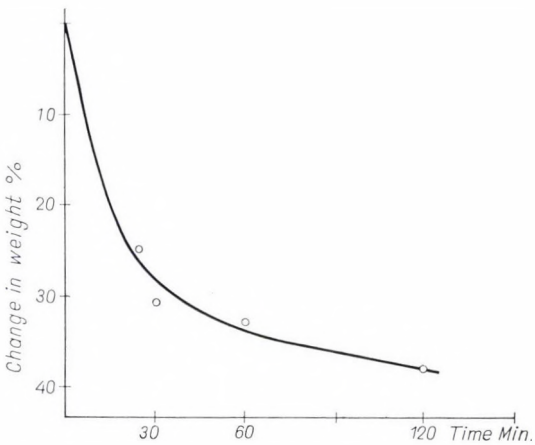

Fig .1. Relative changes of muscle wet weight in 4 times hypertonic solution

2. Changes of resting potential in hypertonic solution

Fig. 2 shows a series of measurements of resting potentials made in different fibres of the same sartorius muscle, as a representative example of a series of 9 muscles. The dotted line represents the earlier published result (Tigyi, Fan Shih-fang, 1962) of a similar series obtained on the sartorius muscle of Bufo asiaticus. The similarity of the time course of the two series shows that no essential difference exists between the two species in this respect. One hour after beginning the exposure to hypertonic solution, the resting potential shows an average decrease to 52 $\pm$ 3 mV from the original value of 97 $\pm$ 3 mV, a decrease by a factor 1.86. The resting potential at 120 minutes has recovered to 71 $\pm$ 3 (Each of these values is the average of 10 different measurements).

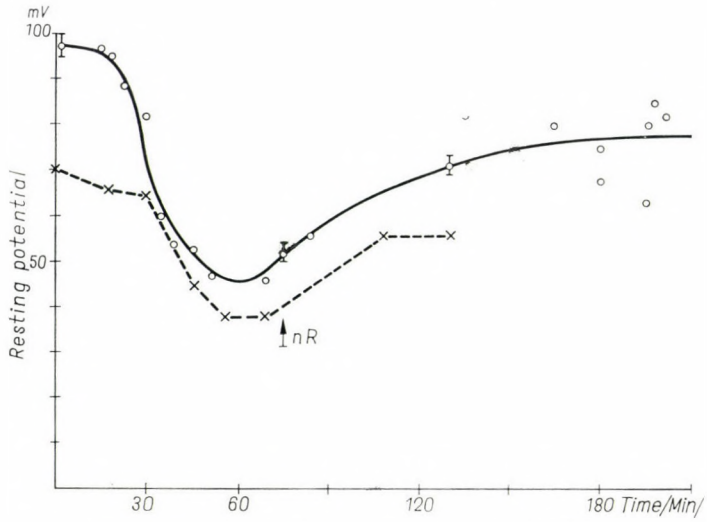

Fig. 2. Changes of the resting potential in 4 times hypertonic solution. The muscles were put in the hypertonic solution at 0 min; at the 75th minute the hypertonic solution was changed for a normal Ringer one. Dotted line: Bufo asiaticus (Tigyi, Fan Shih-fang, 1962). Straight line: Rana pipiens

3. The potassium content and exchange

The potassium content and exchange were studied by the determination of total muscle potassium, assuming that it gives a good approximation of the amount of cell potassium. The relative values of the extracellular potassium concentrations were approximated by the changes in the inulin space and by the potassium concentration of the external solution, because from this point of view we can assume that our system is approximately in the steady state. We used ^{42}K isotope for replacing the KCl of the Ringer's solution in order to be able to estimate possible changes in the K-movement.

Table 1 shows the results of a series of experiments in which one of the parallel muscles was placed in normal Ringer's, the other in hypertonic solution. The muscle weights were nearly identical at the beginning of the experiment, and the muscles placed in normal Ringer's do not show a significant change in their weight. We have therefore indicated in the table only the weights at the end of the experiment. We can see that the most extreme difference from the control in potassium content is less than 15 per cent. In agreement with this, the shift in the specific activity of ^{42}K is about 20 per cent on the average.

Table 1

Changes of water-potassium-content and K^{42} specific activity in sartorius muscles

No	Weight after incub. mg		K-content mg		K^{42} spec. activity $\frac{cpm}{mg}$	
	n	4x	n	4x	n	4x
1	218	170	0.11	0.10	36.4	44.9
2	248	214	0.12	0.10	27.3	43.8
3	132	100	0.08	0.07	33.0	41.0
4	184	130	0.09	0.09	18.2	26.6
5	270	192	0.15	0.14	13.3	18.3
6	238	178	0.13	0.11	14.3	26.8
7	188	120	0.06	0.06	92.0	100.0
8	192	132	0.06	0.06	72.5	93.0
9	234	174	0.12	0.12	8.1	11.2
10	182	122	0.09	0.07	9.3	15.4
11	184	130	0.08	0.08	18.1	15.6
12	180	140	0.08	0.08	79.0	84.0
13	150	114	0.06	0.05	70.0	86.0
14	142	102	0.06	0.07	83.5	60.0
15	168	118	0.08	0.07	36.0	44.5
16	188	124	0.08	0.07	43.5	49.0
17	240	178	0.11	0.10	43.5	53.0
18	170	120	0.08	0.07	55.5	71.5
19	160	120	0.06	0.07	55.5	55.5
20	230	174	0.12	0.10	42.5	59.2
21	158	110	0.08	0.07	43.5	51.0
22	230	160	0.11	0.10	40.0	51.7
Mean	195	142	0.09	0.08	42.4	54.6
St. error	±8	±7	±0.01	±0.01	±5	±5

4. The changes in the inulin space

From the point of view of the membrane theory it is very important to know how the ratio of the extracellular to intracellular spaces changes during exposure to hypertonic solution in order to be able to calculate the potassium concentrations. To obtain information about the extracellular space, we have measured the changes in the inulin space, which behaves similarly to the albumin space in frog sartorii (Tasker et al., 1959).

Muscles weighing not more than 100 mg were incubated at 2 °C for 12 hours in normal Ringer's containing inulin-C^{14}: the control muscle was then placed in normal solution at room temperature and the other in hypertonic solution at the same temperature. A comparison of the average values of 8 experiments lasting

Table 2

Change of the inulin space after 60 min in hypertonic solution

Treatment	Wet weight mg		Decrease per cent	C^{14}-counts Min. 100 mg of orig W W	Decrease per cent
	Beg.	End			
nR	74±5	73±5		1019±27	
4x	75±5	47±4	35	931±37	9

60 minutes is shown in Table 2. In hypertonic solution there is the usual 30 per cent decrease in the water content, accompanied by about a 10 per cent decrease of inulin-C^{14} radioactivity/100 mg of original wet weight. To picture the real change of the extracellular space, we have to make a correction for the changes in the total muscle weight. For this calculation we need the value of the inulin space in the normal muscle. This value according to our present measurements is 25.2±0.5 (per cent of wet weight), in good agreement with the results of Tasker et al. (1959). The new ratio of inulin space in the muscle treated with hypertonic solution expressed in per cent is:

$$25 \frac{iM}{Im} = 35 \text{ per cent}$$

(i and I are the inulin-C^{14} activities of muscles kept in the hypertonic and normal Ringer's, respectively, M is the muscle weight in Ringer's, m the same in hypertonic solution). This means a significant relative increase of inulin space.

5. Changes in chloride content

According to the classic theory, the difference between the external and internal chloride concentrations reflects the passive distribution of chloride ions according to the membrane potential (Hodgkin, 1958; Hodgkin, Horowicz, 1959; Kernan, 1964). In our experiments we have followed the changes in the total chloride content by comparison with the normal control using ^{36}Cl isotope. Table 3 presents the results of 8 parallel experiments. In these the muscles were preincubated for 12 hours at 2 °C in ^{36}Cl-labelled normal Ringer's. After this preincubation one group was incubated at room temperature for 30 minutes, the other for 60 minutes, in isotope-free normal Ringer's and hypertonic solution. The results show a 37 per cent to 38 per cent water loss, accompanied by a 30–35 per cent difference in chloride loss. The relatively small differences between the two groups allow us to conclude that the 60 minute values cannot be too far from the steady state.

Table 3

Changes in the chloride content of muscles in hypertonic solution after 30 and 60 minutes treatment

Time in 4x R. sol min	Wet weight mg		Decrease %	Cl-36 activity	Decrease %
	Beg.	End		Min. 100 mg orig W. W.	
0	66±5	67±4	—	1373±68	—
30	67±4	42±2	37	900±96	34
0	85±7	86±7	—	1104±60	—
60	86±7	53±5	38	786±32	29

Discussion

The present experiments are one of a series of experiments which have shown that the present formulation of membrane theory – in spite of its attractive mathematical formulation – has some difficulties in the explanation of some experimental facts (e.g. Tobias, 1950; Desmedt, 1953; Grundfest et al., 1954; Falk, Gerard, 1954; Shaw et al., 1956; Stephenson, 1957; Stämpfli, 1959; Koketsu, Kimura, 1960). In some of the experiments published by these authors, it is a fact that the muscles of nerves were investigated in an irreversibly damaged state. We have chosen a state of the muscle in which all of the essential functions are reversibly restorable. In our experiments, the muscles can produce a normal action potential, contraction and tension after return to normal Ringer's. The external ionic medium was not changed in the course of the experiments, osmotic concentration was elevated only by adding sucrose.

The resting potential decreases to nearly half of its control value under our experimental conditions. Using the classical formula of membrane theory for K concentration in the initial state

$$97 = 58 \log \frac{K_i}{K_o}$$

which is equivalent to a ratio of 48.7 between the inside and outside potassium concentrations. After 60 min the resting potential had fallen to 52 mV, which corresponds to a ratio of 7.9 i.e. we should expect a 6.2 fold decrease in the ratio of potassium concentrations. As Table 1 shows the maximum individual changes in the potassium content are less than 15 per cent. At the same time the maximum relative increase in the inulin space is about 40 per cent (Table 2), which does not allow a high enough elevation of external K concentration to satisfy the equation.

This calculation neglects the elevation of internal potassium concentration caused by the water loss, but it would increase the discrepancy between the theory and experimental values. We also did not consider the role of sodium. On the one hand sodium is not essential according to the theory, and on the other hand, our earlier measurements show a shift of less than 20 per cent (Tigyi, 1964).

Kernan's (1964) results have shown that the external concentration of Cl ions had a very essential influence of the resting potential (in an additive direction), when the muscles were soaked previously in sucrose solution (6 hours), and the internal chloride concentration was extremely reduced. In our experiments the situation differs from that in Koketsu—Kimura's and Kernan's experiments. In their experiments the potassium content was changed significantly and the values of the membrane potential did not drop as they should according to the theory. In our case the membrane potential has dropped to half its usual value, and we did not find the corresponding decrease in potassium content.

What kind of information can we get from the observed chloride and inulin space changes? The internal chloride concentration is given

$$Cl_i = (T_{Cl} - I_{Cl})\ (T_w - I_w)$$

(T_{Cl} is the amount of total chloride, I_{Cl} the amount of chloride in the inulin space, T_w the total water, I_w the amount of water in the inulin space). Assuming — as usual — that the chloride concentration of the extracellular space equals that of the bathing solution, and using the data of Tables 2 and 3, we never get a positive number. (Such a result used to be interpreted as a damage of the membrane; the reversibility of the functions does not support this argumentation.)

If we try to give some explanation for our results we have to emphasize the difference between our earlier results and those of Dydynska, and Wilkie (1963). In many hundreds of experiments (Ernst et al., 1950; Ernst, 1963) we never could see a larger water loss than 50 per cent in a 4 times hypertonic solution, in contrast to Dydynska and Wilkie's results which have shown — in single fibres measured by diameters — that it behaves as an osmometer. C.f. the new data of Blinks (1965).

According to our experiments — done also by measuring the depression of water vapour tension — a significant part of the muscle water exists not in an osmotic but in a bound (by swelling) state (Overton, 1962). If we want to calculate concentrations e.g. for the Nernst equation we have to take into consideration the free osmotic water only. At present lacking the exact quantitative ratio between osmotic and swollen water, this calculation would be only a formalism. But perhaps this way can help us to solve similar problems which we have found in our present experiments.

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Frequency-Modulation in Motor Nerves

Excitation II[1]

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(Received September 30, 1966)

1. The irritability and contractility of the muscle is influenced by its relative water content; in contrast to loss of contractility of the muscle perfused with hypertonic solution (decrease in the relative water content), the perfusion with hypotonic solution (increase in relative water content) results in long lasting "tetanoid" contractions elicited by a single electric shock.

2. The changes in the relative water content bring about, however, in some cases different results; thus, in contrast to the phenomena appearing in muscle perfused with hypertonic solution, similar decrease in the relative water content due to drying is accompanied by increased irritability.

3. In this state motor nerves are able to transform the nerve-impulse induced by a short single electric shock to a series of spikes. The importance of this result is discussed from the point of view of electronbiology and biocybernetics.

Introduction

The temporal and causal connection between excitation and contraction of the muscle is an old problem of myology. Notwithstanding, these two cardinal phenomena could be disconnected because contraction is preceded by the action current denoting excitation. In agreement with the action current, the same can be said about the initial volume constriction of the muscle. Furthermore, concerning disjoining contraction and excitation the data of Demoor and Philippson[2] should be mentioned, according to which muscle perfused with hypertonic solution produces normal action current without any sign of mechanical activity (Ernst, 1963). These results due to a relative decrease of the water content made us raise the question: what phenomena will be produced by a relative increase in the water content brought about by perfusing the muscle with hypotonic solution (Ernst, 1926; Mányi, 1959).

The experimental data obtained by investigating this question started new problems, the experimental investigation of which produced new data, and so on.

[1] First paper: Ernst (1966).

[2] The correct datum: Demoor, V. et Philippson, R. Travaux du lab. Physiol. Inst. Solvay. Tom 9 F. 1. 1908 and F. 2. 1909 (the different datum in this book is a misprint).

Accordingly, not following the known scheme of this periodical we divide the material to be published into chapters dealing with the questions following one after another in a logical order. Else, even this logical order would not be understandable, if we did follow the scheme of this periodical by describing in one single chapter called e.g. "Methods" all the different methods used to produce the different "Results". Similarly, if all experimental "Results" were described in one single Chapter, and all "Discussions" in another, the logical connection between a certain result and its discussion would be less evident.

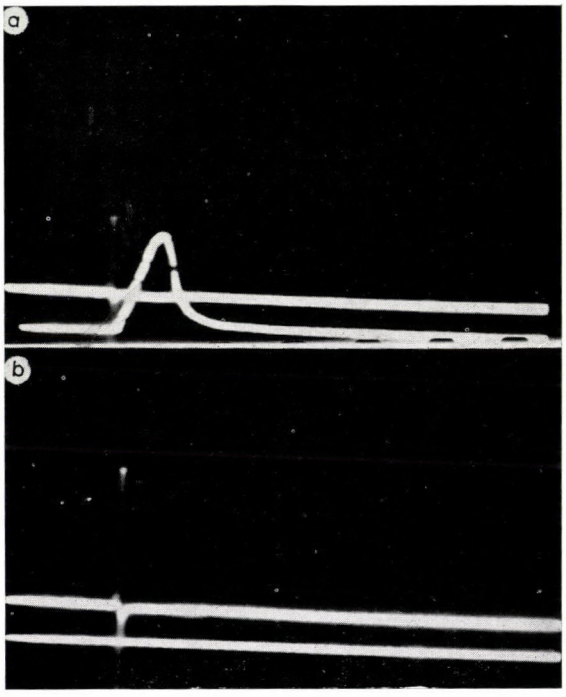

Fig. 1. *a)* free ("isotonic") shortening, *b)* muscle tied up ("isometric"); below: mechanical activity, above: action current

I. Tetanoid contraction due to relative water-richness

1. How are contraction and excitation affected by an increase in the relative water content?

2. The lower extremities (the so-called Läwen-Trendelenburg's preparation) were perfused with normal or 1/4 normal Ringer's solution (3.3 g NaCl, 0.1 g $CaCl_2$ anhydr., 0.1 g KCl, 0.1 g $NaHCO_3$ in 2000 ml bidistilled water). The

right plexus ischiadicus was stimulated with rectangular pulses of 0.1 – 100 Volt and 0.3 ms duration. The action current of the gastrocnemius was conducted by two loops of a fine platinum wire[3] to one pair of plates of the oscillograph and was recorded by the one cathode-ray. After being transformed to an electric signal the mechanical activity was registered (Varga-Mányi, Tigyi, 1962[4]; Mórocz-Juhász, Örkényi, 1966) by the other cathode-ray showing simultaneously time signals of 10 ms. The transit time of both rays could be modified (mostly 10 transits in 15 s, thus the period of one single transit over the screen was ~ 700 ms).

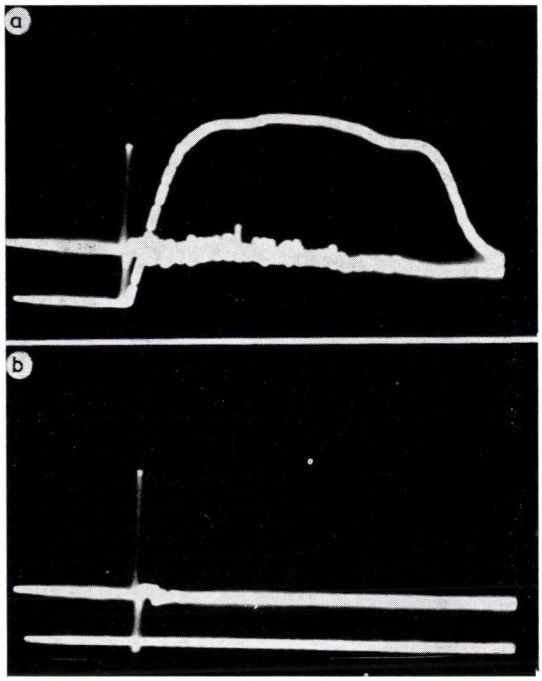

Fig. 2. *a)* free ("isotonic") shortening. *b)* muscle tied up ("isometric"); below: mechanical activity, above: electrical activity

3. Fig. 1*a* shows contraction and action current of the gastrocnemius perfused with normal Ringer's solution. A few minutes after this solution was changed over to a hypotonic one, the muscle performed long lasting "tetanoid" shortening but only one single normal action current followed by some electric impulses of aperiodic and different amplitudes (Fig. 2*a*). In contrast to that, the same muscle being stretched and thus hindered in shortening ("isometric" contraction) produced one single normal action current (Fig. 1*b* and 2*b*).

[3] One from the belly, the other near the Achilles tendon.
[4] See footnote 2.

4.*a)* The first point to be discussed refers to these variable action currents accompanying the mechanical activity (Ernst, 1963). Due to these irregular electrical impulses accompanying the tensionless "tetanoid" shortening, the question can be raised whether the so-called "deformation potential" (Schenck, 1895; Meyer, 1921) could play a role in this phenomenon.

b) The smooth tetanoid shortening is not accompanied by a known series of regular action currents, thus these experiments, too, demonstrate that contraction and excitation can be disjoined from each other.

c) The inability for shortening due to a decrease of the relative water content, on the one hand, and the contrasting result of the "tetanoid" shortening due to an increase in the relative water content, on the other, called our attention to the role possibly played by water in muscular activity. Especially the point of view should be mentioned, from which the electron-shift had been discussed in the recent literature dealing with the migration of protons in a regular chain of water molecules (Krogh, Ussing, 1937; Reynolds, Lumry, 1955; Szent-Györgyi, 1957; Gergely, 1961; Hindmann, 1962; Horne, Axelrod, 1964; Horne, 1964; Ernst, in press).

The experimental results described in this Chapter show that *muscles being relatively rich in water and stimulated with a short single electric shock perform long lasting tetanoid shortenings.* On the other hand, the references quoted above ascribe to the water content of an organ some importance in the process of excitation. Thus, the mechanical and electrical phenomena of the "hypotonic" muscle could be regarded as due to increased irritability accompanying relative water richness.

II. Increased irritability due to relative water shortage

1. This inference, however, contradicts some other older papers dealing with increased irritability due to a decrease of water concentration. Thus, muscles treated with glycerin (Kühne, 1888 and 1890) or dried to a certain degree (Langendorff, 1891; Durig, 1903) have been shown to perform more or less smooth tetanic contractions as a response to single stimuli. (The contrasting results due to relative water shortage of hypertonically perfused muscles have been mentioned above.)

Furthermore, the tetanoid contractions of hypotonically perfused muscles (Chapter I) have been recorded in experiments in which the hind legs were perfused, but the plexus ischiadicus was not and therefore exposed to drying. Thus, the plexus could have caused by its increased irritability those tetanoid contractions which were considered (Chapter I) as being due to relative water richness of the hypotonically perfused muscle.

2. This question was experimentally investigated with a method nearly identical with that described in Chapter I. The hind legs were perfused through

the aorta with *normal* Ringer's solution, the plexus ischiadici stimulated with single electric shocks.

3. Figs 3*a* and *b* show, in the initial phase of the experiment, normal records of contraction and action current *a)* in case of free shortening ("isotonic" at rest length) and *b)* when the gastrocnemius being tied up was prevented from shortening ("isometric" at rest length). In 20–30 minutes ($\sim$ 25 °C) the plexus was dried to a certain degree and the gastrocnemius, perfused with normal Ringer's,

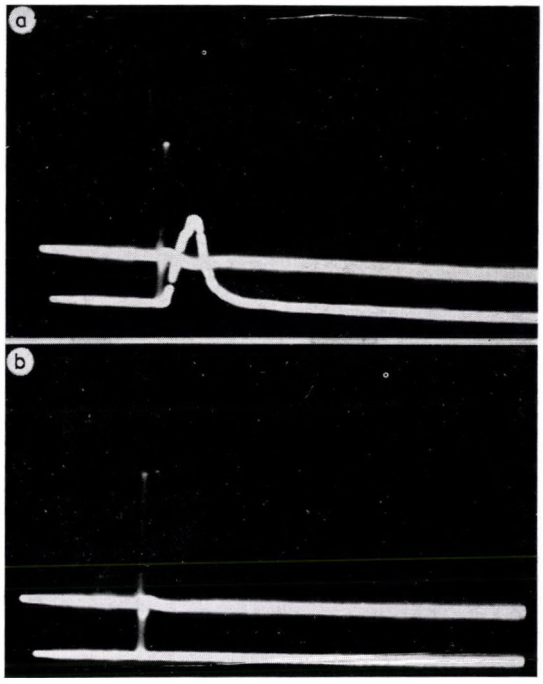

Fig. 3. *a)* free ("isotonic") shortening. *b)* muscle tied up ("isometric"); below: mechanical activity, above: electrical activity

produced tetanoid shortenings and a series of electric impulses (Fig. 4*a*); the same gastrocnemius, when tightened performed, of course, no shortening, notwithstanding, produced similar series of electric impulses (Fig. 4*b*).

4. Since the gastrocnemius has continuously been perfused with normal Ringer's solution, *the tetanic shortening and the series of electric impulses produced by the muscle should be ascribed to the nerve being dried to a certain degree.* This inference is corroborated by the experience that such phenomena never occur if the plexus is continuously wetted with Ringer's solution (see Chapter IV).

III. Disjunction of contraction from excitation

Regarding what has been said in the preceding Chapters I and II we performed experiments in which the muscles were perfused with 1/4 n Ringer's solution and the plexus being wetted with normal Ringer's for the whole duration of the experiment was prevented from drying. Fig. 5 shows, under these conditions too, identical phenomena as described in Chapter I and proves that *mechanical and*

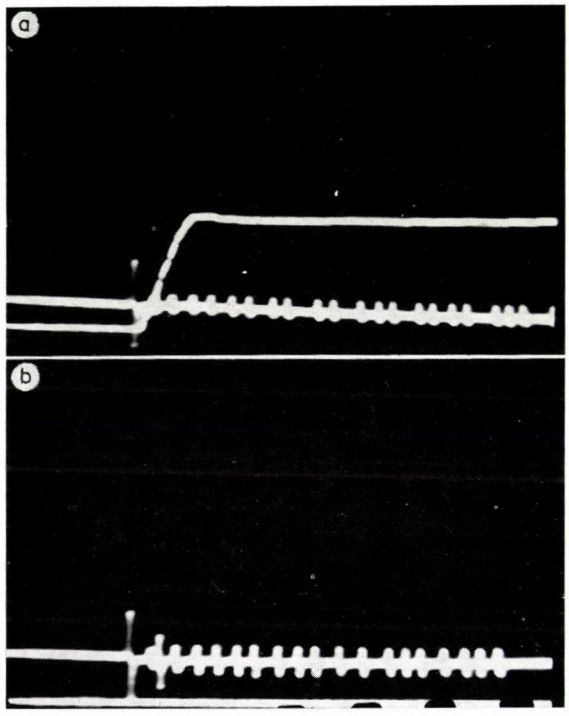

Fig. 4. *a)* free ("isotonic") shortening, *b)* muscle tied up ("isometric"); below: mechanical activity, above: electrical activity

electrical phenomena of the muscle perfused with hypotonic solution *do not behave in the same way*. This demonstrates again the separability of contraction from excitation.

The separability of contraction from excitation can similarly be demonstrated by Fig. 6 showing that the series of electric impulses come earlier to an end than the tetanoid shortening. That is to say the second part of the tetanoid shortening is not accompanied by electric phenomena denoting excitation. That means that electrical activity (excitation) cannot be regarded as being inseparably connected

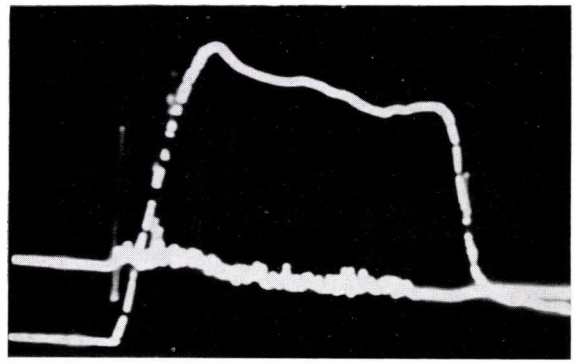

Fig. 5. See Fig. 2 *a*

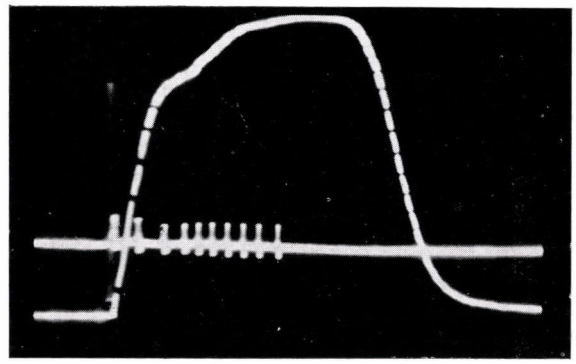

Fig. 6. See Fig. 4 *a*

with contraction. This latter will not be treated further in this paper[5]; the question of excitation however, will be the topic of the following part of this paper.

IV. Frequent spikes on motor nerves due to a short single stimulus

1. In Chapter II the experiments and Fig. 3 and 4 demonstrated that the gastrocnemius perfused with *normal* Ringer's solution produced tetanic shortening and a series of spikes, when the plexus ischiadicus being exposed to drying, was stimulated with a single short electric shock. These results and others of con-

[5] All the less because the number of data and investigations concerning contraction and its mechanism is continually increasing; the immense literature of this question, therefore, should earlier be filtered through a membrane permeable only for data of objective scientific value.

trol experiments made us infer that the frequent spikes produced by the gastrocnemius originated from the nerves of the plexus the irritability of which has been increased due to previous drying. The fact, however, should equally be considered that muscle too can possess a so-called fundamental frequency (Bethe, 1952; Ernst, 1963).

That motor nerves can *propagate* frequent excitatory impulses has been long known, but now the question is to be investigated, better to say to be proved, whether motor nerves are able to transform a single electric sign to a series of frequent impulses.

2. In order to investigate this question experimentally nervus ischiadicus inside the right thigh[6] was fitted on a pair of platinum electrodes serving for the conduction of the action current. Under such circumstances the ischiadic nerve remains normal and does not dry. The gastrocnemius without perfusion was made free for shortening and the skin over the muscle was constantly wetted with normal Ringer's solution. The course of the experiment was the same as described above but the action currents of the nervus ischiadicus were recorded instead of those of the musculus gastrocnemius.

3. Fig. 7*a* shows at the beginning of the experiment normal shortening of the gastrocnemius (below) and the action current of the nervus ischiadicus (above) when the plexus ischiadicus was stimulated with a short single electric shock. The distance between the pairs of electrodes – one pair on the plexus for stimulation, the other on the nervus ischiadicus for the conduction of the action current – was $\sim$ 5 cm, and thus the possibility of the appearance of different waves (α, β, etc.) can be explained. Fig. 7*b* demonstrates tetanoid shortening of the gastrocnemius and a series of action currents of the nervus ischiadicus; these were recorded when the plexus was stimulated as earlier, but after being dried to a certain degree. Fig. 7*c* shows the result produced by a stimulus of greater intensity.

Figs 7*d e*, and *f* demonstrate that the series of frequent spikes originates in the plexus and is to be ascribed to its drying. Fig. 7*d* shows normal shortening of the gastrocnemius and normal action current of the nervus ischiadicus when the plexus was wetted with Ringer's solution in a later phase of the experiment. Fig. 7*e* demonstrates the results in a repeated drying phase, and Fig. 7*f* in a repeated wetting phase.

4. The results of the experiments described in Chapters II and IV indicate that *the motor nerve is able to transform a single impulse (sign) to a series of excitatory impulses of a certain frequency*. This series of impulses is propagated through the nerve to the effector and gives rise to the tetanoid shortening of the gastrocnemius.

[6] In the bottom of the ditch appearing when musculus gluteus magnus and iliofibularis are pulled apart (to a certain degree).

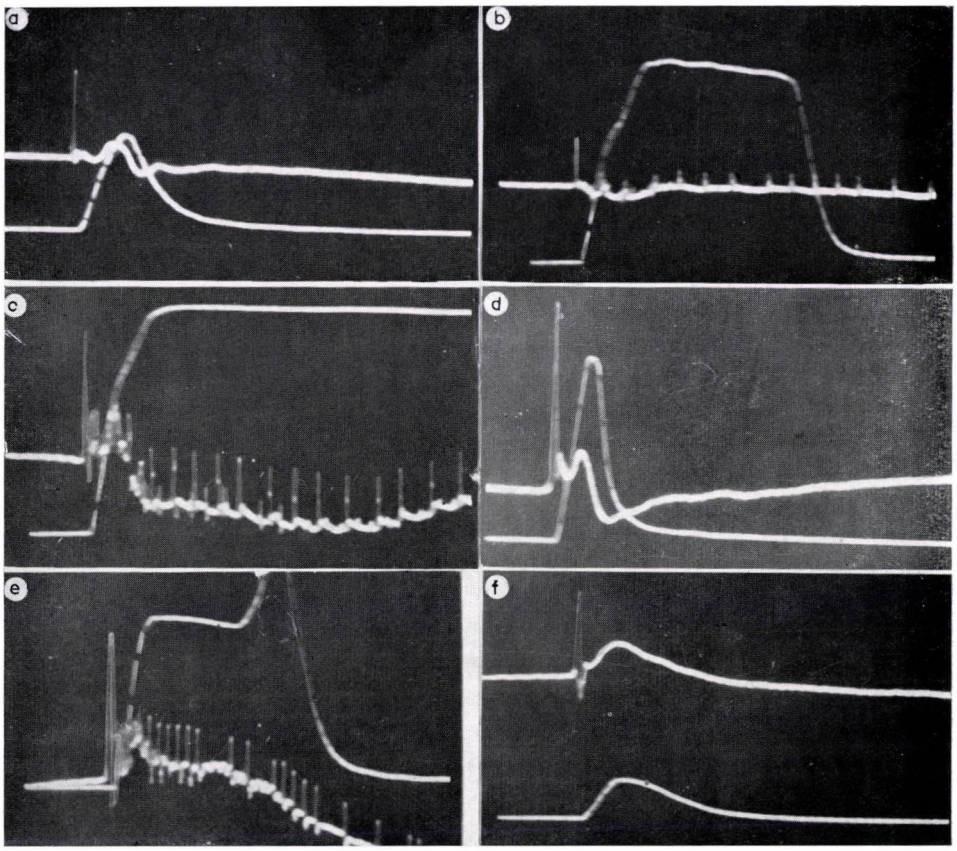

Fig. 7*a—f*. See Fig. 4 *a*: in different phases of an experiment

V. Frequency-modulation as nerve-function

The fact is generally known that sensory nerves transform the generator (or receptor) potential to a series of frequent nerve-impulses (literature e.g. Ernst, 1966). In contrast to that, the motor nerves are known to be able only to propagate but not to originate frequent impulses. Nevertheless, Frey's ingenious intuition is to be mentioned here (Frey, 1883): "Es muss also dem Nerven die Fähigkeit zugetheilt werden, den stetigen Verlauf des constanten Stromes in getrennte Erregungsstösse umzusetzen . . ." ("The ability should be ascribed to nerves to transform the constant course of d.c. into separate impulses of excitation . . .").[7]

[7] Namely, muscle was known at that time to produce tetanoid contraction when the motor nerve was stimulated with d.c.

On the other hand, the circumstance also should be mentioned that Frey's hypothesis has not been particularly promoted by the literature (Fessard, 1936; Katz, 1936; Ernst, 1966).

Therefore, it is perhaps proper that the series of excitatory impulses should be shown to be very variable. Fig. 8 is taken from the same experiment as Fig. 6 and demonstrates a very high frequency due to very great intensity of the stimulus; Figs 7*b* and *c* too show the great variety in the reaction of the drying plexus stimulated with single electric shocks. Figs 9*a*, *b*, *c* and *d* represent different phases of an experiment using equal stimuli and yet displaying different reactions probably

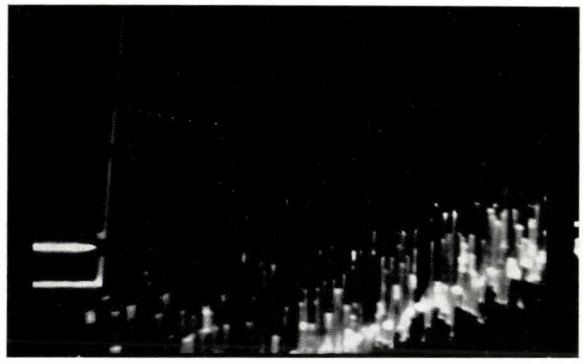

Fig. 8. As Fig. 6, stimulus is of very great intensity

due to different states of the plexus during drying (gastrocnemius was constantly perfused with n Ringer's).

On the basis of what has been said above it can be stated that, besides the sensory nerve, *the motor nerve* also possesses the ability to transform a single excitatory impulse, due to a single short stimulus, to a series of frequent impulses and propagate them.

The formation of frequency, or rather the phenomenon and importance of *automatic rhythmicity* in biology and a detailed discussion of these questions are beyond the scope of this paper. Neither do we want to discuss here *the mechanisms* of the automatic rhythmicity in biology but to emphasize that all the questions mentioned above are in close connection with electronbiology, with the knowledge of semiconductors (Ernst, 1956; 1966) and also with biocybernetics (Ernst, 1967). These topics also surpass the limit of this paper and consequently they are referred to the teams of electronbiology and biocybernetics, resp. which are working in our Institute.[8]

[8] This paper containing descriptions of experimental facts without quantitative analysis is published in this periodical to remain in close connection with papers being published in the future and having been published earlier from our Institute.

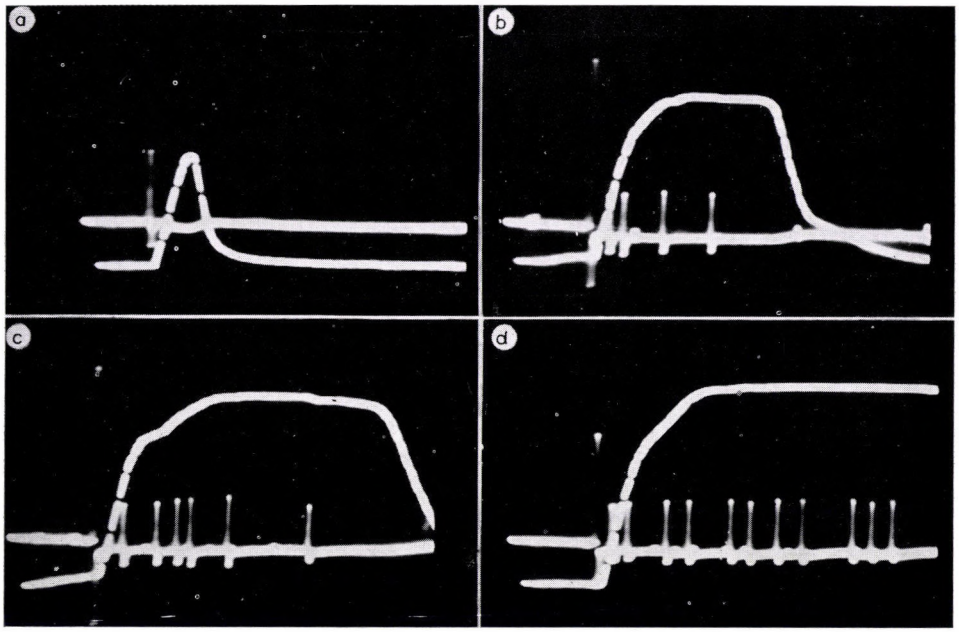

Fig. 9 *a—d*. See Fig. 4.: in different phases of the experiment

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Effect of Visible Light and Ultraviolet Rays on the Stimulus Threshold of Frog Muscle Sensitized by Eosin

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The stimulus threshold of *musculus sartorius* sensitized by eosin decreases significantly under the effect of visible light or ultraviolet rays; the stimulus threshold of muscles containing no sensitizer does not change. Results of recent experiments support the electronic theory of irritation.

Introduction

The cause of the photodynamic effect (Marcacci, 1888) may be interpreted as follows: The photon energy absorbed by the sensitizer passes into the molecular electronic system of biological substances as electron-excitation energy and may induce there different biophysical or biochemical processes (Ernst, 1963). There are about 400 chemicals which are presently known to act as photosensitizers (Santamaria, Primo, 1964). Photodynamic action can appear e.g. in the alteration of irritation, or it can induce spontaneous occurrence of irritation (Lippay, 1929; 1930; 1930a; Lakatos, Kollár-Mórocz, 1966). The present series of experiments is connected with these effects. It has been investigated whether visible or ultraviolet light will influence irritability in the presence of eosin. The purpose of these experiments was to yield new data supporting the electronic theory of irritation (Ernst, 1966).

Methods

1. Experiments with muscles *in situ*.

Eosin (Na-tetra bromine-fluorescein) was introduced into the muscle by perfusion of Ringer solution containing eosin at a low concentration. A Läwen-Trendelenburg's preparation was made by removing the skin from one of the thighs of the frog. The distal end of *musculus sartorius* was made free and was attached by a fine silk thread to the perceiver of a mechanoelectric transducer type RCA 5734. The impulse given by the transducer passed to a cathode-ray oscilloscope. When the deflection of the beam on the screen was 1 cm, the movement of the perceiver (and at the same time the shortening of the muscle) was 0.1 mm (Fig. 1). This

movement of the perceiver took place under the effect of a force of 0.3 g. To illuminate the muscle the apparatus described in a previous paper was used (Lakatos, Kollár-Mórocz, 1966). The illumination intensity was 80,000 lux. For ultraviolet irradiation a high pressure mercury vapour lamp (type HBO 50, Zeiss, Jena) was used. The visible and infrared parts of the spectrum were eliminated by a glass filter of Jena, type UG 1, 1.5 mm thick. Illumination by both visible light and ultraviolet rays covered the total surface of muscle.

Stimulation was carried out by electrical impulses produced by a transistorized generator. The duration of impulses was 1.0 ms and 0.1 ms in the experiments with visible light and ultraviolet rays, respectively. The voltage of impulses could

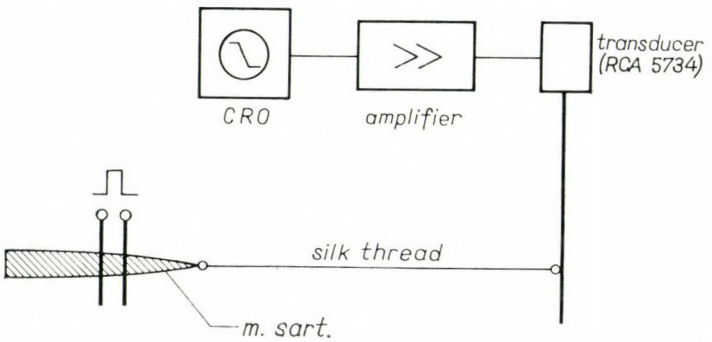

Fig. 1. Sketch of the apparatus used for measuring the stimulus threshold of muscles *in situ*
CRO: cathode-ray oscilloscope

be adjusted with an accuracy of 0.01 V. The stimulating electrodes were made of platinum, their position is shown in Fig. 1. The experiment was carried out as follows:

The frog was perfused with Ringer solution under a water pressure of 25 cm, the stimulus threshold was measured a few times. After perfusion for half an hour the muscle was illuminated for two minutes and just before terminating the illumination the stimulus threshold was measured again. For the next half an hour the frog was perfused with Ringer solution containing eosin in a concentration of 10^{-5} g/cm^3, the stimulus threshold being measured before and during a repeated illumination. In a number of cases two experiments were carried out with the same frog. In such a case the frog was perfused with Ringer solution (without eosin) for two hours after finishing the first experiment in order to remove eosin as perfectly as possible. In two hours the solution trickling out did not show any colour.

2. Experiments with isolated *musculus sartorius*. One half of the excised muscles was placed in Ringer solution and the other half in the same solution but containing eosin in a concentration of 10^{-4} g/cm^3. The preparations were kept at a temperature of $+1$ °C for about 20 hours, and immediately before beginning the experiment at room temperature for about 2 hours. Each muscle was attached

to a holder made of plexiglass (Fig. 2) fixing its proximal end to a pointer which magnified ten times the movement of the muscle. A rubber thread in 0.5 mm diameter ensured a stretching force of 4 g when the pointer was set to zero. A stretching force of 0.2 caused a deflection of 1.0 mm of the end of the pointer, which corresponded to a shortening of muscle of 0.1 mm, therefore this simple method proved sensitive enough to measure the stimulus threshold with an accuracy of 0.01 V. Each muscle was continuously moistened with Ringer solution.

The visible light was produced by a projection bulb of tungstene filament (Tungsram, type 64579, 12 V, 100 W, provided with an elliptic cave mirror in

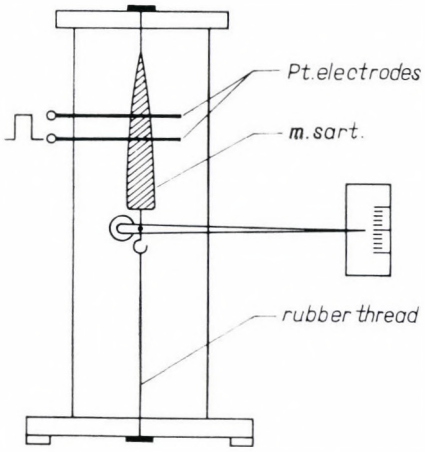

Fig. 2. Sketch of the apparatus used for measuring the stimulus threshold of isolated muscles

order to collect the light of the incandescent filament). The illumination intensity was 100,000 lux. Ultraviolet light was produced by a hydrogen lamp (type H2-0.3 VEB, Berliner Glühlampenwerk) having a quartz lens to collect the rays on an area of 0.13 cm^2; this point was 17 cm from the lens. The light flux was here 0.35 W/cm^2 measured with thermopiles. (The light sources differed from those with which the *in situ* experiments were carried out because of the simultaneity of both series of experiments.) Only the spot of stimulation was irradiated on a surface 5 mm in diameter.The visible and infrared part of the spectrum was eliminated by a glassfilter of Jena, type UG 2. Stimulation was brought about by the use of an electronic impulse generator, the duration of the impulses was 1.0 ms, their voltage could be controlled with an accuracy of 0.01 V. The stimulus threshold was measured immediately before the illumination and during its last period, respectively. As it was measured by a thermistor, the increase of temperature due to the energy of irradiation was not higher than 0.5 °C in case of visible light, and it was not measurable in case of ultraviolet irradiation, neither during the experiments performed with muscles *in situ*, nor in those with isolated ones.

Results

A series of measurements was carried out during every single experiment with muscle *in situ* and the measured voltage of stimulus threshold was plotted against time (Fig. 3). 4 data of measurement were taken into consideration: the

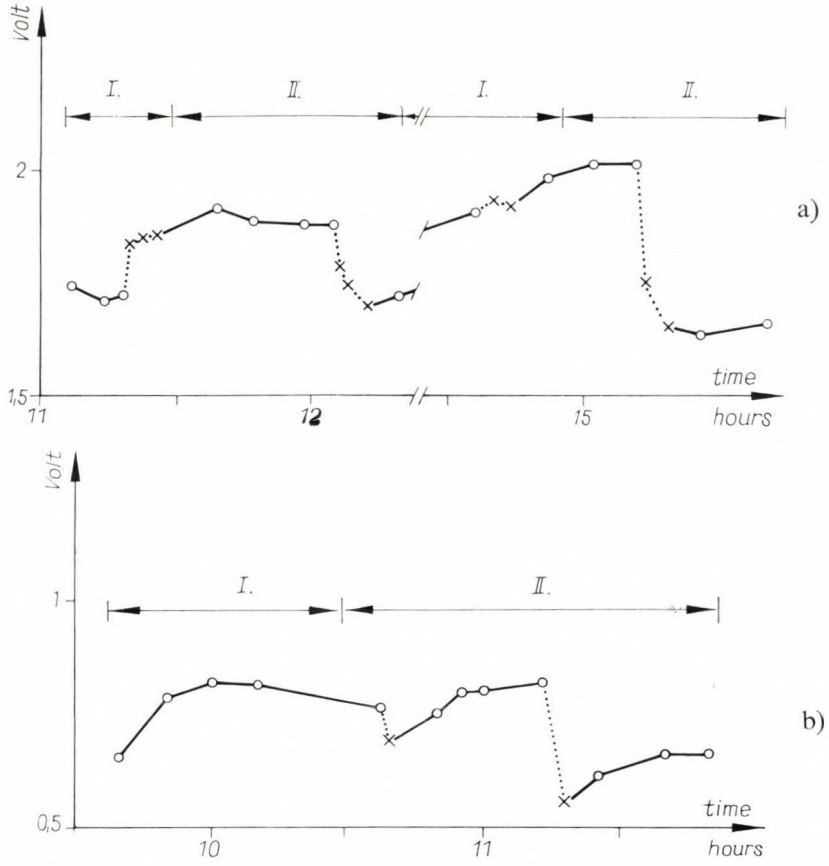

Fig. 3. The time course of experiments with muscles *in situ* performed *a*) with visible light and *b*) with ultraviolet rays. The voltage of stimulus threshold is plotted against time. ○ stimulus threshold without irradiation; × stimulus threshold during irradiation; Dotted line: duration of illumination. Period I: perfused with Ringer solution, Period II: perfused with Ringer solution containing eosin

voltage of the stimulus threshold measured immediately before the illumination, and that measured during the end period of the second minute of the irradiation, both during perfusion with normal Ringer solution and in the presence of eosin. The alteration taking place during irradiation was calculated in each case.

I. In situ experiments

1. 26 frogs were investigated during irradiation with visible light. *a)* During perfusion with Ringer solution a diminution of the stimulus threshold was observed in 10 cases and there was no change in 6 cases. *b)* During perfusion with Ringer solution containing eosin 40 measurements were performed with 30 frogs.* Decreased stimulus threshold was found during the illumination in 31 cases, the stimulus threshold increased in 7 cases, and there was no change in 2 cases.

2. *a)* The effect of ultraviolet irradiation was investigated in 23 experiments using 17 frogs perfused with normal Ringer solution. During the irradiation the stimulus threshold diminished in 8 cases, increased in 9, and did not change in 6 cases. *b)* When perfusion was carried out with Ringer solution containing eosin, the stimulus threshold diminished upon irradiation in 19 out of 24 cases, increased in 2 cases, and did not change in 3 cases. The quantitative data of these experiments are summarized in Table 1.

Table 1

Quantitative results of experiments in situ

The values for the stimulus threshold are given in Volts. Muscles illuminated by visible light were stimulated by electric impulses of 1.0 ms, and the muscles irradiated by ultraviolet rays were stimulated by impulses of 0.1 ms

	Muscles containing eosin		Muscles perfused with Ringer solution		
	Before illumination	During illumination	Before illumination	During illumination	
Mean value of stimulus threshold	0.70±0.39	0.55±0.31	0.70±0.55	0.70±0.56	Irradiation by visible light
Change of stimulus threshold	—0.15±0.25		0.00±0.12		
Significance	$t = 6.01$ ($t = 2.66$ if $P = 0.01$) $n - 1 = 64$				
Mean value of stimulus threshold	1.49±0.61	1.36±0.54	1.34±0.45	1.34±0.48	Irradiation by ultraviolet light
Change of stimulus threshold	—0.13±0.17		0.00±0.19		
Significance	$t = 2.3$ ($t = 2.02$ if $P = 0.05$) $n - 1 = 45$				

* In one part of the experiments the frogs were investigated only during perfusion with Ringer solution containing eosin.

II. Experiments performed with isolated muscles

Results were similar. The only difference was that the voltages of stimulus threshold were lower, but similarly to the experiments carried out with muscles *in situ*, the diminution of the stimulus threshold of muscles containing eosin was about 10 per cent during irradiation.

1. 56 muscles were subjected to the action of visible light. *a)* The stimulus threshold of 24 out of 30 muscles containing eosin decreased during illumination, while it increased in 3, and it did not change in 3 cases. *b)* Out of 26 eosin-free muscles a diminution of stimulus threshold was experienced in 3 cases, an increase was observed in 5 experiments and no measurable change was found in 18 cases.

2. 47 isolated muscles were exposed to the action of ultraviolet rays. *a)* 22 of them contained eosin, 20 of these showed a diminution of stimulus threshold, one exhibited an increase and the stimulus threshold for one remained unchanged. *b)* Of the 25 muscles kept in normal Ringer solution as a control the stimulus threshold diminished in 6, increased in 3, and did not change in 16 cases during irradiation. Quantitative data of these experiments are summarized in Table 2.

Table 2

Quantitative results of experiments performed with isolated muscles
The values of the stimulus threshold are given in Volts

	Muscles containing eosin		Muscles kept in Ringer solution		
	Before illumination	During illumination	Before illumination	During illumination	
Mean value of stimulus threshold	0.41±0.17	0.36±0.14	0.36±0.14	0.36±0.14	Irradiation by visible light
Change of stimulus threshold	—0.05±0.05		0.00±0.02		
Significance	$t = 4.37$ ($t = 2.66$ if $P = 0.01$) $n - 1 = 54$				
Mean value of stimulus threshold	0.40±0.12	0.37±0.11	0.39±0.12	0.39±0.12	Irradiation by ultraviolet rays
Change of stimulus threshold	0.03±0.02		0.00±0.02		
Significance	$t = 4.73$ $n - 1 = 45$ ($t = 2.7$ if $P = 0.01$)				

Discussion

According to our results the stimulus threshold of the *musculus gastrocnemius* of frog decreases under the simultaneous action of eosin and light. This conclusion has been checked qualitatively by the χ^2-test. It was found that $\chi^2 = 33.7$, when illumination was performed with visible light, and $\chi^2 = 15.5$, when ultraviolet rays were used for irradiation. That means in both cases, that the distribution of data obtained by experiments on muscles containing and having no eosin differ from each other at a probability level of 0.001.

The quantitative evaluation has shown, that investigating muscles without eosin content, the average change of the stimulus threshold was less than the accuracy of measurement and therefore cannot be considered as a real change in any experiment using either isolated muscles or muscles *in situ*. The stimulus threshold of muscles containing eosin diminished in every experimental series upon illumination with visible light. In every case a statistical test was used to see, whether the decrease of the stimulus threshold of muscles containing eosin differed significantly from the negligible decrease experienced with the muscles containing no eosin. In every case the decrease of stimulus threshold was found to be significant at a level of $P = 0.01$ (and in a single case at a level of $P = 0.05$). (Tables 1 and 2.) Hence, the statistical tests supported the conclusions drawn from the analyses of single experiments (see e.g. Fig. 3), that visible light or ultraviolet rays decreased the stimulus threshold in the presence of eosin. This result is in accordance with the data presented in a previous paper (Lakatos, Kollár-Mórocz, 1966) in which we reported the stimuli producing effect of light in experiments performed on isolated frog hearts. Experiments carried out with fresh-water mussels (Lábos, Turcsányi, 1966) have shown, that illumination by visible light produced contractions in the presence of different fluorescent dyes (among them eosin). Spontaneous formation of excitation can occur without the use of any sensitizer under the influence of high energy electromagnetic waves (e.g. γ-rays), as it was pointed out by Niedetzky (1966) in a paper on isolated frog hearts. The recent experiments do not give a direct information as to the details of the mechanism of this phenomenon. However, it is safe to suppose that the relatively low photonenergetic visible light and the ultraviolet rays pass their energy to the easily excitable fluorescent dye having an excited state of a long lifetime. In this way the energy is capacitated to migrate to the irritable structure of muscle and to excite the electron system of molecules forming the biologically irritable structure. This process is very likely to be the cause of increased excitability.

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Effect of Ionizing Radiation on the Center of Automatism of Frog Heart

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The automatism of isolated sinus pieces of frog heart stops in K-free solution. The automatism is reestablished in a part of the sinus pieces (40 per cent) in K-free solution containing radioactive Na-24. Thus, ionizing radiation has an effect on the center of automatism. The effective dose was found to be 1 to 130 rad in the experiments.

Introduction

According to the classical investigations of Zwaardemaker (1921, 1923) radioactive radiation influences the heart automatism of cold-blooded animals. Verkhovskaya and Arutunova (1953) as well as Hoitink and Westhoff (1956) have reported similar effects. The effect of radioactive solutions on the activity of isolated frog heart was investigated by using artificial radioactive isotopes. These investigations support Zwaardemaker's results mentioned above (Niedetzky, 1958; Tigyi et al., 1958; Ernst et al., 1959; Niedetzky, Hajnal-Papp, 1963). Thereafter the effect of external γ-radiation on isolated hearts arrested in K-free solution was investigated. It has been shown that the automatism of the arrested frog hearts can be reestablished by external radiation (Niedetzky, 1966). Guttmann (1936; 1936a) has observed a similar effect with ultraviolet radiation.

The rhythmical contractions which sustain the automatism of the frog heart originate from the sinus. From here the excitation propagates first to the auricles then to the ventricle. In addition to the sinus the upper third part of the auricle and the ventricle is also able to produce automatic excitations. Thus, individual parts of the heart are able to function even in an isolated state. Under normal conditions the stimulus of the sinus directs the automatic activity of the whole heart. The function of the sinus to produce stimuli has been reviewed by Cranefield and Hoffman (1958) on the basis of electrophysiology.

The sinus has different structural (Yokochi, 1929) and functional characteristics compared to the other parts of the heart. The glycogen content, the width of the fibre, and the speed of propagation of the heart muscle fibre increases in the order of sinus- ventricle- auricle and Purkinje fibres, the ability for rhythmical

contraction decreases in the same order (Davies, Francis, 1941). On the basis of these differences it can be supposed that the radiation effects on different parts of the heart can be different, too. The data of Bisceglie and Bucciardi (1929) support the same conclusion. They have investigated the effect of Ra-radiation on tissue cultures of embryonic chicken heart. They have found that upon irradiation the force of contraction of the fibrils of the auricle increases, whereas no such effect occurs with the fibrils of the ventricle. Some other data indicate that K in a certain concentration paralyzes the heart muscle and at the same time excites the center of automatism (Kataishi, 1925). According to the data of Rössler (1925) the sinus is not only the center of automatism of the heart, but constantly exerts an inhibitory action on the musculature of the ventricle. According to Ernst's data (1966) the automatic activity of sinus pieces becomes reversibly paralyzed in K-free solution. Ernst and Gábor (1966) have reported from our institute that the acetylcholin sensitivity of sinus pieces kept in K-free solution increases compared to similar preparations kept in normal Ringer solution. The aim of the present experiments is to yield informations concerning the mode of action of ionizing radiation on the sinus.

Methods

The experiments were performed with sinus pieces of frog (Rana esculenta) heart. The frogs were decapitated and the hearts were isolated after destroying the spinal cord. The isolated heart was put into Ringer solution, and the sinus bundle on the right side auricle was excised. The sinus was then cut into as many pieces (usually 3 to 5) as possible. The automatic activity of the sinus pieces stopped for a short time (on the average for 10 to 15 minutes) following preparation. After waiting for half an hour the well functioning sinus pieces were selected and only these were used in the experiment. The experiments were carried out at room temperature. The frequency of activity was controlled in each case before starting the experiment. It was found to be 50/min on the average.

To paralyze the automatic activity of the sinus pieces, isotonic K-free solution was applied. The composition of the solution was the following:

0.68 g NaCl
0.02 g $CaCl_2$
0.02 g $NaHCO_3$ in 100 ml

redistilled water. The solution had a pH of 6.8 to 6.9.

Two kinds of solutions with identical chemical composition were used in the experiments. The only difference between the two solutions was that one of them was inactive, the other was radioactive. In this latter, part of the NaCl was substituted by radioactive $^{24}NaCl$. The well functioning sinus pieces were divided into two groups. The effect of radiation was investigated by two methods.

1. Part of the sinus pieces was placed in K-free inactive solution, the other part was put into an active solution of the same composition. The period of time until the automatism stopped was measured.

2. The sinus pieces arrested in K-free inactive solution were transferred to an active solution of the same composition. The effect of the radioactive solution on the stopped sinus pieces was investigated.

It is impossible to eliminate the effect of mechanical stimulus during the transfer of the pieces. To control this effect an equal number of sinus pieces were placed in an inactive solution and kept under observation.

Solutions of different activities were used for the experiments. The activity of the applied radioactive solution was measured with a GM tube with an end window 2 mg/cm^2 thick. The effective dose was calculated from the activity of the solution and the time necessary to bring about the effect.

The functioning of the sinus pieces was controlled frequently through a magnifying glass and the frequency of activity was determined.

Results

1. The well functioning sinus pieces were divided into two groups, possibly of equal number. One group was placed in an inactive (control) K-free solution, the other one in a radioactive K-free solution. Their activity was controlled frequently. In both K-free solutions the sinus pieces lost their automatic activity after a certain time. The stopping of automatism was reversible in each case. Normal activity restarted within a short period of time after transferring the sinus pieces to Ringer solution. The period of time elapsed until the stopping of the automatism was measured. This period of time differed with the two groups. It was 44 ± 21 minutes with the inactive K-free solution and 58 ± 33 minutes with the radioactive solution. This shows that the automatic activity of sinus pieces will last longer in a radioactive solution than in an inactive one. Nevertheless the difference between the periods characteristic of the two groups is not significant because of the considerable scattering of the results.

148 sinus pieces were investigated in this experimental series. 77 sinus pieces were used in the control group and 71 in the other.

2. The next step was to investigate the effect of ionizing radiation on sinus pieces stopped in K-free inactive solution. To this end the stopped sinus pieces were transferred to a radioactive solution of the same chemical composition. Before this was done, however, the sinus pieces were transferred from the inactive K-free solution to an inactive solution of the same composition to control the effect of mechanical stimulation which is inevitable during the transfer. According to the determinations, the mechanical stimulus of the transfer was ineffective in each case. After controlling the mechanical effect the sinus pieces were placed in the active (Na-24) K-free solution. In addition to above described control experiment on the

effect of mechanical stimulation, an other control experiment was also carried out. Equal numbers of sinus pieces were placed in an inactive solution and in the radioactive solution. The sinus pieces in the radioactive solution and the ones in the inactive solution (control group) were studied simultaneously. In this way, a double control was applied.

In this experiment altogether 154 sinus pieces were used. The effect of radioactive radiation was investigated on 77 objects and the same number were used in the control group.

Out of the 77 sinus pieces placed in radioactive solution 31 ($\sim$ 40 per cent) restarted their activity after a certain time. Out of the 77 sinus pieces placed in inactive solution none started again during the same period of time. These data are summarized in Table 1.

Table 1

Ratio of sinus pieces restarting their activity in radioactive and inactive solutions, respectively

	Restarted	Not started	Total
In radioactive solution	31	46	77
In inactive solution (control)	0	77	77
Total	31	123	154

The value of χ^2 is $\sim$ 39 according to the calculation based on the data of the Table. This value points to a significant difference even if $P = 0.001$ is taken as the probability level. On the basis of these data it is apparent that the effect of radioactive radiation observed with whole hearts can be demonstrated with sinus pieces, too.

Solutions of different activities were used for the experiments. The activity of the solutions varied from 4 to 160 μC/ml. The periods of time elapsing until the reestablishment of the automatism differed extremely from each other. The average value of these periods was 18 $\pm$ 13 minutes. No correlation was found between the period of time elapsed until the restarting and the activity of the applied solution. The value of the correlation coefficient calculated on the basis of the data was $+0.3$.

The value of the effective dose was calculated on the basis of the activity of the solution and the period of time elapsing until the restarting of the automatism. The effective dose varied within a wide range (1 to 130 rad). No correlation was found between the applied dose and the rate of restarting within these dose limits. In other words, the effect is independent of the size of the dose within the dose limits used.

Discussion

The purpose of the experiments was to yield some informations for the interpretation of the effect of radioactive radiation on the restarting of sinus pieces and to investigate the site of action of radiation. It has been established that the effect of radioactive solutions observed with isolated Straub hearts can be demonstrated with excised sinus pieces as well. The automatism of sinus pieces arrested in a K-free solution restarts in a radioactive solution of the same chemical composition. Comparing these experiments with those carried out on whole hearts, a remarkable difference is to be observed in the value of the effective doses. By applying Na-24, the value of the effective dose was found to be 0.1 to 2.4 rad in the experiments performed with isolated Straub hearts (Niedetzky, 1958; Ernst et al., 1959). With sinus pieces the lowest limit of the effective dose corresponds to this value, but in many cases only doses 1 or 2 orders of magnitude higher are effective. This indicates that although the effect of ionizing radiation is on the stimulation center of the heart, the propagating system of the heart or the heart muscle is more sensitive to radiation. On the other hand, in agreement with the data obtained with whole hearts, but even more pronouncedly, it was found that certain sinus pieces had a very different sensitivity to radiation.

The data of the experiments have shown also that the isolated sinus is more sensitive to K-deficiency than the whole heart. The automatism of the sinus pieces placed in K-free solution stopped in every case within an hour. On the contrary the automatism of the isolated hearts kept in K-free solution stopped only after 1 to 4 days (Niedetzky, 1966).

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Effect of γ-Radiation on Isolated Frog Hearts Stopped as a Result of Ca Deficiency

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The mechanical activity of the isolated frog heart becomes reversibly paralyzed in Ca-free solution. In this case the potassium content of the heart significantly decreases and the Na content increases. Such hearts cannot be stimulated either by mechanical or by electric stimuli.

Upon γ-irradiation from a Co-60 radion source the automatism of part of the hearts was reestablished. The effective dosage was found to be ~ 190 rad. Even in long experiments no deleterious effect of this dosage on the mechanical activity of the hearts was observed. The phenomenon may be interpreted as a positive biological radiation effect.

Introduction

It has been demonstrated that the use of radioactive nutrient solutions can lead to the reestablishment of the automatism of hearts which were arrested in a K-rich solution (Niedetzky, 1958; Tigyi et al., 1958; Ernst et al., 1959; Niedetzky, Hajnal-Papp, 1963). External γ-radiation can also re-establish the automatism of hearts which have stopped in a K-free solution (Niedetzky, 1966). The present paper deals with the effect of external γ-radiation on hearts arrested in a Ca-free solution. It is known that from the point of view of heart activity Ca, with its antagonistic effect, plays an important role in addition to K. According to the data of Colle (1926) Ca significantly influences the value of the threshold stimulus of the heart muscle antagonistically to K. When the threshold of the stimulus measured at a normal Ca concentration was taken as 100 per cent upon decreasing the Ca content to the half, the threshold of the stimulus dropped to 84 per cent. According to the data of Clark and White (1928) Ca deficiency, similarly to K excess, decreases the oxygen consumption of the frog heart. Knoll and co-workers (1958) have shown that the isolated frog heart is capable of adaptation in the case of Ca deficiency.

The data of Antoni and co workers (1960) have shown that Ca depletion decreases the contractibility of the heart muscle but, at the same time, electrical stimulatory processes are not affected to a significant degree. The value of the resting potential and the action potential did not decrease measurably in the heart muscle in consequence of Ca depletion. In other words, Ca depletion affects only

the mechanical activity of the heart but not the electrical activity (irritation-production). Adrenalin and ATP neutralize the effect of Ca deficiency. In the opinion of Brücke and Werner (1947) Ca plays an important role in the activity of the nerve end plate.

Thus, according to the above mentioned data Ca depletion does not affect the irritation-production of the isolated heart. Consequently, with hearts arrested as a result of Ca deficiency the direct radiation effect exerted on the heart muscle could be investigated.

Methods

The experiments were performed with frog *(Rana esculenta)* hearts isolated by Straub's method. Blood was completely washed out of the hearts by repeated changes of Ringer solution. After this, the Ringer solution was replaced by a Ca-free solution and the changing of the solution was continued until the automatism of the hearts stopped.

The composition of the Ca-free solution used in the experiments was the following:

6.98 g NaCl
0.20 g KCl
0.20 g $NaHCO_3$ in 1000 ml bidistilled water. The hearts were stored at +2 °C during the night. The experiments were carried out at room temperature.

The automatism of the hearts stopped on the 2nd day following the preparation as a result of the repeated changes of the solution. As far as the time period necessary for the hearts to stop their activity is concerned very small variations (one to two hours) were found with the individual hearts.

The excitability of those hearts which were arrested in a Ca-free solution ceased. The hearts reacted neither to mechanical nor to electrical stimulation.

The arrested hearts were divided into 2 groups. One group was irradiated with γ-rays from a Co-60 radiation source, the other group served as a control. The mechanical activity of the hearts was examined at short time intervals and the number of the hearts which restarted to beat was recorded in both the irradiated group and simultaneously in the control group.

A Co-60 γ-radiation source of an activity equal to 29 g Ra was used for the irradiation of the hearts. (Energy yield: 1.17 and 1.33 MeV.) The dosage rate was estimated to be 135 rad/h at the site of irradiation. The accuracy of this value was controlled by Fricke's chemical dosimetric method.

In order to ensure identical temperature the control hearts were kept, being a lead plate 5 cm thick, in the same room where the radiation source was set up. Here, during the operation of the radiation source the intensity of radiation was 0.2 mr/h.

At the end of the experiment the hearts were out to pieces, rinsed twice with bidistilled water, blotted with filter paper, dried in Ni containers and incinerated in an electrical oven at 450 °C. The ash was dissolved in redistilled water and the Na, K and Ca contents of the solution were quantitatively determined by flame photometry.

Results

Hearts kept in a Ca-free solution stopped on the second day after their preparation while the solution has been changed several times. During this time characteristic changes in the K and Na contents of the heart took place.

The amount of K, Na and Ca was determined in 95 hearts. The Na content of the hearts arrested in a Ca-free solution was found to be 1.67 $\pm$ 0.38 mg/g wet tissue. This is significantly greater compared to the normal value (1.04 $\pm$ 0.12). K content changes in the opposite way. The normal value of the K content of the heart is 1.87 $\pm$ 0.20 mg/g wet weight. The K content of the hearts arrested in Ca-free solution was 1.26 $\pm$ 0.02 mg/g wet weight. The decrease of the K content is also significant. According to the measurements the normal value of the Ca content is 0.04 mg/g wet weight. This value was 0.03 $\pm$ 0.009 mg/g wet weight for hearts arrested under the effect of Ca-free solution. Thus, the Na content of the hearts significantly increases, the K content significantly decreases when this method is applied to paralyze the automatism of the hearts. The Ca content decreases by 25 per cent on the average. The K/Na rate is 0.75 compared to the normal value of 1.79. Table 1 illustrates the above changes in the K, Na and Ca contents.

Table 1

Changes in the Na, K and Ca contents of the heart in the case of Ca deficiency

	Na	K	Ca	K/Na ratio	Number of hearts
	mg/g wet weight				
Normal value	1.04$\pm$0.12	1.87$\pm$0.20	0.04$\pm$0.005	1.79	62
In the case of Ca deficiency	1.67$\pm$0.38	1.26$\pm$0.02	0.03$\pm$0.009	0.75	95

Altogether 290 isolated hearts arrested in a Ca-free solution were further investigated. Out of these 139 hearts were irradiated with γ-rays from a Co-60 radiation source and 151 hearts served as a control. 62 ($\sim$ 45 per cent) of the 139 irradiated hearts started to beat again. 28 ($\sim$ 18 per cent) of the 151 non-irradiated (control) hearts also restarted their activity. This phenomenon points to the existence of adaptation. The data are summarized in Table 2.

Table 2

Effect of γ-irradiation on hearts arrested as a result of Ca deficiency

	Restarted	Per cent	Not started	Per cent	Total
Irradiated	62	45	77	55	139
Control	28	18	123	82	151
Total	90		200		290

The results were statistically evaluated by the χ^2 test. The value of χ^2 is 23.3 as calculated from the data of Table 2. There is a significant difference in the rates of restarting between the irradiated group and the control group even if $P = 0.001$ is taken as the probability level.

The value of the dose rate was 135 rad/h at the site of irradiation. Different time periods elapsed until the hearts restarted to beat, but the fluctuation was comparatively small. The mean value of the time periods between the starting of irradiation and the restarting of the hearts was 1.4 ± 0.4 hours. Accordingly automatism appeared again as a result of an average dose of 189 ± 54 rad.

As a control 100 Straub hearts arrested in a Ca-free solution were irradiated first with visible light according to the method published earlier from our institute (Lakatos, Kollár-Mórocz, 1966). In no case was restarting of activity observed. Afterwards, the above described experiment was performed on the hearts controlled in this way. 59 hearts were irradiated with γ-rays from a Co-60 radiation source, and 41 hearts served as a control. 14 (24 per cent) out of the 59 irradiated hearts started to beat upon γ-irradiation and 2 (5 per cent) of the 41 control hearts started to beat during the same time. There was a significant difference between the irradiated group and the control group as shown by the χ^2 test, taking $P = 0.01$ as the probability level. The dose necessary for the restarting corresponded to the dose which was needed for the restarting of the hearts not pretreated with visible light. It is remarkable that with the hearts irradiated previously with visible light a smaller proportion started to beat upon γ-irradiation than with the group not pretreated with light. This conclusion is valid for the control group also.

The following control experiment was performed to prove that the mechanical activity of the isolated hearts was not affected by the radiation dose necessary to bring about the effect described above:

42 Straub hearts were functioning in normal Ringer solution. 21 out of 42 hearts were irradiated with γ-rays from a Co-60 radiation source and 21 hearts served as a control. The activity of the hearts was registered several times daily for a short time and the period of the spontaneous activity was determined. In the experimental group doses of 700 to 10,000 rad were applied. The mean value of the period of functioning was 4.0 days with the irradiated hearts and 3.5 days

with the control ones. Thus the mean value of the period of functioning was larger in the irradiated group than in the control one. The difference in the period of functioning between the two groups was compared by calculating the significance of the differences and by the *t* test. According to the results there is no significant difference in the period of functioning between the two groups. No far reaching conclusions can be drawn from these data because of the considerably small number of the experiments. All the same, it is indisputable that a dose of about 190 rad used in our experiments has no deleterious effect on the mechanical activity of the heart.

Discussion

As shown by the experiments, hearts which have been arrested in a Ca-free solution started to beat again when irradiated with γ-rays. The hearts stopped in a Ca-free solution cannot be stimulated mechanically and electrically, in contrast to the hearts arrested in a K-free solution (Niedetzky, 1966; Belágyi, in the press). Lakatos and Kollár-Mórocz have reported from our institute that the hearts arrested in Ca-free solution cannot be excited by mechanical stimulation, and contraction does not occur as a result of illumination with visible light (60,000 lux for 2 minutes) even in the presence of a radiation sensitizer (Na-eosin). Part of the hearts arrested in Ca-free solution show adaptation phenomena. This is in agreement with the data of Knoll and co-workers (1958). When the hearts were arrested in Ca-free solution the K content of the heart muscle significantly decreased, and the Na content increased. This shift has the same trend and extent as the changes observed when K-free solution was used (Niedetzky, 1966). In the case of applying Ca-free solution, the heart muscle lost about one third of its K content, while the Na content increased by 50 to 60 per cent on the average.

The value of the dose necessary to restart heart activity was around $\sim$ 200 rad. The dose necessary to restart heart activity is almost the same within the limits of error irrespective of whether the hearts have been arrested in a K-free or a Ca-free solution. Nevertheless, this dose is 2 orders of magnitude higher than the dose which induces the restarting effect in the case of applying radioactive solutions (Tigyi, et al. 1958; Ernst et al., 1959; Niedetzky, 1958). This difference of 2 orders of magnitude makes the interpretation of the results very difficult.

The difference in the order of magnitude of the effective doses in the experiments performed using two different methods refers to the fact, that certain materials sensitize the hearts to the effect of radiation in the case of using radioactive solutions. In an earlier publication (Niedetzky, Hajnal-Papp, 1963) some data have been presented concerning the sensitizing effect.

Nevertheless, the fact that externally applied γ-radiation has the same effect, supports our earlier experimental results obtained by using radioactive solutions. All these data prove indisputably that radioactive irradiation is able to restart the automatism of the arrested heart under certain conditions, even if the arrest-

ing has been carried out with quite different methods. Automatism can be re-established by applying comparatively small doses. Such small doses do not adversely affect the mechanical activity of the heart. The phenomenon is interpreted as a bio-positive radiation effect.

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Symposium on Muscle

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The Symposium was held immediately after the International Biophysical Congress in Vienna (from September 12 to 17, 1966) with the participation of about 50 experts from 14 foreign countries and about 20 Hungarian experts (under the auspices of the International Union of Pure and Applied Biophysics).

F. B. Straub after greeting the members of the congress on behalf of the Hungarian Academy of Sciences held his introductory lecture. He passed in review the results of muscle research in Hungary within the scope of international accomplishments. This well supported the approval of several foreign colleagues who expressed in their letters that such an amount of initiatives and results originated from Hungary that Budapest was the most appropriate place for this congress. In his introductory lecture F. B. Straub explained the motive on which (according to E. Ernst's proposition) the organization was performed. It was namely suggested that in each section only one lecture should be held and the free discussion of the subject should fill out the rest of the time.

Before coming to the description of the summary of contents we should like to note that the daring proposition which seemed rather risky at first, according to which besides the one lecture the rest of the time should pass with discussion, became the most general and greatest success of the symposium. The estimation based on international experiences proved to be right because the great number of short lectures lasting for 10 minutes was not judged to be the method which served scientific progress. It was interesting to experience the pleasure and activity of the participants, how they entered into the debates almost relieved and seemed to enjoy it. (Consequently there was no conversation in groups on the corridor while in the room the lecture was going on.)

The program of the symposium included four main topics:

1. The microstructure and submicrostructure of the striated muscle. Prof. R. Couteaux (Paris) was asked to give the opening lecture, the title of his lecture was: "Structural Aspects of the Striated Muscle." In his lecture he did not give a general review about the present status of the problem but rather described his own latest results. These were about the differences in the fast and slow fibres of the frog muscle, the slow muscles are thicker ($\phi \sim 50\ \mu$), the foldings of the postsynaptic membranes are insignificant, they contain much less cholinesterase, but

they occur more often at the sites of muscle and tendon attachements. No cytochemical data are available concerning the slow process of contraction. Here we mention only Gutmann's data* among those who took part in the following discussion. In his opinion the main difference between the so-called fast and slow fibres is represented by the specialities of the metabolism.

In spite of the fact that the opening lecture did not give a survey of the general status of the problem, this question was in the centre of the debate on the first day. The discussion of the mechanical function in relation with the submicroscopic structure of the *striated muscle fibril* occupied most of the time. Perhaps it is not immoderate to note that results of the Biophysical Institute of Pécs play an important role in the fact that the international research work almost quite naturally considers the fibril as the functional unit of the striated muscle.

H. E. Huxley's well known sliding hypothesis is in the centre of the great international literature concerning the submicroscopic structure of the fibril and the shortening mechanism connected with it. In fact the main argument was about this problem in a direct or indirect way. *30 persons took part in the following debate with 53 comments.* The flashing quick questions and replies produced indeed such a scientific atmosphere which has been missed by so many of us in international scientific meetings several times. Of course a debate is not for its own end, the gained results are important. In this short review naturally the content of the comments cannot be given in detail, but a circumstance must be mentioned. E. Ernst, later N. Garamvölgyi and F. Guba have been arguing against the autocracy of Huxley's hypothesis in Hungary for years, and their comments were made on the basis of the same concept now. Let us characterize the situation before the symposium on muscle by a quotation from Huxley's lecture given in Vienna a week earlier: "The active sliding motion of actin filaments along myosin filaments during contraction is brought about by processes occurring at active sites situated on cross-bridges which extend out sideways from the myosin filaments." Well, the same Huxley's opinion was the following on the last session of the symposium on muscle: "The sliding hypothesis is not for the explanation of everything . . . the supposed activity of the cross-bridges does not belong to the scope of this hypothesis." In the following we shall see what could have brought about this change.

In addition to the lecturer the following participants took part in the debate (in succession): H. E. Huxley (Great Britain), A.G. Szent-Györgyi (USA), K. A. Edman (Sweden), X. Aubert (Belgium), W. Hasselbach (West Germany), R. E. Davies (USA), R. A. Rinaldi (USA), J. Hanson (Great Britain), J. Gergely (USA), F. Guba (Hungary), R. Rice (USA), A. Biró (Hungary), A. Strickholm (USA), M. Dydynska (Poland), I. I. Ivanov (USSR), R. T. Tregear (Great Britain), N. Garamvölgyi (Hungary), K. Marujama (Japan), G. Hoyle (USA), E. Ernst (Hungary), K. Laki (USA), E. Wilkie (Great Britain), W. Drabikovszki (Poland), W. Johnson (USA), E. Gutman (Czechoslovakia), F. A. Sreter (USA), A. Kövér (Hungary), B. Csillik (Hungary), Sz. Virágh (Hungary), E. Varga (Hungary).

* The whole text of the Symposium on Muscle will be published later.

2a) The second day of the Symposium on Muscle consisted of the discussion of the biochemistry of the muscle, if biochemical and biophysical data could have been separated from each other in the field of muscle research. The forenoon of the second day passed by the lectures of S. Ebashi (Japan) and I. I. Ivanov (USSR) and the discussion of the lectures. The author of this paper cannot undertake the professional evaluation of this question, but it seems appropriate to emphasize that although the biochemical discussion of the muscle protein has increased by several new details in the last two decades, it has not exceeded the level of the Szent-Györgyi – Straub conception. On the contrary, the definite direction of the myosin-actin-ATP hypothesis seems to get lost in such myosin details the importance of which remains in darkness from the point of view of the main muscle problem.

Besides S. Ebashi and I. I. Ivanov the participants of the debate were the following: J. Hanson (Great Britain), J. Gergely (USA), E. Drabikowski (Poland), K. Maruyama (Japan), F. Guba (Hungary), D. Kominz (USA), A. G. Szent-Györgyi (USA), K. A. Edman (Sweden), H. E. Huxley (Great Britain), F. A. Pepe (USA), N. Garamvölgyi (Hungary), G. P. Pinaev (USSR), A. Szöőr (Hungary). (Altogether 36 contributions from 15 debaters.)

2b) The afternoon of the second day of the symposium was occupied by the discussion of the anorganic constituents of the muscle. The opening lecture was held by E. Page (USA) instead of A. Fleckenstein who became seriously ill in the last weeks before the symposium. This resulted in a certain displacement from the original program because the mammalian heart constituted the backbone of the lecture, but the debate discussed the anorganic constituents of the muscle, all the same. In any case E. Page's lecture also contained such data, which were closely related to the muscle. According to these data the membrane hypothesis of today is not by all means the only right hypothesis because the changes in the so-called resting potential do not run parallel with the changes in the K content. Furthermore, 12 to 25 per cent of the K content behaves differently from the rest of the potassium. Referring to the Hodgkin-Huxley hypothesis he emphasized: the increasing Na influx and K efflux have not been proved unambiguously with mammalian heart muscle.

46 contributions were made on the part of 16 participants in the extensive debate. The participants were the following: J. Tigyi (Hungary), J. Gergely (USA), G. Hoyle (USA), D. Wilkie (Great Britain), S. Ebashi (Japan), E. Varga (Hungary), W. Sleator (USA), A. Srickholm (Sweden), X. Aubert (Belgium), M. Dydynska (Poland), E. S. Benson (USA), E. Ernst (Hungary), W. J. Bowen (USA), K. A. Edman (Sweden), F. A. Sreter (USA). It is characteristic of the vivacity of the debate that E. Page and J. Tigyi made a comment 7 times each, D. Wilkie and A. Strickholm 4 times each, W. Sleator, W. J. Bowen, E. Varga and E. Ernst 3 times each. Neverhtless, the K-problem remained an open question.

3. The main topic planned for the third day of the symposium was "The mechanical activity of the striated muscle". Instead of this F. Jöbsis (USA) in his opening lecture lectured about the chemism existing together with the mechanical

activity, or rather, about a few problem belonging to this subject. His investigations were mainly performed on the sartorii of toads and frogs. Although in this way he deviated from the debated basic problem of the lecture, still his statements about Ca, pH, and especially about the energy expenditure raised great interest and produced a very aminated discussion. Among these statements were the unambiguous rejection of Hill's "activation heat" and "active state". At last, let me quote one of his sentences: "The amount of tension is directly related to the concentration of an enzyme-substrate complex whereas shortening seems to be an end product of the reaction sequence." This unambigously considers muscle tension and muscle shortening as two different processes.

The details of the discussion consisting of 58 comments are neglected this time, too, owing to lack of space, but two data must be mentioned which have arisen during the debate. One of them is cited from R. E. Davies' (USA) comment: ". . . it is impossible that heat of shortening have come directly from the breakdown of ATP", and further ". . . . the efficiency for external work reaches values of as much as 90 per cent . . ." The other datum is W. J. Bowen's (USA) according to which in a certain way pretreated ". . . rabbit psoas muscle fibres lost their ATP-ase activity but not their ability to undergo ATP-induced shortening . . ." and on this basis he emphasized the role of change in electric charge in the process of shortening. Anybody may look up the articles of E. Ernst and his co-workers and will find in them these statements or statements like these made one or more decades ago.

In addition to the above mentioned three colleagues 18 others took part in the debate: S. Ebashi (Japan), H. E. Huxley (Great Britain), J. Tigyi (Hungary), A. Strickholm (Sweden), A. Kövér (Hungary), W. Hasselbach (West Germany), J. Gergely (USA), J. R. Bendall (Great Britain), J. Hanson (Great Britain), K. A. Edman (Sweden), F. Brinley (USA), D. Wilkie (Great Britain), A. Spronck (Belgium), W. Johnson (USA), M. Dydynska (Poland), X. Aubert (Belgium), R. T. Tregear (Great Britain), V. I. Vorobjev (USSR).

4. The opening lecture of the fourth main subject was presented by D. Wilkie (Great Britain). The title of his lecture was: "Energetic Aspects of Muscular Contraction." He accounted quantitatively for the observed energy output (heat + work) in terms of the measured breakdown of phosphocreatine in muscles poisoned with iodoacetate (0 °C, N_2-pressure). The heat production was measured thermoelectrically. He referred to the data of Davies quoted earlier according to which only ATP breakdown occurs in muscles poisoned with 2.4-dinitrofluorobenzene and the measured amount accounts for neither the activation heat nor the shortening heat, and drew the conclusion that: ". . . only a small part of the heat observed in a muscle that is shortening rapidly can be accounted for by the ATP or PC split up to that time." He maked an effort to improve the uncertain energetical picture with the help of considerations concerning the thermodynamics of irreversible processes, but emphasized as conclusion the uncertainty and the necessity of further experimental work.

Out of the 47 comments of the active debate I would like to mention Tregear's (Great Britain) data, according to which the sites which perform the ATP splitting appear to be activated by the stretch of glycerinated muscle fibre. V. I. Vorobjev (USSR) apart from presenting a similar result emphasized that: ". . . ATP-ase activity of myosin or actomyosin solutions is increased upon the action of hydrodynamic field." This is the right place to quote from Davies' above-mentioned comment concerning the amount of ATP breakdown associated with activation: ". . . and 25 per cent of this occurred after the last pulse had been given (50 pulses/sec)." These data can be summarized in two points: 1. Mechanical effect increases ATP-breakdown without contraction or after contraction. 2. Actomyosin and myosin solutions also have an ATP-ase effect, thus the submicroscopic function of Huxley's cross bridges is not a condition of ATP breakdown. (Vorobjev did not mention Engelhardt.) Those who have studied this question may take all these as the justification of E. Ernst's repeated standpoint, because the situation recognizably begins to resemble the story of the hypothesis concerning the relationship between lactic acid production and contraction. Then it was revealed that lactic acid production exists without contraction, and there is contraction without lactic acid production.

The participants in the discussion besides the above mentioned were: J. Tigyi (Hungary), D. Wilkie (Great Britain), X. Aubert (Belgium), E. Ernst (Hungary), G.Hoyle (USA), J. Gergely (USA), E. Page (USA), A. Oplatka (Israel), F. Jöbsis (USA), J. C. Rüegg (West Germany), W. J. Bowen (USA), W. Hasselbach (West Germany), H. E. Huxley (Great Britain), A. Strickholm (Sweden), R. A. Rinaldi (USA).

5. The opening lecture of the last plenary session was presented by E. Ernst. The title of his lecture was: "Facts, Implications, Perspectives." After the usual greetings and expressing his gratitude, he emphasized especially 3 points. 1. Experimental facts cannot be substituted by assumptions, conceptions, analogous figures, or attractive description of models. 2. Although the submicroscopic, moreover the molecular data are indispensably necessary for the exact description of muscle activity, it must not suppress the fact that the muscle is a macroscopic, work producing system. The suppositions concerning the submicroscopic or molecular dimensions must not contradict the macroscopically proved data. 3. The participants of the symposium on muscle should debate *a few* of the discussed problems and should try to give a short account of either their agreement or their dissent. In this way 1 or 2 debated topics could be established for the next symposium on muscle instead of a series of short comments.

After the lecture 18 participants discussed the questions in 38 comments. R. E. Davies (USA) proposed as further development of the fundamental idea of the lecture that a list should be made of the most important experimental facts (he has already started to do so) and asked the participants to write and send him those facts (together with the references) which need explanation. The final list should be published by the editorial of the Information Exchange Group of the

United States and should be distributed. He agreed with the lecturer concerning the spread of hypotheses because "even a clever man like Huxley" is not able to oversee the whole field. Huxley's modest and attractively moderate attitude has been mentioned already (in the debate following this lecture). Bendall's (Great Britain) reaction was very interesting. He referred to the Empedocles motto of the symposium. "Each of us believes only what he has encountered during his random wanderings and nevertheless prides himself of having found the whole." Then as it were for approval he quoted a sentence of Occam (who lived at the end of the Middle Age and can be looked upon as a materialist philosopher considering the circumstances of that age): "Entities should not be multiplied beyond necessity." The participants of the debate were in addition to the above: F. Guba (Hungary), P. Kominz (USA), A. G. Szent-Györgyi (USA), G. Hoyle (USA), J. Gergely (USA), J. Hanson (Great Britain), R. A. Rinaldi (USA), D. Wilkie (Great Britain), W. Sleator (USA), N. Garamvölgyi (Hungary), E. S. Benson (USA), K. A. Edman (Sweden), X. Aubert (Belgium), E. Gutmann (Czechoslovakia), W. J. Bowen (USA).

6. According to the original plan an excursion was made on the whole fifth day of the symposium. The purpose of this was partly that the participants present may have the opportunity to discuss some questions with those they wished (travelling by bus lasted 5 to 6 hours). This opportunity was fully utilised by the participants but of course I cannot give an account of that. All I know is that E. Ernst and I had a long discussion with D. Wilkie about the energetics of muscle activity and especially about the phenomena of heat production. The "fundamental" myothermic data of Hill were treated in a moderately negative manner in the lectures and in the comments. In this discussion we came to the conclusion that the present data on myothermic heat measurements are not quite satisfactory for the quantitative description of the energetic conditions related with the mechanical activity of the muscle. This agrees with the standpoint of the biophysical group of Pécs which has been taught for several decades. (No decision was made as to the reliability of the methods used for measurement: the thermoelectric and the calorimetric measurements.)

Summing up we may say without any exaggeration that the initiative proved to be good. According to this initiative the international symposium should cover only a few fundamental problems, make an effort to elucidate them and should not supply a torrent of diverse short lectures. Everybody liked this concept, although it was not kept strictly by everybody because part of the time was taken by lecture-like "comments" which did not serve the clearing of the main problem. It is true, that the invited lecturers were not informed that they should present a comprehensive survey of the problem, and so, the opening lectures represented rather the personal results of the lecturers. Still, the discussion turned to the main problem most of the times and this justified the view of the organization.

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Chromatographic Heterogeneity of Pig Pancreatic Amylase

Short communication

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According to the observations of Berk et al. (1965), heterogeneity of serum amylases can be attributed to differences in the origin of these proteins. Norby's (1964) investigations revealed heterogeneity of salivary amylase but homogeneity of pancreas amylase. Marchis-Mouren (1959) has characterized amylase as a homogeneous protein on the basis of its behaviour upon chromatographic fractionation of pancreatic juice from pig and dog on DEAE-cellulose. Szabó and Straub (1966), however, observed heterogeneity of crystalline amylase from pig pancreas upon chromatography on DEAE-Sephadex. The question arises whether the heterogeneity observed by these latter authors could have resulted from the procedures of isolation and recristallization.

In the experiments presented here heterogeneity of native amylase as present in the aqueous extract of pancreas was investigated by chromatography on DEAE-cellulose column. The properties of the two fractions of the crystalline enzyme prepared from the same aqueous extract by the procedure of Hatfaludy and Straub (1966) were also compared.

For the preparation of the native extract chilled pig pancreas obtained from the slaughter-house immediately after the animal had been killed freed from fatty connective tissue, chopped and homogenized twice in 3 volumes of a 0.01 *M* $CaCl_2$ solution for 1 min each time. The homogenate was centrifuged at 0 °C for 15 min at 15,000 x g and the clear supernatant was dialyzed against the buffer used for the equilibration of the ion exchange column.

Crystalline enzyme was prepared by the method mentioned above and was recrystallized 4 to 5 times from urea solution. The specific activity of this preparation agreed whith that of "pure" amylase.

Chromatography of the proteins was performed on a 15×1 cm DEAE cellulose column (Whatman), in the hydroxyl form, equilibrated with 0.01 *M* Tris-HCl buffer, pH S. As amylase is resistant to proteolytic enzymes in the presence of Ca^{2+} ions the buffer solution was made 0.001 *M* with respect to $CaCl_2$. According to our earlier unpublished results, quantitative recovery of amylase from DEAE cellulose columns and good raproducibility of the fractionation can

be accomplished in the presence of β-mercaptoethanol. In the present experiments, therefore, all chromatographic runs were performed in the presence of 2.10^{-4} M β-mercaptoethanol. The column was charged with 10—50 mg protein. The protein was eluted with the equilibrating buffer followed by a linear NaCl gradient in the same buffer. 6 ml-fractions were collected the OD_{280} values of the fractions were determined and their amylase content assayed according to the method of Smith and Roe (1949). In order to determine the K_M values, amylase activity was measured by the method of Bernfeld (1948).

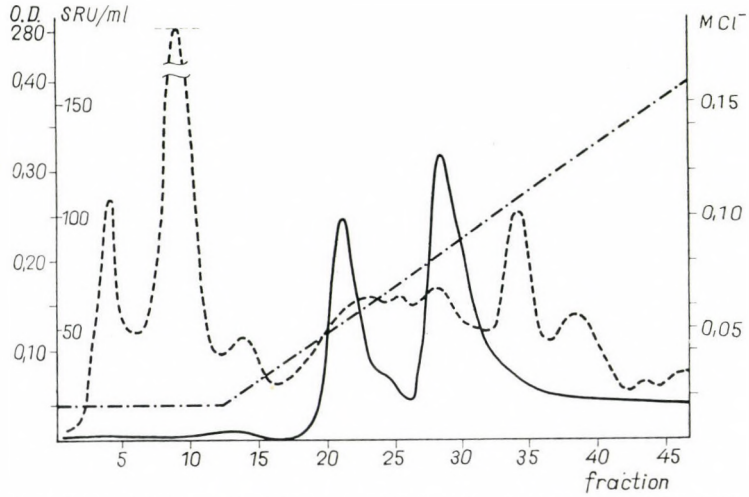

Fig. 1. Chromatogram of an aqueous extract of pancreas. 15 × 1 cm DEAE-cellulose column charged with 45 mg protein in 2 ml. Amylase content: 7840 Smith-Roe units. 6 ml-fractions collected. Recovery: 82%.- - - - - - - - -: OD_{280} ——————: amylase activity, units/ml; —·—·—·—·—·—·: Chloride concentration

Upon chromatography of the native extract of pancreas, amylase appears in the eluate in the form of two distinct peaks. As can be seen in Fig. 1 both peaks are eluted at elevated Cl^- concentrations.

The enzyme crystallized from urea solution is also separated into two fractions under identical conditions. The two active fractions are uted at different Cl^- concentrations upon rechromatography.

The rechromatographed iso-enzymes exhibited the same pH-optima (pH 6.9) and their K_M values were also identical, corresponding to 0.84% soluble starch in both cases.

The above experiments suggest that the heterogeneity found by Szabó and Straub (1966) in crystalline amylase can be observed also in the native enzyme unde the conditions of chromatography applied by us.

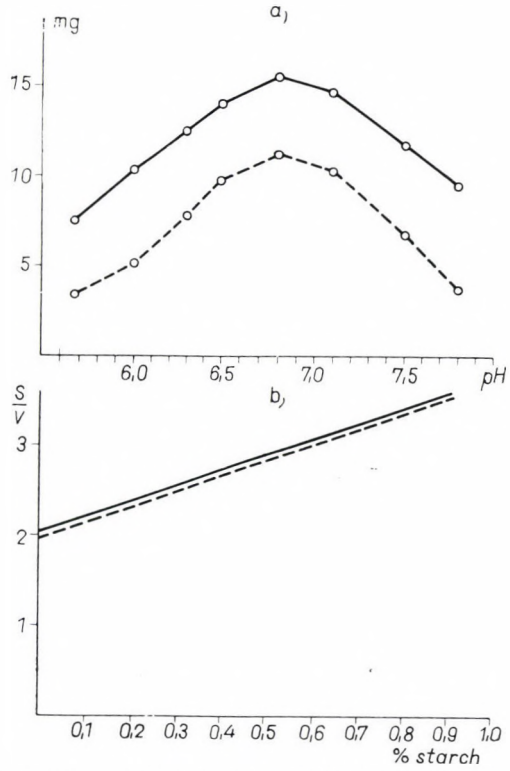

Fig. 2. pH optima and K_M values of the two amylase fractions obtained after rechromatography of the recrystallized enzyme. A.: pH dependence of amylase activity. The pH of the phosphate buffer was varied under the conditions of the assay of Smith and Roe. - - - - : first amylase fraction; ——————: second amylase fraction. B.: Dependence of amylase activity upon substrate concentration, plotted according to Lineweaver and Burke

The identity of the pH-optima and of the K_M values of both proteins, indicates an essential identity of their structure. The two forms of the molecule probably differ only in the charge distribution on their surfaces.

Further studies are in progress to investigate in detail the properties of these iso-enzymes.

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Localization of $^{32}PO_4$ in Isolated Muscle Fibril

Preliminary Report

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PO_4 labelled with ^{32}P was supplied in vivo with the food and its localization was investigated in the fibril of bee's muscle by light and electron microscopic autoradiography. The experiments were carried out partly with a liquid emulsion prepared by us, and partly with an Ilford L-4 liquid emulsion. The evaluation of the light microscopic experiments was carried out by counting the grains in the microscope and the electron microscopic experiments were evaluated by counting the grains on the electronmicrographs.

The thorax muscle of bee (Apis mellifica) was used in the experiments. 0.3 to 0.6 g honey was placed on a little aluminium dish to which carrier-free (^{32}P) Na_2HPO_4 was mixed. The total activity of this mixture was 700 to 1000 μCi. The little aluminium dish was placed in a 500 ml glass jar and 2 to 3 bees were put into it. The bees were allowed to live for 48 to 50 hours and were killed afterwards.

For light microscopic examinations the thorax muscle of the bee was dissected into fibrils on a slide with the help of two needles. After measuring the activity with a G. M. counter the preparations were covered with liquid emulsion according to Leblond's coated method (Bélanger, Leblond, 1946). In some experiments a liquid emulsion described by Kolocšek et al. (1956) was used and in some others an Ilford L-4 liquid emulsion.

The slides were placed in a lightproof box over $CaCl_2$ for exposure at 2 °C. The time of exposure was chosen such that from the actual activity of the preparation 10^8 beta/cm^2 could be obtained for the total surface of the emulsion (Boyd, 1955).

After exposure the slides were developed in Ansco 47/A developer at 20 °C for 7 minutes. This was followed by fixing for 4 minutes and rinsing for 5 to 6 minutes. After drying the preparations were evaluated by counting the grains in the light microscope. Drying was performed at room temperature.

For electron microscopic examinations the thorax muscle was slightly stretched and fixed in the vapour of a 5 per cent OsO_4 solution for 1 hour. This was followed by the removal of water by the use of an alcohol series of gradually increasing concentrations. The fixed and dehydrated muscles were embedded in araldit and methacrylate. Sections about 300 Å thick were cut from the specimen

by a Porter-Blum ultramicrotome (the interference colour is gray on water). The sections were picked up over grids, stained with phosphotungstic acid dissolved in alcohol, and covered with a formvar film about 100 Å thick. Grids fixed in this way were covered with a film of emulsion which contained a monolayer of silver-haloid grains and were made of Ilford L-4 emulsion. The monolayer film of emulsion was prepared according to the description of Caro and van Tubergen (1962) from the twice diluted solution of Ilford L-4 emulsion with the help of a copper wire loop 4.5 cm in diameter and 0.1 mm thick.

Exposure and development were performed in the same way and under the same circumstances as for the light microscopic examinations. After drying, the grids were carefully lifted from the slides and exposures were taken in a Tesla electron microscope at an accelerator voltage of 60 kV.

First it was investigated whether the PO_4 provided in vivo was incorporated into the muscle fibril. At a 640 fold magnification those areas were chosen, in which one or two isolated fibrils occurred. The size of the area was 45,200 μ^2 the area of the fibril was 400 to 1000 $-\mu^2$. All the grains in this area and those in the fibril were counted. The number of grains in the area was 400 to 600 and that in the fibril amounted to 20 to 40. From these data the density of grains was calculated for the fibril and for the background. The density of grains in the fibrils was found to be 5 to 7 times greater than that in the background.

Thereafter the grains in the fibrils were investigated as follows:

1. The percentage distribution of the grains in certain bands of the fibril was established by counting the grains. The isolated fibrils were examined in the light microscope at a 640 fold magnification. The grains were counted along the entire length of the fibril (30 to 80 grains, depending on the length of the fibril) and their location in the individual bands of the fibril was recorded. The results are shown in the first column of Table 1.

2. To evaluate the experiments, a more objective method was looked for because of the subjective factor involved in counting the grains. The microdensitometric evaluation of films obtained by photographing the single fibrils seemed a

Table 1

Percentage distribution of grains in individual bands of the fibril as revealed by light microscopic and electron microscopic examinations

Band of the fibril	Percentage distribution of grains in the fibril	
	Light microscope	Electron microscope
A-band	77.0±2.1	74.5±3.1
Z-line	17.2±1.8	16.6±2.3
I-band	5.8±1.1	8.9±1.6

semiquantitative method fitting for this purpose. This method provided in some cases good and evaluable results.

3. The autoradiographic experimental series performed at the electron microscopic level makes it possible to obtain a higher resolution and a more precise

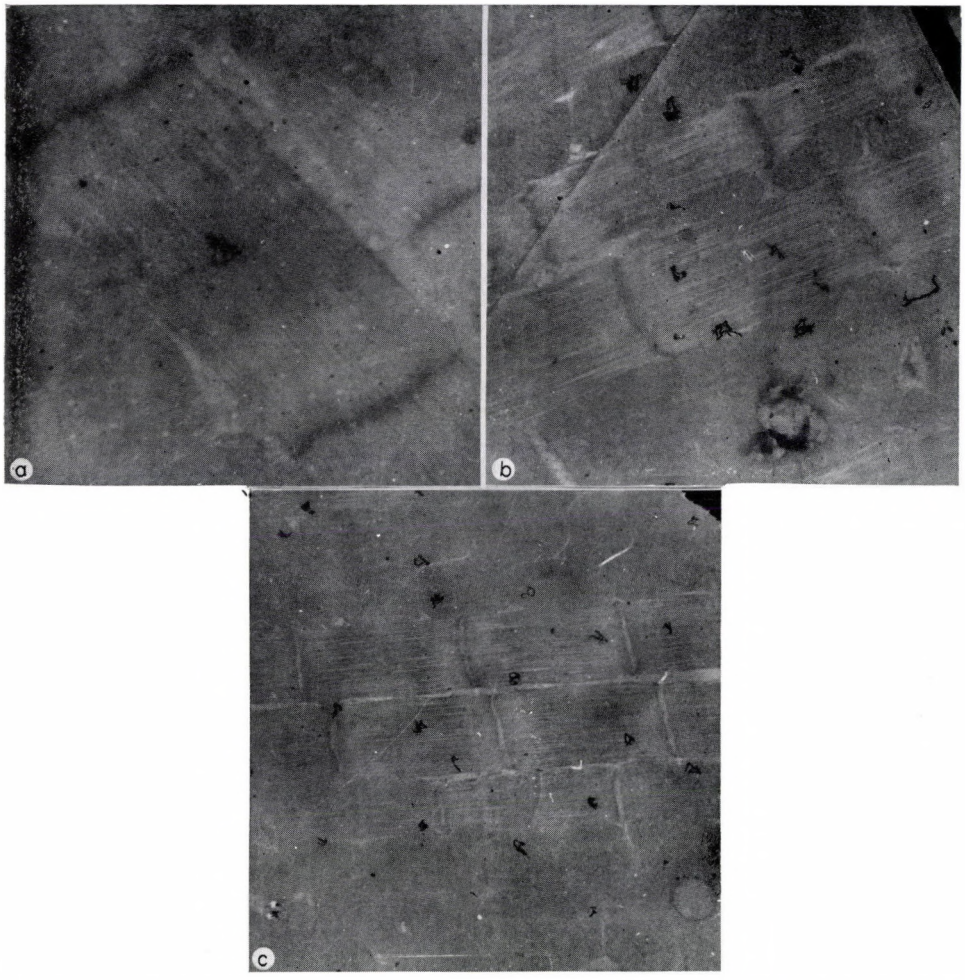

Fig. 1 *a—c*. Electron microscopic autoradiograms of a striated muscle fibril labeled with ^{32}P. Time of exposure: 30 days, development in Ansco 47/A developer for 5 minutes. In the case of Fig. *a* N = 21,600, *b* N = 11,000 and *c* N = 7400

determination of localization. The number of observable grains was considerably greater than in the light microscopic experimental series. According to our results the density of grains in the fibril was 0.261 ± 0.021 grains/μ^2 which is

about 5 times as great as the density of grains found in the light microscopic experiments.

The experiments were evaluated by counting the grains on the electronmicrograph. Altogether 661 grains were counted on 40 electronmicrographs at a 10,000 to 15,000 fold magnification. From this 105 grains were found outside the fibril and 556 grains were localized in the fibril and attached to different bands. The grains belonging to the individual bands of the fibril were counted and their percentage distribution calculated. This distribution is shown in the 2nd column of Table 1. The errors belonging to the average values are given, too. As it can be seen this is in good agreement with the distribution calculated in the experiments performed with the light microscope.

The experiments show that the A-band contains most of the PO_4 introduced in vivo, most probably in a protein bound state. Further experiments are necessary to ascertain this assumption. A significant amount might be found in the Z-line also. The grains in the I-band may originate either from the A-band or from the Z-line because taking into account the error their number is near the significance level of 5 per cent accepted in biological experiments and the beta particles of P-32 have on energy of 1.7 MeV.

The distribution shown in Table 1 should be modified to some extent if we consider the proportion of the areas represented by the individual bands. Even so the minimum number of grains in the I-band does not change, but it is essential to correct for the area of the Z-line. If this is done, the number of grains in the Z-line will be equal to the number of grains in the A-band.

The relatively low density of grains observed in the experiments can be explained by the fact, that PO_4 was incorporated under in vivo circumstances and so the activity incorporated was limited.

Acknowledgement

Authors express their thanks to Professor E. Ernst and to Associate Professor J. Tigyi for encouragement in this work.

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Submicrostructure of the Muscle and Moiré Patterns

Preliminary Report

I. ACHÁTZ

Biophysical Institute, Medical University, Pécs.

(Received April 12, 1967)

Up to the present the results of submicrostructural muscle research have been obtained almost solely from electron microscopic investigations. Electron micrographs, however, are to be evaluated with caution. In a debate on the principles of submicrostructural investigation Ernst (1963) has expressed the view that a two dimensional picture is not identical with a three dimensional structure and further, that the actual structure of the muscle can be revealed only by the simultaneous application different methods.

Electron micrographs of the striated muscle display great variations (Achátz, 1966). These are the following: *a)* only identical filaments can be observed, *b)* a thick filament is followed by a thin one, *c)* a thick filament is followed by two thin ones, *d)* two thick filaments are followed by two thin ones (Ernst, Benedeczky, 1962) etc. Pictures may be also seen in several cases where a given filament is not of uniform thickness, but exhibits periodical thickenings (e. g. Ernst, in press).

The question may be raised: What kind of effect is exerted on the muscle during the preparation for electron microscope to cause this variation? Hodge et al. (1954) have suggested that the above mentioned phenomena are caused by sectioning different layers of filaments with the ultramicrotome.

We have turned our attention to the moiré patterns (e.g. Menter, 1958) which are used extensively in studies on crystal line structures. Moiré phenomenon is produced upon the sliding of two grated structures with respect to each other. The mathematical basis of this phenomenon will not be discussed here in detail. Rather an account will be given on such patterns in which the two planes of gratings are parallel. In the model shown in Fig. 1 the thickness of the lines and the grating constant of the gratings are equal, but because of the distance of the two parallel planes *the lower lines appear to be thinner on the photograph.* In Fig. 2 the two gratings are composed of lines of uniform thickness, but the grating constants are different. As a result of parallel sliding the lower and upper line-systems are partly summarized leading to the appearance of thicker lines. Thus, the result obtained is similar: *A thinner line is located between two thicker ones.* In Fig. 3 the line-systems of the two gratings intercept in an angle. Here the lines

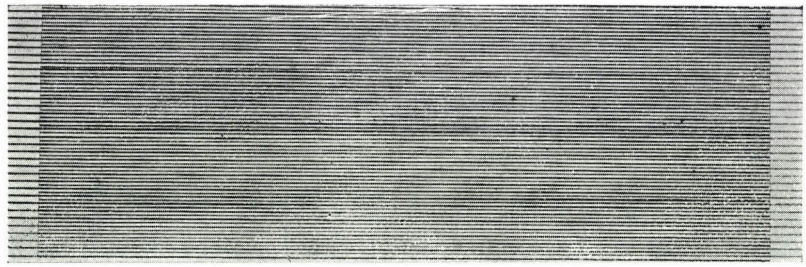

Fig. 1. Moiré pattern produced by two parallel grating-planes. Here the thickness of the lines and the grating constant are equal

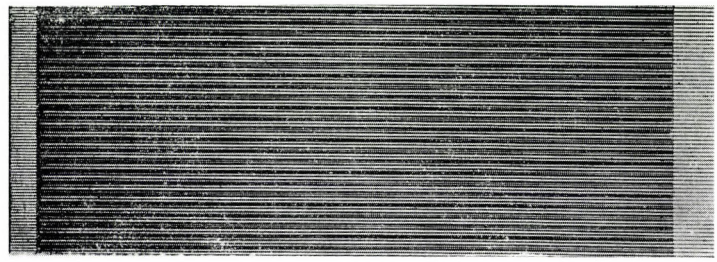

Fig. 2. Moiré pattern produced by two parallel grating-planes. Here the thickness of the lines is equal but the grating constants are different

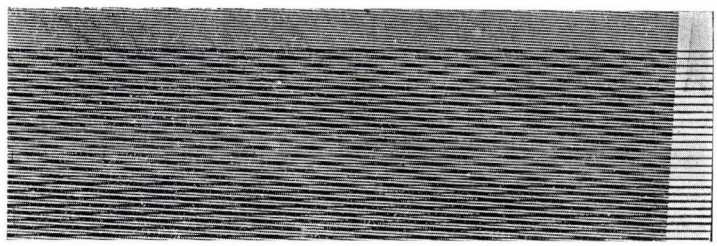

Fig. 3. Moiré pattern produced by two parallel grating-planes. Here the lines intercept in an angle of about 2.5 degrees

of the gratings exhibit periodical thickenings. The models are the photographic copies of two gratings placed on each other. The individual gratings which served for the construction of the models can be seen without covering on the margins of the photographs.

As far as electron micrographs are concerned it seems to be possible that during preparation (e g. in the course of sectioning) two layers of filaments slide along each other, either in such a way that certain lines remain parallel or that they intercept in a certain angle. Phenomena similar to those shown in the Figures

can be produced in this way and they are the possible models of the patterns seen in the electron micrographs of the muscle. Of course, the models are not perfect, they do not contain in full measure all characteristics of the optical image seen in the electron microscope. It must be taken into consideration that the gratings may slide not only in parallel planes, but also in planes which intercept in an angle. This may also produce similar patterns to those described in the present paper. The direction of light may also play a role in the formation of the patterns.

Acknowledgement

The author is indebted to Professor E. Ernst for calling his attention to this problem and for his advice.

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Studies on Proteins and Protein Complexes of Muscle by means of Proteolysis

III. Tryptic Digestion of Myofibrils

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(Received January 6, 1967)

The tryptic digestion of myofibrils and the influence of Ca ions on this process was studied by measurement of base consumption and by studying the properties of the protein fractions going into solution and remaining undissolved, respectively, during this process.

In Ca free milieu addition of trypsin caused a nearly complete dissolution of myofibrils. The presence of acto-H-meromyosin in the digest was demonstrated.

In the presence of 0.01 M Ca ions in spite of considerable peptide bond splitting there remained a great proportion of undissolved protein. When these undissolved proteins were extracted by Hasselbach—Schneider solution ultracentrifugation of the extract showed that hardly any myosin remained unfragmented.

The conclusion is drawn that the organized arrangement of myosin molecules in the myofibril does not interfere substantially with their accessibility to trypsin.

Introduction

Proteolytic fragmentation added considerably to our understanding of the architecture of the myosin molecule (Mihályi, Harrington, 1959, Lowey, Cohen, 1962, Young et al., 1964).

Changes in the higher, secondary and tertiary structures of a protein molecule are sensitively reflected in changes in susceptibility to proteolytic attack (e.g. Harrington et al., 1959, Szabolcsi et al., 1959). It is thus conceivable that the structure of myosin built in the myofibrils in an organized fashion could be studied with the help of proteolytic enzymes.

The starting point of the experiments we are to report in this paper was the assumption that proteolytic disintegration of the myofibrils could give information on two questions:

first on eventual conformational differences in myosin molecules in situ and in an isolated state respectively, and

second on the way they are assembled in the myofibril to form the A-rods. (See e.g. Huxley 1960, 1963.)

Due to several favourable circumstances it may be expected that the interpretation of the results would not present insuperable difficulties. Under the experimental conditions used for the fragmentation of myosin, actin is completely

resistant to proteolysis (Mihályi, Szent-Györgyi, 1953a, Laki et al., 1962, Biró, Göbel, 1961 unpublished) whereas tropomyosin is easily disintegrated to peptides (Laki et al., 1962, Milstein, 1966). Regarding the small amount of tropomyosin it can be expected that digestion of myofibrils would reflect in the first place the degradation of the myosin component. Furthermore in actomyosin *solutions* the attachment of myosin to actin is of no conspicuous influence on the proteolysis of myosin (Mihályi, 1953, Mihályi, Szent Györgyi, 1953a, b). Therefore we assumed that differences in the proteolytic behaviour of miofibrils as compared to unorganized myosin (or actomyosin) are caused by the organized structure of the myofibrils. Rather unexpectedly, in the course of the experiments presented in this paper, we were unable to detect any serious influence of the organized arrangement of myosin molecules on their proteolytic behaviour.

Materials and Methods

Myofibrils were prepared according to Perry [1952] and kept in 0.02 M KCl + 0.02 M borax-borate buffer pH 7.09. This solution will be designated in the following as "borate-KCl".

Myosin was prepared according to Portzehl, Schramm and Weber [1950], myosin-B according to A. Szent-Györgyi [1947].

Myofibril "ghosts" were prepared as follows: 100 ml of myofibrils (150–180 mg total protein) were centrifuged and extracted for 1–2 hours with 12 ml of Hasselbach–Schneider solution (Hasselbach, Schneider, 1951) in the cold with gentle stirring. The extraction was repeated four times. The final precipitate was washed with borate-KCl and suspended in the same milieu. The amount of extracted protein as measured in the combined extracts was 55–65 per cent of the total, the specific ATP-ase activity of the ghosts was lower than 0.04 μmole P/mg. min.

Trypsin was the same lyophilized preparation of "Kőbányai Gyógyszergyár" the standardization of which is given in our previous paper (Biró, Bálint, 1966). We used a crystalline soybean inhibitor (Worthington).

pH-state measurements were done with a Radiometer TTT1 + SBU + + SBR2 assembly. In general, digestion was carried out in 10 ml borate-KCl containing a total of 60 mg protein and 0.5 mg trypsin. For titration we used approx. 0.03 N NaOH. Before the addition of trypsin the reaction mixture was adjusted to pH 8.2, and was observed for 20–40 minutes with continuous rinsing of the gas phase with N_2 in order to detect any eventual drift. The reaction was started by the addition of 1 ml of a 0.05 per cent trypsin solution. Proportional band setting was constantly 0.1 during recording.

Viscosity measurements were carried out at 0 °C, in a vol. of 4 ml with an Ostwald type viscosimeter having outflow time of 0.73 minutes for 0.5 M KCl.

The conditions of the ATP-ase test: 4 mM ATP, 4 mM $CaCl_2$, 50 mM tris-HCl buffer, pH 7.0, 0.5 M KCl; protein 0.5–1.5 mg/ml, total vol. 2 ml, 22°, 3–5 min.

Inorganic phosphorus was estimated according to Fiske and Subbarow (1925), protein content by the biuret method of Gornall et al. (1949).

Results

Under the experimental conditions used for the proteolysis of myosin *suspensions* (Biró, Bálint, 1966) myofibrillar suspensions were easily digested by trypsin.

In Fig. 1. are shown the pH-stat records of the digestion of myofibrils in borate-KCl and in the presence of different concentrations of Ca ions. As it can be seen, myofibrillar proteins were digested by trypsin at a considerable rate.

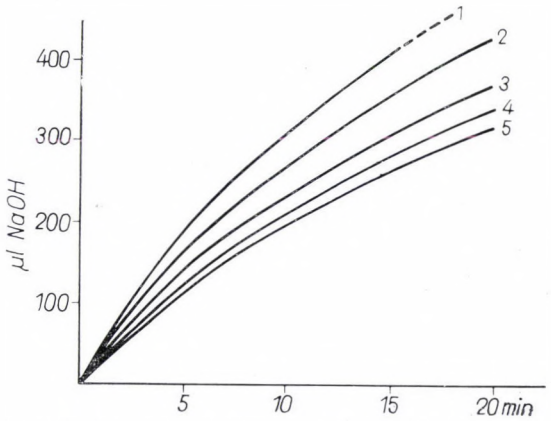

Fig. 1. Digestion of myofibrils by trypsin in the presence of various concentrations of Ca^{++} pH-state records. For experimental conditions see the "Methods". Additions: 1 — none; 2 — 0.001 M $CaCl_2$; 3 — 0.002 M $CaCl_2$; 4 — 0.003 M $CaCl_2$; 5 — 0.03 M $CaCl_2$

With increasing Ca concentrations we obtained an increasing inhibition of proteolysis much reminding of the inhibition caused by Ca in myosin suspensions (Biró, Bálint, 1966).

In accordance with the observation of Szent-Györgyi and Holtzer (1963) we observed a practically complete dissolution in the very first minutes of proteolysis of myofibrils (in the absence of Ca). It should be noted that we used ten times lower trypsin to protein ratios than the authors cited, but the pH of the reaction mixtures was substantially higher, 8.2 instead of 7.0.

We have to suppose, that during digestion the greater part of actin went into solution as well as the other myofibrillar proteins. Actin makes up about 20 per cent of the myofibrillar proteins. The amount of protein undissolved after digestion rarely exceeded 5 per cent of the total as measured after centrifugation at 30,000 g for one hour. On the other hand, if the supernatant fluid was centrifuged for 2 hours at 105,000 g both protein concentration and ATP-ase activity decreased

considerably. When centrifugation at 105,000 g was carried out in the presence of ATP, the amount of sedimenting protein as well as that of the ATP-ase activity were considerably less (Table 1).

Table 1

Differential centrifugation of myofibrils digested by trypsin in borate-KCl

Myofibrils (14 mg/ml) were digested at pH 8.2 and at 22°, for 15 minutes with trypsin (50 μg/ml); digestion arrested by addition of inhibitor (60 μg/ml final concentration). Protein content and ATP-ase activity were tested in the supernatants of subsequent centrifugations as indicated in the table

Conditions of centrifugation	Protein (mg/ml)	Specific ATP-ase activity, μmole P/mg.min.
10,000 g, 20 min.	2.96	0.39
30,000 g, 40 min.	2.96	0.33
105,000 g, 180 min.*	0.95	0.11
105,000 g, in presence of ATP, 180 min.*	1.37	0.35

According to Fig. 2, the supernatant fluid obtained after centrifugation at 30,000 g shows a marked drop of viscosity upon the addition of ATP. This experiment suggests that the protein in question is mainly acto-HMM, since the observed drop of viscosity is in quantitative agreement with the value found by Mihályi and Szent-Györgyi (1953b) for trypsin treated actomyosin.

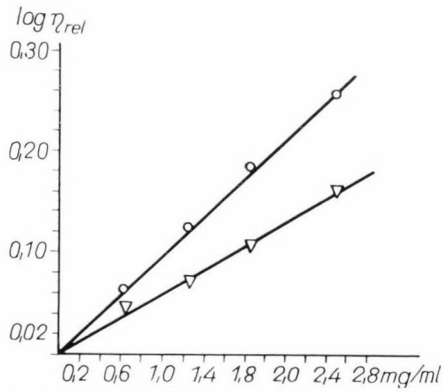

Fig. 2. Viscosimetry of proteins dissolved by tryptic digestion of myofibrils in borate-KCl. Experiments were done with the 30,000 g supernatant from the experiment referred to in Table 1. ○: before, ▽: after addition of 1 mM ATP

*Before centrifugation at 105,000 g the samples were complemented to contain 0.5 M KCl and to 0.5 M KCl + 2.2 mM ATP, resp.

The analytical ultracentrifugation of the digest revealed a major, extremely fast component leaving behind a slow component (shown in Fig. 3 upper curve) with an s_{20} of 2.29. This again shows the presence of acto-HMM, together with some LMM.

Taking into account these data it was rather unexpected to find (Table 2) that when myofibrils freed from myosin were digested under similar conditions, very little protein became solubilized (14.6% in the experiment presented). All our attempts to extract substantial amounts of actin from myofibrillar "ghosts" failed. An attempt was made to extract also untreated and trypsin-treated ghosts

Table 2

Digestion of myofibril "ghosts"

"Ghosts"prepared by extraction of myofibrills with Hasselbach—Scheider solution as described in "Methods", were suspended in borate-KCl to a concentration of 11.7 mg/ml. Trypsin concentration 0.075 mg/ml, digestion at 22°. At the times indicated 4 ml aliquots were added to 8 ml borate-KCl containing 0.8 mg trypsin inhibitor. The suspension was centrifuged and protein content estimated both in the supernatant and in the precipitate

Time min.	Protein, mg in 4 ml sample	
	supernatant	precipitate
0	0.8	47
5	6.0	41
10	6.4	36
20	7.5	33
30	7.0	32.5

with myosin solutions, HMM solution, total digests of myosin obtained at high or low ionic strength as extractants. Extraction times were extended to 24 hours. The expected dissolution of actin was tested by viscosimetry (drop of viscosity upon addition of ATP) and by measuring protein concentrations.

These findings are in contrast with the results of Hama et al. (1965). The discrepancy can be explained by some secondary aggregations brought about by the very drastic extraction of myosin we applied (see: Methods). It should be noted that actin in our ghost preparations was not highly denatured: when dialyzed saltfree we observed nearly complete dissolution, i.e. actin depolymerized.

In an other series of experiments we investigated the digestion of myofibrils in the presence of Ca. When Ca is present, the suspension does not go into solution. It remains opaque after prolonged digestion even in the case of the lowest Ca concentrations used in the pH-state experiments shown in Fig. 1. It could be demonstrated that myosin, although it remains undissolved, becomes fragmented when myofibrils are treated by trypsin in the presence of Ca.

Proteolysis as followed in the pH-state was conducted to an extent which leads in the Ca free system to complete dissolution. The proteins remaining undis-

solved were extracted by Hasselbach–Schneider solution. This extract showed in the ultracentrifuge a quite complex picture (Fig. 3 lower curve). The nature of the faster components could not be revealed by ultracentrifuge experiments. On the basis of their s_{20} values the two components may represent myosin and HMM or HMM and subfragment 1 (see: Mueller, Perry, 1961). The slower, rather homogeneous component on the other hand is in all probability an LMM-like fragment ($s_{20} = 2.54$). The amount of this component is 25–30% of the total (by area measurement). Hence we must assume that these proteins (which remained undissolved during digestion) contain but negligible amounts of unfragmented

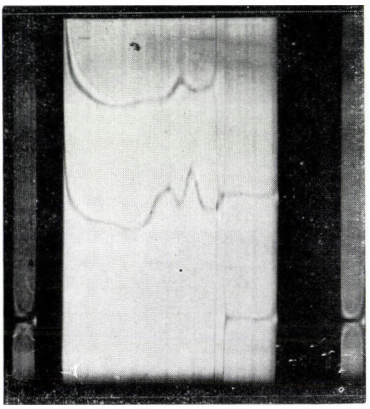

Fig. 3. Ultracentrifugation pattern of protein fractions obtained from digested myofibrils. Upper curve: 30,000 g supernatant of digested myofibrils (in borate-KCl). Lower curve: Hasselbach—Schneider extract of the precipitate fraction of myofibrils digested in the presence of 0.01 M $CaCl_2$. Centrifugation at 59,780 rpm (256,000 g) at 20 °C; photographed 53 minutes after reaching full speed; bar angle 55°. Digestion was carried out essentially as in the pH-state experiments (see: "Methods") at 22° for 18 minutes. Extraction of the precipitate: the precipitate of 10 ml suspension was extracted once with 5 ml once with 3 ml of Hasselbach—Schneider's solution for two hours with gentle stirring. The combined extracts were treated with an aminodiacetate resin in order to remove Ca, then dialyzed 24 hours against 0.5 M KCl + 0.01 M borate of pH 8.2. The supernatant of the Ca free digest was dialyzed in the same way. Both samples were diluted before centrifugation to a protein concentration of 5.5 mg/ml

myosin. This reasoning would be questionable if during digestion substantial amounts of HMM did go into solution, as in this case the relative amount of LMM would increase. The experiment shown in Fig. 4 indicates, however, that the solubilization of ATP-ase active components during digestion in the presence of Ca is negligible.

This experiments show that myosin of the myofibrils can be fragmented "in situ". Even when myosin remains in the myofibrillar structure, the trypsin sensitive regions are reached by the enzyme.

A further support of the finding, that the organized arrangement of the myosin molecules in the myofibril has no conspicuous influence on its proteolysis

is offered by the experiments presented in Figs 4 and 5 showing the solubilization of proteins and the specific activity of the dissolved fraction when myofibrils and myosin-B, respectively are digested in the presence of different amounts of Ca ions. As it is quite evident there is practically no difference in the behaviour of "organized" and randomly aggregated actomyosin, respectively. The insolubility of the myosin fragments under the circumstances of the experiment is caused

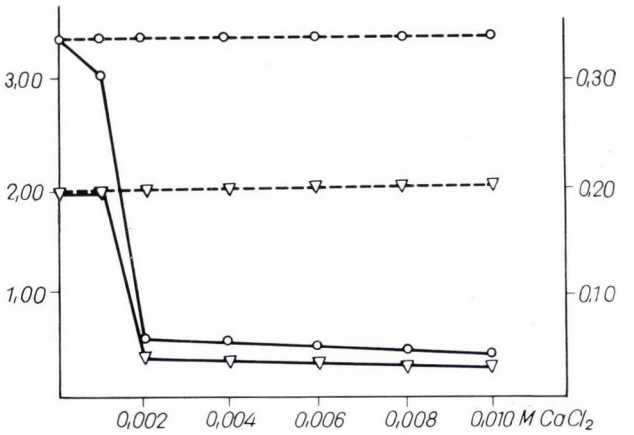

Fig. 4. Solubilization of protein and specific ATP-ase activity as a function of Ca concentration in myofibrils. To 10 ml samples containing 35 mg protein in 0.02 M BO_3 NaOH buffer of pH 8.2 and $CaCl_2$ as indicated on the abscissa 0.3 mg trypsin was added. Digestion was carried out for 18 minutes at 22° and was stopped by the addition of 0.5 mg inhibitor in 0.2 ml volume. After half an hour standing and subsequent centrifugation at 10,000 g for 30 minutes ATP-ase activity and protein content of the supernatants were tested. ○: (left hand ordinate), protein content mg/ml; ▽: (right hand ordinate) specific activity, μmole P/mg. min. Dotted lines with the respective symbols: protein content and ATP-ase activity of the *unseparated* samples

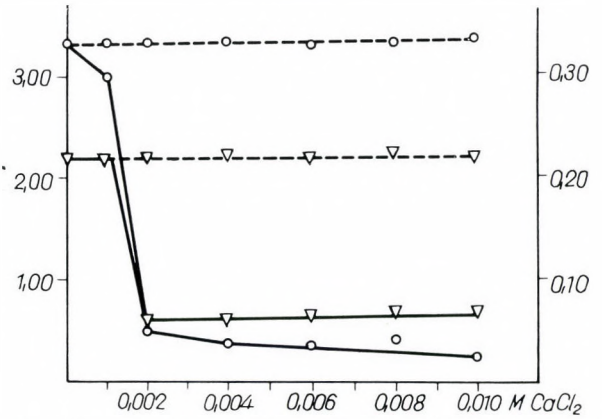

Fig. 5. Solubilization of protein and specific ATP-ase activity as a function of Ca concentration in myosin-B

mainly by the *presence* of actin and not by the structured arrangement of the myofibrillar proteins.

In a previous publication of this series (Biró et al., 1966) it has been reported, that when myosin suspension (in a medium of low ionic strength) is digested in the presence of 0.01 M $CaCl_2$, in spite of considerable proteolysis, a relatively low per cent of the protein and of ATP-ase activity goes into solution, similarly to the experiments shown in Figs 4 and 5. When, however, a similar experiment is carried out with myosin (Fig. 6) the solubilization of protein decreases much more

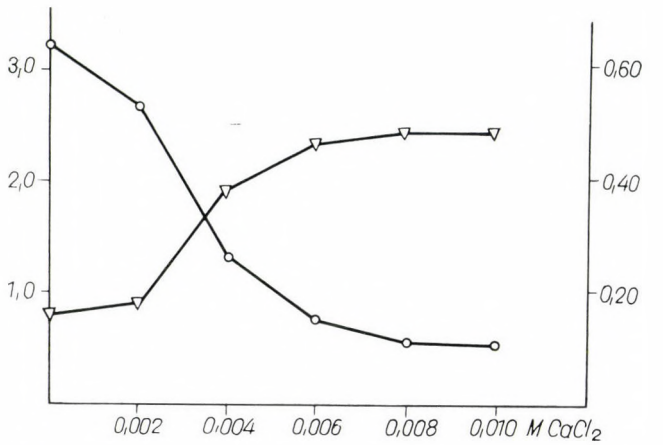

Fig. 6. Solubilization of protein and specific ATP-ase activity as a function of Ca concentration in myosin. Note that the scale of the right hand ordinate is doubled relative to the foregoing analogous diagrams

gradually than in the case of actomyosins, although at 0.01 M $CaCl_2$ the low solubility found with actomyosins is approximated. This experiment gives further support to the view that in the case of the actomyosins it is the complex formation with the actin-component which plays the decisive role in preventing the fragments from dissolving.

Discussion

Our experiments described in this paper deserve some comments with regard to the acessibility of myofibrillar myosin to proteolysis. We failed to find any real difference between the digestion of the myosin in structured arrangement in the myofibril and in unorganized state. The experiments suggest that in the myofibril the HMM-parts of all myosin molecules are connected to actin filaments as in unstructured actomyosin suspensions and solutions.

The myosin containing A-rods of the myofibril are suggested, mainly from eiectronmicroscopic observations, to consist of myosin molecules bundled together tlghtly. According to Huxley (1960) they contain some 400 myosin molecules. Tight aggregation of protein molecules can strongly interfere with proteolytic attack as in the case of tobacco mosaic virus monomers, which are easily digested by chymotrypsin and subtilisin but are completely resistant when assembled into virus rods (Kleczkowski, Van Kamen, 1961). This resistance to proteolysis is due to the screening of sensitive surfaces of the monomer by aggregation. In the case of A-rods, with the tight aggregation suggested by accepted electronmicroscopic studies, it is hard to imagine an arrangement with all the trypsin sensitive sites on the surface as our proteolytic experiments would suggest. Although the proteolytic methods used in our experiments are rather crude a tool in comparison with the electronmicroscopic procedures, they have the great advantage that they allow the study of unfixed preparations. It is not quite impossible that the structure seen in the electron microscope represents the agglomeration of an ordered structure, which in an unfixed state is much looser. The isolation of A-rods from the myofibril or the aggregation of myosin to form "artificial A-rods" as described by Huxley (1963) is not in contradiction to this assumption, as in these studies the aggregates were finally observed in a dehydrated fixed state. The generally accepted picture of the myofibrillar architecture is brilliantly supported by the low-angle X-ray data (Huxley, 1953, Elliot et al., 1963, Elliot, 1964) partly obtained on untreated live muscles. Although the results are in complete agreement with the electron-microscopic investigations with regard to the filament lattice structure, the X-ray data do not give direct information concerning the diameter of the filaments occupying the scattering centers. The exact share of the myosin molecules of the myofibril in the building up of the scattering centers found in the A-rod positions is not known. Elliot et al. (1963) e.g. in a quantitative treatment of the intensity ratios of the diffraction pictures take tentatively the double values of the filamentdiameters deduced from electronmicroscopy as the basis of their calculations. On the ground of this reasoning it seems to us not quite unjustified to consider a model which places only a limited portion of the myosin molecules (most probably the LMM segments or part of them) in the position corresponding to the A-rods, with a considerable part spreeding over loosely to the actin-filaments. In this arrangement the trypsin sensitive regions of all myosin molecules would be freely exposed. This suggestion based on the proteolytic experiments is in good agreement with the picture deduced by Pepe (1966) from his very refined antibody-staining experiments.

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Ion and Amino Acid Absorption by Unicellular Algae

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The uptake of cations (K^+ and Na^+), an anion (Br^-) and an amino acid (glycine) by Scenedesmus (and in some cases, Chlorella) has been studied to determine the basic characteristics of absorption processes. In one part of the experiments synchronous cultures were used.

The time curves of absorption begin with a rapid exchange process followed for an extended period by a constant absorption rate.

The concentration curves of uptake can be divided into three groups: 1/following saturation at high concentration there is another rise in the bromide absorption by Scenedesmus; 2/potassium and sodium uptake by Scenedesmus and bromide uptake by Chlorella give a simple saturation curve over the concentration range examined; 3/glycine absorption by Scenedesmus shows only traces of saturation. The order of the K_m values of the uptake processes of Scenedesmus is as follows: K, Na, Br.

K and Na ions inhibit each other's uptake. Only part of the K absorbed is exchangeable; an initial rapid phase can also be observed in the exchange process.

According to preliminary experiments with synchronous Scenedesmus cultures the V_{max} of bromide absorption rises more or less continually during the light period of the cycle, simultaneously with the growth of cells.

Introduction

The use of synchronous cultures (Tamiya, 1966; Pirson, Lorenzen, 1966) in the study of the life cycle of cells has come into prominence since the early 50's. In recent years attention has particularly been focused on the timing of enzyme synthesis for it is closely connected with the mechanism of regulation (Halvorson, 1964).

Both indirect and direct proofs (Fox, Kennedy, 1965; Kolber, Stein, 1966) support the idea that special proteins in or on the lipid membrane operate as transport mediators. Their synthesis and activity are supposed to be regulated by mechanisms similar to those operating in the regulation of enzyme synthesis and activity.

Based on these general considerations we began to study in 1964 the changes in the biological transport processes taking place during a cell cycle. Unicellular algae were chosen to serve as a material in view of the fact that 1. their synchronisation can be easily accomplished if the natural environmental factors (e.g.

light-darkness) are changed adequately, 2. and because the short life cycle and rapid uptake phenomena in yeasts (and especially in bacteria) present particular methodological difficulties.

Naturally, the selection of unicellular algae led to certain problems apart from the advantages mentioned earlier. One of the problems we had to face at the beginning was that we had hardly any data available on the transport processes in unicellular algae. Earlier data published by Scott (Scott, 1943; 1944), Knauss and Porter (1954) referred to simple diffusion processes. Other papers only casually touched upon absorption processes (Wedding, Erickson, Black, 1959, 1960; Kylin, 1964a, b, c; Shrift, Sproul, 1963) or discussed the problem of the uptake of toxic inhibitors (Wedding, Blackman, 1961) or reported too high concentrations (Taylor, 1960a, b). Papers (Kylin, 1964a, b, c, 1966; Schaedle, Jacobson, 1965, 1966; Nielsen, 1965; Cseh, Szabó, 1965; Shrift, 1966) discussing some aspects of these processes albeit not without contradictions, appeared when our examinations were well under way.

In this paper data on some fundamental characteristics of the transport processes of unicellular algae will be presented and preliminary observations of the changes in the parameters of bromide uptake during the cell cycle of synchronous cultures will be described.

Materials and Methods

The experiments were carried out with two algae strains supplied by the Biological Research Institute of the Hungarian Academy of Sciences, Tihany, and listed in the collection as Scenedesmus obtusiusculus Chod. No. 5618 and Chlorella vulgaris Beyer No. 7K. In the early 60's extensive physiological research was done in the Biological Research Institute (Felföldy, 1964) with these two strains. According to Prof. Prat (Prague) strain No. 7K is to be regarded as Chlorella zopfingiensis Doens (personal communication).

The algae strains were grown on solid agar media containing inorganic salts (a modified Knop–Pringsheim solution supplemented with trace elements) 0.5 per cent glucose and 0.2 per cent peptone. To produce the algae for the experiments a modified Knop–Pringsheim solution (from now on K–P solution) supplemented with trace elements was used in liquid cultures. The composition of the modified K–P solution was the following:

KNO_3	1000 mg/l
$Ca(NO_3)_2$	100
$MgSO_4 \cdot 7H_2O$	100
K_2HPO_4	200
$FeCl_3$	1
H_3BO_3	0.0143
$MnCl_2 \cdot 7H_2O$	0.0090

$ZnSO_4 \cdot 7H_2O$	0.0056
$CuSO_4 \cdot 5H_2O$	0.0020
K_2MoO_4	0.0045

In the majority of experiments algae grown for 5 to 7 days in 500-ml liquid cultures in one litre flasks were employed. The air used to aerate the liquid cultures contained approximately 5 per cent CO_2. The cycle consisted of a 14-hour light and 10-hour dark period with an illumination of 8000–9000 lux (F. 29 fluorescent tubes made by the Hungarian United Incandescent Factory). The temperature of the cultures was 22–24°C.

To establish synchronous cultures we adopted the method described by Tamiya and Morimura (1964) and made use of the experience gained from a preliminary experiment in the Biological Research Institute (Tihany) (Felföldy, personal communication). One-week-old liquid cultures were exposed for 4 to 5 days to an illumination of 800 to 1000 lux. The 45 by 380 mm tubes used for the synchronous culture were inoculated with algae grown under reduced light. The inoculum contained 2–3 million cells/ml. The intensity of the illumination was raised to 14,000 lux (F. 29 fluorescent tubes supplemented with candescent bulbs); the temperature was kept at 20–22°C with water cooling. The first cell division could be detected after 24 hours, when with a dark period, we started to alternate the regular 14 : 10 hour light : dark cycles. The cell number was determined daily both at the beginning and end of the light period and it was kept at the initial value by appropriately diluting the cultures. The increase in cell number varied from 3.5 to 4.8, the theoritical value is $N = 4$, which, taking into consideration possible errors of counting, be regarded satisfactory. The preparatory and synchronous cultures were aerated with air enriched with 5 per cent CO_2.

At the beginning of an uptake experiment the cells of an aliquot taken from the cultures were centrifuged and taken up in filtered K–P solution. In this alga suspension the number of cells was again determined and set at a value of 50–100 million/ml. Before the beginning of the uptake experiment the alga stock suspension was shaken for less than 20 minutes either in diffuse light, or under illumination by F. 29 fluorescent tubes (3000 lux), or in complete darkness. In the latter case the stock suspension was centrifuged and prepared in darkness. When the absorption of K and Na was studied, the alga suspension was prepared using a solution containing 100 mg/l of $Ca(NO_3)_2$ and 100 mg $MgSO_4$ (from now on Ca–Mg solution).

50 ml aliquots kept at 22°C and continually shaken were used in the uptake period. The solutions were prepared from filtered K–P solution, or from Ca–Mg solution in the K and Na experiments. Each sample contained approximately 50 million cells. On the basis of cell counting in parallel samples the data were calculated to 10^{10} cells. The uptake experiments were carried out either in diffuse light, or at 3000 lux or in complete darkness.

At the end of the uptake period the alga suspension was filtered through a MF 50 membrane filter (Membranfilter Ges., Göttingen) and washed with 20 ml

solution. (The composition of the liquid used for washing depended on the objective of the experiment and the ion used for further details see the respective experiments.) The filtering and washing took less than one minute.

In the K leakage experiments, absorption took place from a K^{42} labelled solution and the alga suspension was filtered and transferred together with the membrane filter to a solution containing inactive K^+ ions. At the end of the leakage period the suspension was filtered through a new membrane filter.

In previous experiments (Böszörményi, Cseh, Felföldy, Szabó, 1962) we used the MF 50 membrane filter to measure photosynthesis with C^{14} bicarbonate and concluded that it did not bind even traces of the labelled bicarbonate. In the present experiments, however, the filter was found to bind various labelled ions and amino acids in a quantity disturbing the measurements. For this reason the filtered algae could not be measured together with the filter. After filtration and washing the wet algae could be quantitatively removed from the filter. In the case of gamma radiating isotopes (Na^{22}, Na^{24}, K^{42} and Br^{82}) the algae were collected from the filter, were suspended in distilled water and the radioactivity was measured with a well-type scintillation measuring head. In the case of C^{14} labelling (glycine$-1-C^{14}$) the algae were suspended in a drop of water placed in the middle of an aluminium planchet and then the preparate was carefully dried under an infrared lamp. In this case the radioactivity was measured with a windowless GM tube.

Each experiment was repeated two or three times and the data given in the figures represent the average values.

Results

Uptake and leakage time-curves

The bromide uptake by Scenedesmus in darkness is linear at all the three, concentrations (0.01, 0.1 and 1 mM/examined Fig. 1). It is noteworthy that although after the absorption period the samples were washed with a 1 mM series the curve shows an intercept of 0.03 μequ./10^{10} cells. Potassium absorption by Scenedesmus examined at 0.003 mM concentration is also linear both in the light and in darkness Fig. 2). The c(urve obtained in the light starts with an intercept of approximately 0.2 μequ./10^{10} cells. When the uptake was determined in darkness at $1-2$ °C the values did not rise for one hour and remained within the limits of $0.05-0.1$ μequ./10^{10} cells.

As we reported earlier (Cseh, Szabó, 1965) the rate of glycine absorption by Scenedesmus remains approximately linear for an extended period. The intercept was found to be greater in the light than in darkness.

A part of the K^{42} ions is exchangeable if the cells are transferred to 0.01 mM Ca$-$Mg solution containing KCl (Fig. 3). Two phases can be distinguished on the exchange curve.

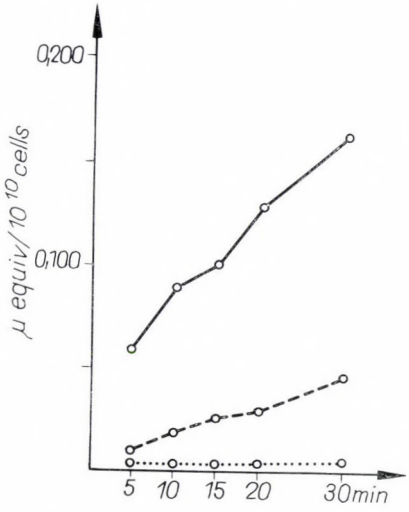

Fig. 1. Time curves of bromide uptake by Scenedesmus. Absorption from filtered K—P solution in darkness, KBr concentration 0.01 ·····, 0.1 --------- and 1 mM ———. Washing with filtered K—P solution containing 1 mM KBr

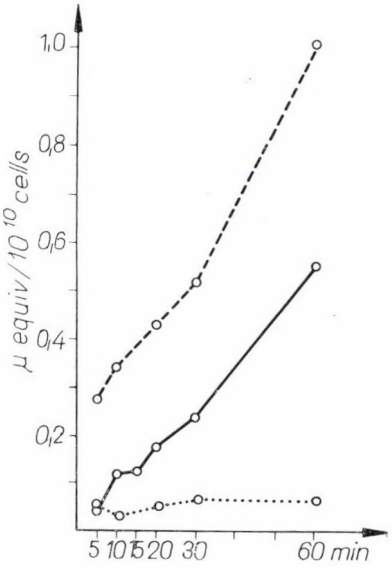

Fig. 2. Time curves of potassium uptake by Scenedesmus. Absorption from Ca—Mg solution, KCl concentration 0.003 mM. In darkness, at 22°C ——; illumination: 3000 lux, at 22°C --------; in darkness at 1—2°C ·····

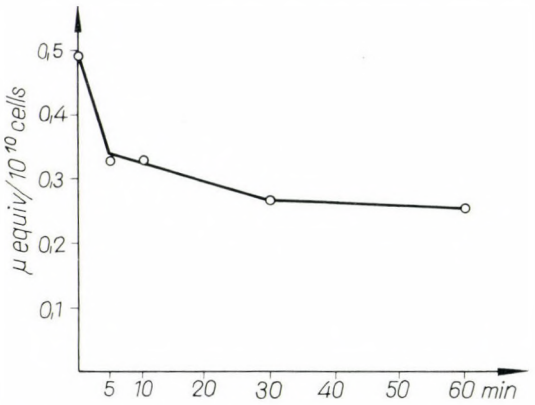

Fig. 3. Time curve of potassium leakage by Scenedesmus. Absorption from 0.01 mM Ca—Mg solution containing K^{42} Cl, in diffuse light, 30 minutes. Leakage in 0.01 mM inactive Ca—Mg solution containing KCl, in diffuse light. Washing with Ca—Mg solution

Concentration curves

Several concentration curves of the bromide uptake by Scenedesmus in 20-minute experiments were obtained both in the light and in darkness with different algae. In almost all cases the concentration curves can be divided into two linear parts, with a kind of saturation step between 1 and 3 mM (Fig. 4). The stimulation of absorption taking place with an illumination of 3000 lux was 50 to 90 per cent higher than that of the uptake in darkness. The extent of stimulation is roughly constant over the whole concentration range. Two series of the experiment presented in Fig. 4 were carried out with cells obtained from the beginning of the light period of a synchronized culture and two series with cells gained from the end of the light period. There is no difference between the two species either in the general pattern of the curve or in the extent of stimulation by illumination. From the beginning to the end of the period of illumination the rate of uptake (calculated on a cell basis) was found to rise by 300 per cent.

The concentration curve of bromide uptake by Chlorella failed to show the two separate stages observed in the case of Scenedesmus (Fig. 5). Over a higher concentration range, however, the curve indicates a kind of saturation. The stimulatory effect of light was found lower in samples obtained from the end of the light period, and it was also comparatively smaller over a higher concentration range. From the beginning to the end of the light period the rate of uptake calculated on a cell basis rose by only 200—250 cent.

In the case of Scenedesmus the saturation of the rate of potassium absorption took place at a concentration much lower than the rate of bromide uptake (Fig. 6). The K_m of the bromide absorption is about 0.5 mM, with that of the potassium uptake varying around 0.004 mM. In 20-minute experiments the rate of sodium

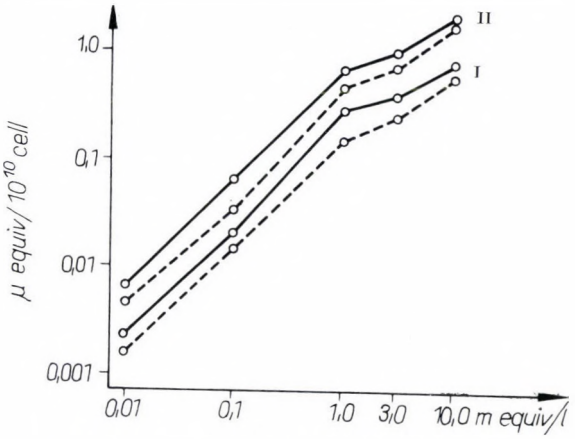

Fig. 4. Concentration curves of bromide uptake by Scenedesmus. Synchronous culture: I young cells from the end of dark period (8^h), II aged cells from the middle of light period (18^h). Absorption from filtered K—P solution, 20 minutes illumination: 3000 lux (———), or in darkness (---------). Washing with 1 mM KBr

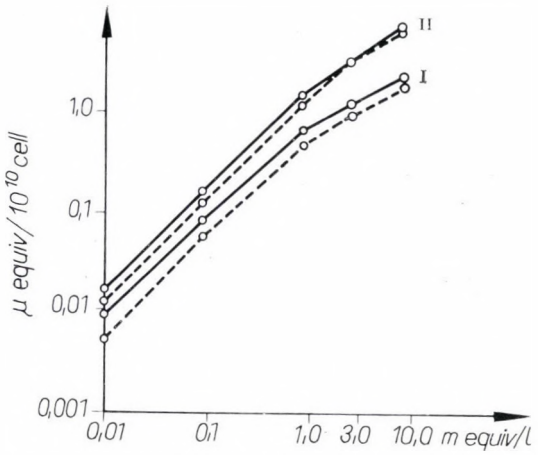

Fig. 5. Concentration curves of bromide uptake by Chlorella. Synchronous culture: I young cells from the end of dark period (8^h), II aged cells from the middle of light period (18^h). Absorption from filtered K—P solution, 20 minutes, illumination: 3000 lux (———), or darknes (---------). Washing with 1 mM KBr

absorption was lower than that of potassium and bromide uptake, and the K_m of sodium uptake was intermediate between the K_m-s of the two other ions.

In accordance with our data published earlier (Cseh, Szabó, 1965), the uptake of glycine showed only a slight deviation from a straight line over the concentration range examined.

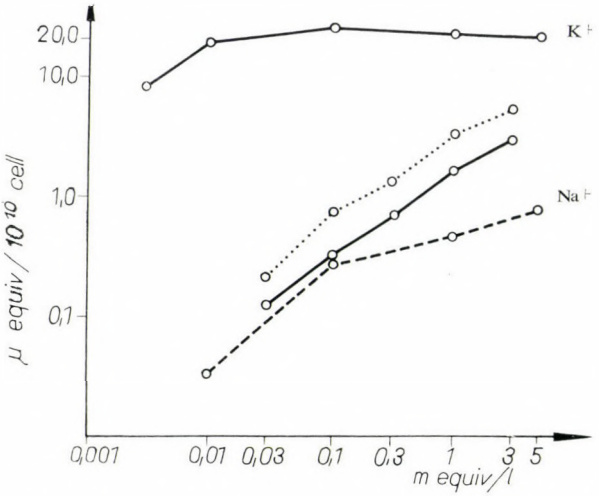

Fig. 6. Concentration curves of potassium (———), natrium (---------) and glycine uptake by Scenedesmus. K and Na absorption from Ca—Mg solution, in diffuse light, 20 minutes. Washing with Ca—Mg solution. Glycine uptake from filtered K—P solution, 3000 lux 5 (———) and 20 minutes (·····). Washing with 0.1 mM filtered K—P solution containing inactive glycine

K and Na competition

To study the competition between K and Na ions concentration curves were taken (5 mequ./l) in both the presence and the absence of the respective competitive ion. The concentration curves of the absorption of both ions (Figs 7 and 8) show, in accordance with Fig. 6, that the K_m of K uptake is much lower than that of Na uptake. Three parallel experiments were carried out with a one hour interval between them. (The data also indicate that the rate of K and Na absorption by algae kept on Ca – Mg solution decreased during storage.)

Na and K mutually inhibit each other's uptake. Surprisingly, the extent of inhibition is approximately the same, although, as mentioned earlier, there is a great difference between the rates of absorption of the two ions. At 0.01 mequ./l the inhibitory effect amounted to 90 – 92 per cent. (The concentration of the inhibiting ion was 500 times higher.) It is interesting to note that at a concentration of 5 mequ./l Na was found to have a stronger inhibitory influence on the absorption of K than vice versa.

Changes in bromide absorption during a synchronous cycle

The changes in the concentration curve (between 0.01 – 10 mequ./l) of bromide uptake by the synchronized culture of Scenedesmus were examined during a complete cycle, with samples taken every three hours. The uptake period took

20 minutes in darkness. The concentration curves obtained at 9 different points of time are qualitatively similar: after a constantly rising phase saturation tendency can be observed between 1 and 3 mequ./l followed by another rise. There is no essential change in the K_m of the uptake process, but its V_{max} has risen by 300 to 350 per cent if samples taken at the beginning and at the end of the light period

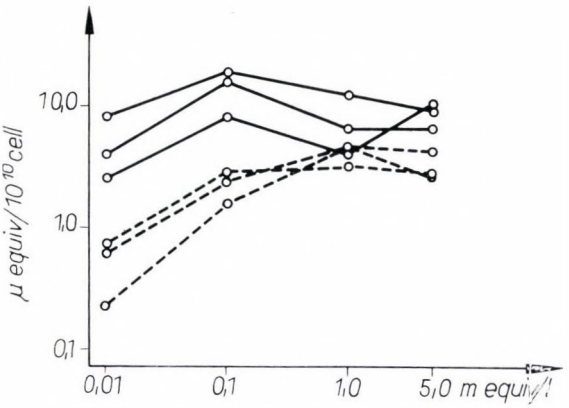

Fig. 7. Effect of sodium ion on potassium uptake by Scenedesmus. Absorption from Ca—Mg solution, in diffuse light, 20 minutes without (———), or with 5 mM NaCl (---------). Washing with Ca—Mg solution

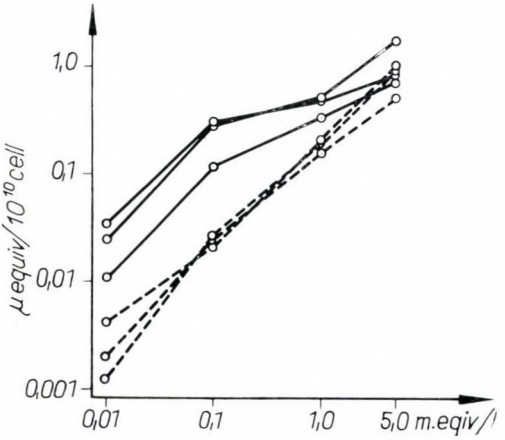

Fig. 8. Effect of potassium ion on sodium uptake by Scenedesmus. Absorption from Ca—Mg solution in diffuse light, 20 minutes without (———), or with 5 mM KCl (---------). Washing with Ca—Mg solution

are compared (Fig. 9). For the time being reasons for the slight decrease observed in the case of the 18-hour sample cannot be given, but a transitional error in aeration is likely to be responsible for the decline. During the dark period a decreasing trend in the rate of absorption could be detected. The fact that the end of the dark period the uptake at low concentrations rose may account for

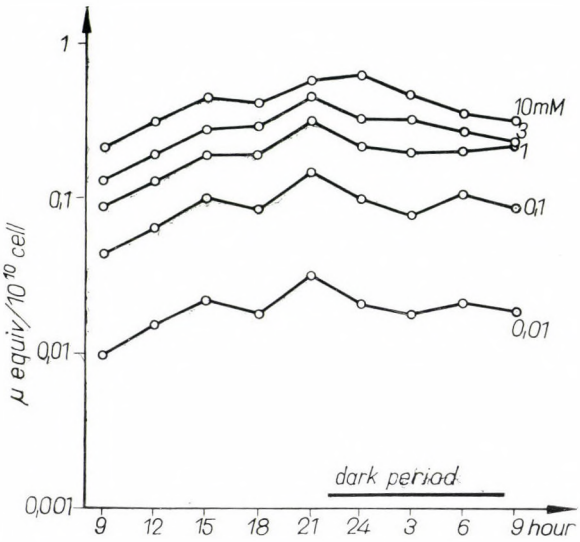

Fig. 9. Changes in bromide uptake by Scenedesmus during a synchronous cycle. Absorption from filtered K—P solution, 20 minutes, in darkness. Washing with 1 mM filtered K—P solution containing KBr

a slight change in the K_m. The initial value could not be obtained completely at the end of the cycle, a phenomenon likely to be attributed to the fact that full use was made of the culture and the sampling at 9 o'clock of the subsequent day was not preceded by the usual dilution step.

Discussion

Time curves of ion uptake by plants are generally characterized by an initial rapid phase followed by a phase of constant rate. Differences according to the objects and types of ions used can be observed between the two phases, but the methods of examination can also be responsible for these changes. For instance in the case of alkali cations (Ca-free solutions) the time curve begins with a rapid initial phase. This is usually explained either by the assumption that ion exchange

takes place on the negative groups of cell walls, or that a certain quantity of ions is being bound to special carriers (e.g. Fried, Noggle, Hagen, 1958). The presence of a rapidly exchangeable cation fraction in unicellular algae was reported by Scott (1944) and Cohen (1962a b,). According to Schaedle and Jacobson (1965) in Ch. pyrenoidosa this fraction reaches a value of 20–25 μequ./100 g dry weight and its exchange is completed within one minute.

Comparatively few authors have studied the possible occurrence of a rapid phase of anion absorption. Nielsen (1965) has reported Ch. pyrenoidosa, extrapolated from one minute measurements, can bind a measurable quantity of chloride ions. With Sc. obtusiusculus, extrapolated from five minute measurements, the quantity of bromide bound can reach 0.03 μequ./10^{10} cells. Similarly, the rapid anion uptake can be also considered an ion exchange (although the number of positive by changed groups in the cell wall is presumably small), or as a process taking place in the loosely bound electric double layer, or as a result of a reaction with special carriers (e.g. Laties, 1959).

In literature on biological transport the absorption curves saturating in the function of concentration are generally considered a proof of mediated uptake, but the linear concentration dependence does not necessarily prove diffusion; it can mean that the K_m of the mediated transport process in question is much higher than the concentration range under examination (Christensen, 1962). Considering to saturation character of the concentration curves of absorption we regard bromide uptake by Scenedesmus and Chlorella and the alkali ion transport of Scenedesmus as mediated absorption processes. In our view, the reason for Schaedle and Jacobson (1965) finding the uptake by Ch. pyrenoidosa of potassium independent of the concentration is due to the fact that they worked over a concentration range too high compared to the K_m. Like in the case of iodide uptake (Böszörményi and Cseh, 1964) or amino acid absorption (Cseh and Böszörményi, 1966) by excized roots. The concentration curve of glycine absorption by Scenedesmus shows only signs of saturation. This particular type of curve can be attributed to mediated transport of a relatively low rate and to diffusion. The concentration curves of a large number of transport processes have been found take of a complex character: they consist of a mediated process and diffusion or of two or more mediated processes. Of the absorption processes studied in unicellular algae only bromide uptake by Scenedesmus appears, on the basis of the concentration curve, to be a complex one. Presently available data are not detailed enough to enable us to take a position on the character (diffusion or mediated) of the process occurring at higher concentrations.

According to Kylin (1964a, b, c; 1966), in a Scenedesmus species a separate Na secretion process can be detected in addition to mediated potassium absorption. Our data (mutual competition between Na and K) suggest that the K and Na influx of Sc. obtusiusculus can take place through a common mediated absorption system. But they do not exclude the possibility that there is a separate Na secretion system; moreover, we possess certain (unpublished) "irregular" Na influx time curves that support Kylin's assumption. Studies on the timing of enzyme synthesis

in the synchronous cultures of different microorganisms have shown that the process is confined as a rule to certain phases of the cycle (Halvorson, 1964; Halvorson, Tauro, Epstein, Smith, 1966), although the reason being this sequentional enzyme synthesis has not yet been decided (e.g. Masters, Donachie, 1966; Goodwin, 1966). Knutsen (1965) has observed that the inducibility of nitrite reductase in Ch. Pyrenoidosa is confined to a short phase of the light period, and Johnson (1965) has reported that the synthesis of thymidilate kinase takes place during a similarly short period.

According to our preliminarly data the bromide absorption capacity (the V_{max} of the rate of uptake) in Sc. obtusiusculus gradually increases during the light period. The assumption can be made that the growth of the cell surface has a part to play in the rise of this capacity. It is also possible, however that the increase in the V_{max} can be ascribed to the synthesis of a protein component of the mediated transport system, or to changes in the energy pool that can be utilized for transport.

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Control by Tricarboxylic Acid Cycle Intermediates of the Oxidation of Pyruvate to Acetyl-CoA

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1. When the tricarboxylic acid cycle is blocked in liver mitochondria by trans-aconitate or malonate, the oxidation of pyruvate to acetyl-CoA is increased.

2. The effect of tricarboxylic acid cycle intermediates on the oxidation of pyruvate to acetyl-CoA was studied in a system which contained oxalate plus trans-aconitate. Under these conditions pyruvate can only be metabolized via acetyl-CoA, and the tricarboxylic acid cycle is blocked simultaneously. It has been found that (*a*) malate, fumarate, succinate, and 2-oxoglutarate have an inhibitory effect, (*b*) increasing the concentration of the tricarboxylic acid cycle intermediates results in an enhanced inhibition (*c*) succinate inhibits only if it is oxidized, (*d*) there is a direct correlation between the number of oxidative steps required for the conversion of an intermediate to oxaloacetate and the extent of its inhibitory action, and (*e*) the inhibition is overcome by 2,4-dinitrophenol.

3. It is suggested that there is a competition for the electron transport chain between the oxidation of tricarboxylic acid cycle intermediates and the oxidative decarboxylation of pyruvate and that the observed inhibition of the oxidation of pyruvate to acetyl-CoA is the consequence of this competition.

4. The rate-limiting factors in the in vitro conversion of pyruvate to acetyl-CoA are discussed. We suggest that the observed inhibition plays a regulatory role in the in vivo utilization of pyruvate and, consequently, in the operation of the tricarboxylic acid cycle.

Introduction

We have reported in a previous communication (König et al., 1964) that the utilization of pyruvate by isolated rat liver mitochondria is inhibited in the presence of fumarate. Haslam and Krebs (1963) found in studies with labelled substrates that there was a competition between the oxidation of pyruvate and that of 2-oxoglutarate in homogenates of rat liver, and that 2-oxoglutarate was oxidized preferentially. They suggested a number of mechanisms to account for this phenomenon, and assumed that the preferential oxidation of 2-oxoglutarate might play a role in the regulation of the formation of ketone bodies from pyruvate. Berry (1965) has shown by the use of carboxyl labelled pyruvate that in isolated rat liver cells the oxidation of pyruvate to acetyl-CoA is inhibited by fumarate. In our previous studies with liver mitochondria (König, Szabados, 1966) we have made a similar observation with different techniques. We have found that both

fumarate and succinate inhibit the utilization of pyruvate by liver mitochondria even in the presence of oxalate, that is, under conditions when the conversion of pyruvate to oxaloacetate is blocked (Losada et al., 1964; Seubert, Huth 1965; Mildvan et al., 1966). This observation indicates that it is the conversion of pyruvate to acetyl-CoA which is inhibited by fumarate and succinate. The same inhibition may play a regulatory role in the operation of the tricarboxylic acid cycle, and this has lead us to a more detailed investigation of the underlying mechanism. A short abstract of part of these studies has already been published (König, Szabados, 1966). In this paper we come to the conclusion that the intermediates of the tricarboxylic acid cycle inhibit the oxidative decarboxylation of pyruvate by being simultaneously oxidized, and thereby competing for the electron transport chain under conditions, when the activity of the electron transport chain is the limiting factor.

Materials and Methods

Preparation and incubation of rat liver mitochondria, the materials used and the measurement of oxygen uptake have already been described (König et al., 1964). In those experiments in which oxygen uptake was not determined, the reaction mixtures were incubated in 25-ml Erlenmeyer flasks with continuous shaking. The standard reaction mixture contained in a final volume of 3 ml 10 mM potassium phosphate buffer, pH 7.5; 3 mM $MgCl_2$; 2 mM ATP–Na; and 250 mM sucrose. The concentration of substrates and inhibitors is given in the tables. Pyruvate was determined after Koepsell and Sharpe (1952), citrate according to Schneider et al. (1956) and acetoacetate according to Walker (1954). An additional control was used for the determination of acetoacetate in the presence of malonate. In this control the amount of formazan formed from malonate was determined according to Walker, and the acetoacetate value was corrected accordingly. Nitrogen was determined by a micro-Kjeldahl method.

Results

We have started from the assumption that, if the conversion of pyruvate to acetyl-CoA is inhibited in the presence of tricarboxylic acid cycle intermediates, a blocking of the cycle should result in an increased formation of acetyl-CoA from pyruvate. To test this hypothesis the tricarboxylic acid cycle was blocked by inhibiting the isomerization of citrate catalyzed by aconitase. For this purpose, we used trans-aconitate which is a competitive inhibitor of aconitase (Saffran, Prado, 1949).

Table I shows the effect of 1 and 8 mM trans-aconitate on the metabolism of pyruvate in liver mitochondria.

There is no marked change in oxygen uptake in the presence of trans-aconitate. On the other hand the utilization of pyruvate is increased in the pres-

Table 1

Effects of trans-aconitate and oxalate on pyruvate metabolism by liver mitochondria

Standard reaction mixture. Substrate: 8.33 mM pyruvate. Amount of mitochondria: 1.82 mg N per 3 ml reaction mixture. Incubation at 37 °C for 35 min. The values refer to 3 ml reaction mixture

Addition(s)	—	trans-aconitate			1 mM oxalate
		1 mM	8 mM	8 mM + 1 mM oxalate	
Oxygen uptake μatoms	12.1	11.5	12.6	11.3	9.2
Pyruvate utilization μmoles	12.2	14.6	19.3	14.5	11.7
Acetoacetate production μmoles	1.14	2.36	3.16	4.33	4.13
Citrate accumulation μmoles	1.50	2.06	4.55	1.45	0.04

ence of trans-aconitate, and this increase is dependent upon trans-aconitate concentration. Increased pyruvate utilization is accompanied by increased production of acetoacetate and increased accumulation of citrate. These results support our original assumption: if the tricarboxylic acid cycle is blocked, the utilization of pyruvate via acetyl-CoA is increased. This would be the main reason for the ketogenic effect of trans-aconitate. The greatly increased accumulation of citrate indicates that the synthesis of oxaloacetate from pyruvate is unimpaired. Pyruvate utilization and acetoacetate production are also significantly enhanced when the tricarboxylic acid cycle is inhibited with malonate. In the latter case succinate will accumulate instead of citrate.

If oxalate is added to a system containing trans-aconitate, that is, we inhibit the formation of oxaloacetate from pyruvate, the utilization of pyruvate and the accumulation of citrate decrease considerably. In contrast, there is a further increase in the formation of acetoacetate. There is also a slight decrease in oxygen uptake. The inhibition of pyruvate carboxylase by oxalate can account for all these observations. The results further demonstrate that there is a considerable synthesis of oxaloacetate from pyruvate in isolated rat liver mitochondria (König et al., 1964) as well, as in homogenates (Haslam, Krebs, 1963).

In a system containing oxalate plus trans-aconitate, pyruvate can only be metabolized via acetyl-CoA, and the citrate which is formed from added oxaloacetate precursors can not be further metabolized in the tricarboxylic acid cycle. This system is, therefore, suitable for a study of the effect of added tricarboxylic acid cycle intermediates on the conversion of pyruvate to acetyl-CoA. A further methodical advantage of this procedure is that the extent of conversion of pyruvate to acetyl-CoA can be measured by determining pyruvate utilization.

The effect of succinate and malate on pyruvate metabolism in the presence of oxalate plus trans-aconitate is shown in Table 2.

Table 2

Effects of malate and succinate on pyruvate metabolism by liver mitochondria in the presence of oxalate plus trans-aconitate

Standard reaction mixture. Substrate: 8.33 mM pyruvate. All reaction mixtures contained also 1 mM oxalate + 8 mM trans-aconitate. Amount of mitochondria: 1.46 mg N per 3 ml reaction mixture. Incubation at 37 °C for 45 min. The values refer to 3 ml reaction mixture

Addition	—	1 mM L-malate	1 mM succinate
Oxygen uptake μatoms	12.4	13.4	13.9
Pyruvate utilization μmoles	14.8	11.9	11.0
Acetoacetate production μmoles	3.93	2.17	2.03
Citrate accumulation μmoles	1.51	2.53	2.39

Both intermediates of the tricarboxylic acid cycle inhibit the oxidation of pyruvate to acetyl-CoA. Oxygen uptake is slightly increased. Formation of acetoacetate is decreased, while the accumulation of citrate is increased.

In our next experiment we have studied the extent to which the above effects of intermediates of the tricarboxylic acid cycle depend upon their concentrations.

It is seen from Table 3 that both malate and succinate inhibit the conversion of pyruvate to acetyl-CoA more effectively in a higher concentration. As expected, when the concentration of the tricarboxylic acid cycle intermediate is higher, less acetoacetate is formed and more citrate is accumulated.

With respect to the mechanism of the inhibition further information was gained in experiments in which the effect of some tricarboxylic acid cycle inter-

Table 3

Effects of two different concentrations of malate and succinate on pyruvate metabolism by liver mitochondria in the presence of oxalate plus trans-aconitate

Standard reaction mixture. Substrate: 8.33 mM pyruvate. All reaction mixtures contained also 1 mM oxalate + 8 mM trans-aconitate. Amount of mitochondria: 1.27 mg N per 3 ml in Expt. 1, and 1.47 mg N per 3 ml in Expt. 2. Incubation at 37 °C for 45 min in Expt. 1, and for 40 min in Expt. 2. The values refer to 3 ml reaction mixture.

Addition	—	1 mM L-malate	2 mM L-malate	1 mM succinate	2 mM succinate
Expt. 1					
Pyruvate utilization μmoles	12.9	10.3	9.6	—	—
Acetoacetate production μmoles	4.33	2.41	1.67	—	—
Citrate accumulation μmoles	1.37	2.51	3.42	—	—
Expt. 2					
Pyruvate utilization μmoles	13.0	—	—	9.7	8.0
Acetoacetate production μmoles	3.35	—	—	1.78	1.07
Citrate accumulation μmoles	1.12	—	—	1.91	2.17

mediates on the oxidation of pyruvate to acetyl-CoA was compared. The inhibition of pyruvate utilization by 1 mM L-malate, fumarate, succinate and 2-oxoglutarate in a system containing oxalate plus trans-aconitate is shown in Table 4.

Table 4

Comparison of the inhibitory effect of some tricarboxylic acid cycle intermediates on the oxidation of pyruvate to acetyl-CoA by liver mitochondria

Standard reaction mixture. Substrate: 8.33 mM pyruvate. All reaction mixtures contained also 1 mM oxalate + 8 mM trans-aconitate. Amount of mitochondria: 1.11 mg N per 3 ml in Expt. 1, and 1.22 mg N per 3 ml in Expt. 2. Incubation at 37°C for 45 min in Expt. 1, and for 50 min in Expt. 2. The values refer to 3 ml reaction mixture

Addition	–	1 mM L-malate	1 mM fumarate	1 mM succinate	1 mM 2-oxoglutarate
Expt. 1					
Pyruvate utilization μmoles	11.3	10.1	10.2	9.1	7.7
Inhibition of pyruvate utilization	0%	11%	10%	19.5%	32%
Expt. 2					
Pyruvate utilization μmoles	12.1	11.0	10.7	9.7	8.3
Inhibition of pyruvate utilization	0%	9%	11.5%	20%	31.5%

From the data it is concluded that there is a direct correlation between the extent of inhibition of the conversion of pyruvate to acetyl-CoA exerted by an intermediate, and the number of oxidative steps required for its conversion to oxaloacetate. Support to this conclusion comes from the observation that succinate does not inhibit the oxidation of pyruvate to acetyl-CoA in the presence of oxalate plus malonate in contrast to the inhibition observed in the presence of oxalate plus trans-aconitate (Table 5).

Table 5

Effects of succinate on pyruvate metabolism by liver mitochondria in the presence of oxalate plus trans-aconitate or malonate

Standard reaction mixture. Substrate: 8.33 mM pyruvate. Amount of mitochondria: 1.22 mg N per 3 ml. Incubation in Erlenmeyer flasks at 37 °C for 50 min. The values refer to 3 ml reaction mixture

Additions	1 mM oxalate + 8 mM trans-aconitate		1 mM oxalate + 8 mM malonate	
		+ 1 mM succinate		+ 1 mM succinate
Pyruvate utilization μmoles	12.1	9.7	12.7	12.5
Acetoacetate production μmoles	3.01	1.66	4.34	4.14
Citrate accumulation μmoles	1.14	2.02	0.01	0.49

This means that succinate is no longer inhibitory when its oxidation is inhibited. In the presence of oxalate plus malonate succinate has practically no influence either on the formation of acetoacetate or the accumulation of citrate.

As a next step we wanted to decide whether the oxidation of tricarboxylic acid cycle intermediates would also inhibit the oxidative decarboxylation of pyruvate when the activity of the electron transport chain is increased by uncoupling. Therefore, we studied the effect of tricarboxylic acid cycle intermediates on the oxidation of pyruvate to acetyl-CoA in the presence of DNP. The results of two experiments of this type are shown in Table 6.

Table 6

Action of 2,4-dinitrophenol on the effects of tricarboxylic acid cycle intermediates on pyruvate metabolism by liver mitochondria in the presence of oxalate and trans-aconitate

Standard reaction mixture. Substrate: 8.33 mM pyruvate. All reaction mixtures contained also 1 mM oxalate + 8 mM trans-aconitate. Amount of mitochondria: 1.32 mg N per 3 ml in Expt. 1, and 1.36 mg N per 3 ml in Expt. 2. Incubation at 37 °C for 45 min in Expt. 1, and for 40 min in Expt. 2. The values refer to 3 ml reaction mixture.

Addition(s)	–	0.01 mM DNP	1 mM succinate	1 mM succinate + 0.01mM DNP	1 mM L-malate	1 mM L-malate +0.01 mM DNP
Expt. 1						
Oxygen uptake μatoms	10.0	10.5	12.2	19.0	—	—
Pyruvate utilization μmoles	13.4	13.4	11.0	15.6	11.4	16.0
Acetoacetate production μmoles	3.56	5.19	2.07	4.59	2.29	4.50
Citrate accumulation μmoles	1.26	1.35	2.39	3.58	2.83	3.88
Expt. 2						
Oxygen uptake μatoms	9.9	—	11.3	17.9	11.2	17.4
Pyruvate utilization μmoles	11.4	9.2	8.7	12.7	9.5	13.4
Acetoacetate production μmoles	2.81	3.53	1.38	3.91	1.57	3.80
Citrate accumulation μmoles	1.06	1.31	1.78	2.98	2.25	3.47

From among a great number of replications, DNP slightly inhibited in some experiments the conversion of pyruvate to acetyl-CoA in the presence of oxalate plus trans-aconitate. The reason for this inhibition is not clear. However, in the presence of DNP irrespective of whether, or not it inhibited the above reaction succinate and malate increased rather than decreased the oxidation of pyruvate to acetyl-CoA, as compared to the control, which contained only oxalate plus trans-aconitate. At the same time there was a significant increase of oxygen uptake and an enhanced formation both of acetoacetate and citrate. In those experiments in which DNP did not inhibit, upon the addition of a dicarboxylic acid there was a stoichiometric relationship between the increment in the oxidation of pyruvate to acetyl-CoA and the increment in the synthesis of citrate. The main conclusion which can be drawn from these experiments is that in the

presence of DNP tricarboxylic acid cycle intermediates do not inhibit the conversion of pyruvate to acetyl-CoA.

Discussion

The results show that all intermediates of the tricarboxylic acid cycle used in our experiments inhibit the conversion of pyruvate to acetyl-CoA in isolated rat liver mitochondria. The question to be discussed is the possible mechanism of this inhibition.

Haslam and Krebs (1963) have suggested that there are three possible mechanisms which may account for the preferential oxidation of 2-oxoglutarate in the presence of pyruvate: (1) There is a competition between the oxidation of pyruvate and 2-oxoglutarate for a component of the electron transport chain, (2) there is a competition for a common cofactor involved in the oxidative decarboxylation of both substrates, and (3) there is a competitive inhibition of pyruvate oxidase by 2-oxoglutarate at the level of the active centre.

Evidently, mechanisms (2) and (3) can be ruled out in the case of succinate, fumarate and malate, since the presence of trans-aconitate in our system prevents the formation of 2-oxoglutarate from these dicarboxylic acids. Of course, it is still possible that these two mechanisms contribute to the inhibition by 2-oxoglutarate. It is a further possibility that the inhibition of the oxidative decarboxylation of pyruvate by intermediates of the tricarboxylic acid cycle is the result of an allosteric effect. The observation, however, that succinate is no longer inhibitory when its oxidation is inhibited by malonate provides convincing evidence against an allosteric mechanism. In the same time this fact indicates that the inhibitory effect of tricarboxylic acid cycle intermediates on the conversion of pyruvate to acetyl-CoA is due to a mechanism which involves a competition for the electron transport chain between the oxidation of these intermediates and the enzyme complex catalyzing the oxidative decarboxylation of pyruvate. This assumption is further supported by the following experimental evidences: (1) The oxidation of pyruvate to acetyl-CoA is increased when the tricarboxylic acid cycle is blocked by either trans-aconitate or malonate. (2) In the presence of oxalate plus trans-aconitate the extent of inhibition is directly related to the number of oxidative steps required for the transformation of the added intermediate to oxaloacetate. (3) The inhibition is overcome if the activity of the electron transport chain is stimulated by DNP.

One should also consider that there is a difference in the penetration rate of different members of the cycle into the mitochondria (Chappell, 1966), since this might determine the extent to which they inhibit the oxidation of pyruvate to acetyl-CoA. However, we have found a strong correlation between the number of oxidative steps required for the transformation of an intermediate to oxaloacetate and its inhibitory effect. This correlation indicates that differences in permeability do not play a decisive role under our experimental conditions.

It follows from the above discussion that a competition between the oxidation of tricarboxylic acid cycle intermediates and the oxidative decarboxylation of pyruvate can only occur if their simultaneous oxidations are limited by the activity of the electron transport chain. Of course, there may also be a number of further limiting factors (Krebs, 1959). Therefore, it is worth while to discuss some other possible factors, which will limit the oxidation of pyruvate to acetyl-CoA in mitochondria when the experimental conditions are changed.

When pyruvate is added to liver mitochondria, acetyl-CoA and oxaloacetate are generated which, in turn, yield citrate upon condensation. Citrate is further oxidized in the tricarboxylic acid cycle and in this way the oxidative decarboxylation of pyruvate is inhibited. Accordingly, if the oxidation of citrate is inhibited with trans-aconitate the conversion of pyruvate to acetyl-CoA will increase. Apparently, the oxidation of pyruvate to acetyl-CoA is not limited by the activity of the electron transport chain in a system which contains oxalate plus trans-aconitate, that is, in which pyruvate can only be metabolized via acetyl-CoA, since under these conditions DNP does not increase the utilization of pyruvate. More likely it is the rate of utilization of acetyl-CoA for the synthesis of acetoacetate, which is the limiting factor. It has recently been reported that pyruvate dehydrogenase is inhibited by acetyl-CoA (Garland, Randle, 1964, Shepherd, Garland, 1966). The situation is different if a tricarboxylic acid cycle intermediate is also added to the system containing oxalate plus trans-aconitate. The intermediate will be oxidized simultaneously, and the oxidative decarboxylation of pyruvate will be limited by the activity of the electron transport chain. This results in a decreased oxidation of pyruvate to acetyl-CoA. If DNP is also added to the system oxygen uptake will markedly increase and, therefore, the activity of the electron transport chain will no longer be the limiting factor. Under these conditions, the utilization of acetyl-CoA will again be the limiting factor. Acetyl-CoA will be used not only for the production of acetoacetate, but also for the synthesis of citrate. Consequently, the rate of oxidation of pyruvate to acetyl-CoA will increase to the same extent as that of the condensation of acetyl-CoA with oxaloacetate produced by the oxidation of the added tricarboxylic acid cycle intermediate. The greatly increased synthesis of citrate in the presence of DNP plus a dicarboxylic acid can be accounted for by both a stimulated oxidation of the dicarboxylic acid to oxaloacetate, and a higher activity of the condensing enzyme due to a low ATP/ADP ratio (Hathaway, Atkinson 1965, Shepherd, Garland, 1966).

It was suggested (Keech, Utter 1963, Krebs 1964, Krebs et al., 1964) that in addition to the actual ATP/ADP ratio, acetyl-CoA contributes to the control of the synthesis of oxaloacetate from pyruvate, and this way to the regulation of glyconeogenesis and the operation of the tricarboxylic acid cycle. According to a generally accepted view the rate of cellular respiration is limited in vivo by the phosphate acceptor through the activity of the electron transport chain. Therefore, the observed feedback inhibition of the oxidation of pyruvate to acetyl-CoA, which is due to the oxidation of tricarboxylic acid cycle intermediates,

might also operate in vivo. The extent of this feedback inhibition may be even significantly greater at physiological levels of the metabolites than in our model systems. In other words, this feedback mechanism may be an important component of the complex regulatory mechanism discussed above. The concentration of tricarboxylic acid cycle intermediates in tissues is constant under normal conditions (Krebs, Lowenstein, 1960). If, for some reason, they do accumulate – e.g. glyconeogenesis is arrested, or their utilization in other synthetic processes is reduced – their oxidation will be increased. This results in a decreased rate of production of acetyl-CoA from pyruvate and, consequently, the rate of oxaloacetate synthesis is reduced. In this way the normal level of the tricarboxylic acid cycle intermediates can be rapidly restored.

Note added in proof. After this paper had been submitted for publication, the work of R. J. Haslam (in Regulation of Metabolic Processes in Mitochondria. Tager J. M., Papa S., Quagliariello E., Slater E. C. (editors), Elsevier Publishing Co., Amsterdam 1966. p. 108) came to our knowledge, in which some results similar to ours were obtained with a rat-liver homogenate system.

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A Heat Resistant Factor from E. coli Involved in the "Transfer Reaction" of Protein Synthesis

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Two complementary factors were separated by protamine fractionation from the p-hydroxy-mercuri-benzoate treated 105,000 g supernatant of disrupted Escherichia coli B cells. Both factors were necessary for the polyuridylic acid directed polyphenylalanine synthesis on purified Escherichia coli B. ribosomes. The factor precipitated by protamine appeared to be a heat sensitive protein (partially purified), whereas the other one present in the supernatant following protamine treatment was extremely heat resistant. This factor was purified and some of its physico-chemical characteristics were established. Based on its stability and precipitation characteristics, ultraviolet absorption spectrum and its sensitivity to proteolytic enzymes it seems to be a polypeptide. This factor proved to be specific for bacterial ribosomes. Its possible biological role in protein synthesis is discussed.

Introduction

One of the most important steps in protein biosynthesis is the so-called "transfer reaction". This term – to become sooner or later out of date – implies a series of reactions, comprising the synthesis of peptide bonds. It takes place on the surface of ribosomes and involves the interaction between messenger RNA carrying the structural information and aminoacyl-tRNA's bound in proper order to the messenger–ribosomal complex. Apart from the above-mentioned components (ribosomes, messenger RNA, aminoacyl-tRNA's) the transfer process requires some accessory factors, such as monovalent cations (NH_{4+} or K^+), Mg^{++} ions and GTP. Last, but not least, two or more protein factors, so-called "transfer enzymes" or "polymerization factors" are needed for the active process. These are partly dissolved in the cell sap (105,000 g supernatant) and partly bound to the ribosomes. The presence of these factors has been demonstrated in extracts from rat liver by Fessenden, Moldave (1961); Gasior, Moldave (1965a);

Abbreviations used: ATP = adenosine 5' triphosphate; ADP = adenosine 5' diphosphate; GTP = guanosine 5' triphosphate; UDP = uridine 5' diphosphate; PEP = phosphoenolpyruvic acid; PEP kinase = phosphoenolpyruvic acid kinase; poly U = polyuridylic acid; poly A = polyadenylic acid; EDTA = ethylene diamine tetraacetate; Tris = Tris-(hydroxymethyl) aminomethane; tRNA = transfer ribonucleic acid; TCA = trichloroacetic acid; PVS = polyvinyl sulfate; p-MB = p-hydroxy-mercuri-benzoate

Gasior, Moldave (1965b); in rabbit reticulocytes by Bishop, Schweet (1961); Arlinghaus, Schaeffer, Schweet (1964); from cells of Escherichia coli B by Allande et al. (1964); Conway, Lipmann (1966). The partially purified protein fraction from animal tissues characterized by transfer activity has been resolved later into two complementary factors. At first two, and later three cooperating "polymerization factors" have been isolated from the E. coli supernatant fraction in Lipmann's laboratory. The actual role of these factors in the transfer process is not yet known and it is not even clear which of the steps of the transfer process is enzyme-dependent or needs another accessory factor.

The elucidation of the role of transfer factors is difficult because the overall process has not yet been adequately resolved into its component reactions.

We have studied some aspects of the transfer process in a ribosomal system isolated from E. coli B cells. It was possible to separate a heat resistant factor from the 105,000 g supernatant solution of these cells which in cooperation with a thermolabile protein factor of the same origin is necessary for polyuridylic acid directed phenylalanine polymerization on the E. coli ribosomes using aminoacyl-tRNA as donor.

Materials and Methods

1. E. coli B cells were grown under aerobic conditions on the following medium: KH_2PO_4, 3.0 g; $Na_2HPO_4 \cdot 7H_2O$, 15.0 g; NaCl, 3.0 g; Na-citrate (tribasic), 3.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; Casamino Acid (Bacto), 4.0 g; Yeast Extract (Bacto), 4.0 g; glucose 5.0 g; water ad 1000 ml; pH 7.2.

Cells were harvested in the early logarithmic phase of growth. All subsequent procedures were carried out at 0–4°.

The cells were washed once with 0.15 M KCl then suspended in a "standard buffer solution" (the composition of the "standard buffer solution" was the following: 0.01 M Tris-HCl; 0.01 M Mg-acetate; 0.002 M mercaptoethanol; pH 7.5). The cell paste collected by centrifugation at 5000 g for 20 min was either used immediately or stored at −20° in the frozen state.

2. The cells were broken by grinding with commercial Amylum Tritici (according to our experience better results were achieved with this method compared to grinding with alumina). The cell paste was mixed with starch in a weight ratio of 1 : 1.2–1.5 and ground in a chilled mortar or in a polyethylene tube until it became a very viscous mass. For the purpose of decreasing its viscosity, deoxyribonuclease was added to the cell mass (1 μg/ml extract). The ground cell mass was then taken up in the "standard buffer solution" (3–3.5 volumes of the original cell weight) mixed thoroughly at least for half an hour (until the clusters disappeared). The starch and whole cells were removed by centrifugation at 9000 g for 25 min and the supernatant was clarified by centrifuging at 30,000 g for 45 min. Ribosomes were sedimented from the supernatant by centrifugation at 75,000 g for 3 hours in the No. 30 rotor of the Spinco Model L centrifuge. The

combined supernatant was stored in a refrigerator at $-20°$. This supernatant served as the starting material for the isolation of transfer factors.

3. Purification of E. coli ribosomes was carried out by the procedure of Allande et al. (1964). The turbid layer above the ribosome pellet was removed by rinsing the sediment with 0.01 M Tris HCl buffer pH 7.8 containing 0.002 M Mg-acetate. The ribosomes were suspended in the same buffer solution (1/3 of the original volume). The suspension was gently stirred at low speed in a Potter-homogenizer. It was clarified by centrifuging at 15,000 g for 10 min. The risobomes were pelleted by centrifugation at 105,000 g for 1 hour in the No. 40 rotor of the Spinco Model L centrifuge. The supernatant was discarded and the washing of the ribosomes was repeated twice in the same way. The ribosome pellet was suspended in a 0.01 M Tris HCl buffer solution (pH 7.8) containing 0.01 M Mg-acetate. The suspension was spun again at 15,000 g for 10 min. The ribosomes free of transfer enzymes retained their activity for at least one month if kept frozen.

In some cases the crude ribosome fraction was washed twice with 0.01 M Tris HCl buffer (pH 7.8) containing 0.5 M NH_4Cl and 0.002 M Mg-acetate. In these experiments at least 3 hours were necessary for the precipitation of the ribosomes at 105,000 g. The last washing and subsequent treatment of ribosomes was the same as mentioned above.

4. tRNA was isolated from E. coli B cells by the method of v. Ehrenstein and Lipmann (1961). Charging of tRNA with ^{14}C labeled phenylalanine (47.3 mCi/mmole) or in some cases with ^{14}C labeled lysine (91 mCi/mmole) was carried out using the procedure of the same authors (v. Ehrenstein and Lipmann, 1961) with some slight modification. 100 mg tRNA was charged with 10 μ Ci ^{14}C phenylalanine (or lysine) in a reaction mixture of 5 ml using 0.3 ml of the 105,000 g supernatant fluid of E. coli (protein content nearly 5 mg) as a source of aminoacyl-tRNA synthesizing enzymes. Following the deproteination by phenol treatment, the nucleic acids were precipitated from the aqueous phase with 2.5 volumes of ethanol. The precipitate collected by centrifugation at 5000 g for 5 min was transferred to a chilled Büchner funnel, washed with 20–30 ml of 80% ethanol at $-10°$ and dried by air suction. The precipitate was dissolved in 10 ml distilled water, the insoluble material was removed by centrifugation and the supernatant was lyophilized in one milliliter aliquots and stored in scaled vials at $-20°$.

Specific radioactivity of the charged tRNA was 12,000–18,000 cpm/mg nucleic acid (measured in a windowless Friesecke–Hoepfner gas flow-counter).

Charged tRNA was dissolved in distilled water immediately before use.

5. Polyuridylic and polyadenylic acids were synthesized from UDP or ADP respectively using a polynucleotide phosphorylase preparation partially purified from Micrococcus lysodeicticus (Basilio, Ochoa, 1963). The sedimentation constant of the poly U was about 7 S, that of poly A about 8 S.

6. The "soluble enzyme fraction" from rabbit reticulocytes containing the aminoacyl-tRNA synthetases was prepared by the procedure of Allen and Schweet (1962).

7. The activity of transfer enzymes was essayed by two alternative methods:

a) The method of Allande, Monro and Lipmann (1964). An 0.5 ml reaction mixture contained 0.4–0.5 mg of ribosomal protein, 20 μg of polyuridylic (or polyadenylic) acid, 0.2 mg of ^{14}C phenylalanine (or lysine) charged tRNA (3–4000 cpm), 0.5 μmoles ATP, 0.1 μmole GTP, 6.5 μmoles Mg-acetate, 40 μmoles NH_4Cl, 50 μmoles Tris-HCl, 3 μmoles mercaptoethanol, and transfer enzymes. The pH of the mixture was 7.8. (Assay method I.)

The reaction was started after a 3-minute preincubation by the addition of charged tRNA. The incubation period was 10 min at 30°. The process was stopped by precipitation of proteins with 2 ml of 10% trichloroacetic acid.

b) A simplified method was introduced in the course of this work using a "soluble enzyme fraction" of animal origin as aminoacyl activator and measuring the incorporation of free amino acids (for details cf. the "Experimental results" of this paper). (Assay method II.)

In cases when polylysine synthesis was investigated, hydrolysis with 1 ml of 10% TCA was followed by the addition of 4 ml of "tungstate mixture" (50 ml of 3.5% sodium tungstate dissolved in 1 M acetate buffer pH 4.8 was mixed with 950 ml of a 1% TCA solution). After standing for 10 min the precipitate was centrifuged, thoroughly homogenized and washed three times with the "tungstate mixture" and finally with ethanol.

The protein precipitate suspended in distilled water was plated on aluminium planchets 2 cm in diameter and dried under an infrared lamp. The amount of plated protein was weighed. The radioactivity of the samples was measured in a Friesecke–Hoepfner gas flow-counter. The specific radioactivities were calculated per mg of ribosomal protein. The results were corrected by substracting incorporated counts in samples without transfer enzymes. These blank values are indicated in the legends to the individual figures.

8. Preparation of the 75,000 g supernatant fluid. The first steps of purification of the transfer factors were essentially the same as those in the procedure of Allande, Monro and Lipmann (1964). Nucleic acids were precipitated with streptomycin and the proteins were fractionated by stepwise addition of solid $(NH_4)_2SO_4$. The pH of the solution in the course of fractionation was kept constant by the addition of solid Tris. The bulk of the transfer activity was precipitated between 40% and 60% saturation with ammonium sulfate. This precipitate was dissolved in the "standard buffer solution" (1/10 of the original volume of the 75 000 g supernatant) and dialyzed against the same buffer overnight. The dialyzed material (designated as "40%–60% A. S. fraction") in the frozen state retained its activity for 1–2 months.

9. Protein was determined by the Folin method (Lowry, 1951).

Tris (hydroxymethyl) amino methane (Leith) was purified by recreystallization from hot water. 2-mercaptoethanol was purchased from Fluka A. G. Phosphoenolpyruvic acid (PEP) was obtained from REANAL (Budapest) in the form of Ba–Ag salt and was transformed to the K-salt. Pyruvate-kinase was prepared from rabbit muscle by the method of Bücher and Pfleiderer (1955).

ATP; Na salt; GTP, Na salt; UDP and ADP as Ba salts were obtained from REANAL, Budapest. ^{14}C phenylalanine (47.3 mCi/mmol) and ^{14}C lysine (91 mCi/mmol) were obtained from the Institute of Isotopes, Budapest. Protamine sulfate was obtained from Insulinwerke, Copenhagen. DEAE cellulose for column chromatography was a product of W and R Balston, Ltd., London. Calcium phosphate gel was prepared according to Keilin and Hartree (1938). Polyvinylsulfate was synthesized by the procedure of Nomura, Hosoda and Nishimura (1958).

Experimental Results

I. Species specificity of transfer enzymes

In agreement with literary data, species specific differences were found with respect to transfer enzymes extracted from the tissues of higher organisms (rat liver, calf liver, rabbit reticulocytes) on the one hand, and transfer factors of bacterial origin on the other.

105,000 g supernatant fractions of the above mammalian tissues were inactive when combined with purified E. coli ribosomes in the amino acid polymerization test. The reciprocal phenomenon was also valid: transfer enzymes of E. coli cells did not cooperate with the ribosomes of animal origin. However, it could be shown that E. coli tRNAs were actually charged with amino acids (at least in the case of phenylalanine and lysine) by the "soluble enzyme fraction" prepared from rabbit reticulocytes or rat liver. This observation rendered it possible to construct a simplified system for testing the transfer enzymes. This system containing E. coli ribosomes and a soluble enzyme fraction from mammalian cells does not require the addition of charged tRNA as the free amino acids are activated in the reaction mixture by the mammalian "soluble enzyme fraction".

On the basis of this principle the following test system was constructed and used for the E. coli transfer enzyme assay in addition to "assay method I." described in "Methods". 0.5 ml reaction mixture contained 0.3–0.4 mg of E. coli ribosomes, 20 μg poly U (or poly A), 0.3 mg "soluble enzyme fraction" from rabbit reticulocytes (see "Methods") 5 μmoles PEP, 10 μg PEP kinase, 0.5 μmoles ATP, 0.1 μmole GTP, 3 μmoles mercaptoethanol, 6.5 μmoles Mg acetate, 40 μmoles NH_4Cl, 50 μmoles Tris HCl buffer, 10 μg E. coli tRNA (uncharged!) ^{14}C phenylalanine 0.05 μCi/or lysine 0.1 μCi and transfer factors isolated from E. coli cell sap. (Assay method II.)

Incubation was carried out at 30° for 20 min. Subsequent procedures were the same as in the case of the other test method.

It should be noted that although the quantitative results obtained by the two different methods differed from each other qualitative correlations were satisfactory. For routine tests the simplified method was used and from time to time the results were controlled with the other one.

II. Purification and fractionation of the 75,000 g supernatant solution

Transfer enzymes are known to be sensitive to $-SH$ reagents. Therefore it was assumed that treatment of the transfer enzymes with p-hydroxymercuribenzoate would alter their electric charge so as to make their separation easier. The fractionation of the p-MB treated proteins was achieved by protamine sulfate.

Protamine fractionation was carried out as follows: to 1 ml of the "40–60% AS fraction" (see "Methods") with a protein content of about 10 mg per ml 1 ml of 0.5 M Tris HCl buffer pH 7.8 and 0.3 ml of a 1.10^{-2} M p-MB solution (pH adjusted to 8.2 with solid Tris) were added. After standing for 20 min at 0°, 0.4 ml of a 1% protamine sulfate solution was added dropwise (a slight excess of protamine sulfate is important, otherwise the active protein remains in the supernatant and the two factors do not separate). After thorough mixing the precipitate was centrifuged at 2000 g for 5 min. The supernatant was kept. ("Protamine supernatant.")

The precipitate was washed with 4 ml of 0.1 M Tris HCl buffer pH 7.8 and after centrifugation it was dissolved in 0.5 ml of the following buffer solution: 2 M NH_4Cl; 0.05 M Tris HCl (pH 7.8), 0.1 M mercaptoethanol. Then 0.4 ml of 1% polyvinyl sulfate was added to precipitate the protamine and the mixture was diluted with 1 ml distilled water. The PVS-protaminate precipitate was removed by centrifugation. 50–75 mg dry weight calcium phosphate gel washed with 0.1 M Tris HCl buffer pH 7.8 containing 0.01 M mercaptoethanol was added to the opalescent supernatant, mixed thoroughly and stirred at 0° for 10 min. After centrifugation the gel was washed with 5 ml of the above Tris HCl buffer. The active protein fraction was eluted from the gel with 1 ml of 0.1 M K_2HPO_4 containing 0.005 M mercaptoethanol. The protein content of the solution was about 2 mg per ml. The enzyme obtained by this procedure is relatively labile: its activity is destroyed even by freezing; standing in a refrigerator at 0° resulted in a loss of more than 50% of its activity within 3 days.

20–30 μg of the freshly prepared enzyme saturated the assay systems, it had, however, to be complemented with a factor contained in the "protamine supernatant".

In preliminary experiments the protamine supernatant was treated as follows: mercaptoethanol was added to a final concentration of 0.01 M; the excess of protamine was removed by the addition of 0.2 ml of 1% PVS.

In the experiments with these factors (purified "protamine precipitate" and "protamine supernatant" factor) it was found that although both fractions exhibited a slight transferase activity when tested separately, their combination resulted higher incorporation than expected on the basis of additivity (Fig. 1). Fig. 1 shows also that neither of the two factors could be substituted by the "soluble enzyme fraction" isolated from rabbit reticulocytes.

Cross-contamination of the fractions was reduced in the course of further purifications.

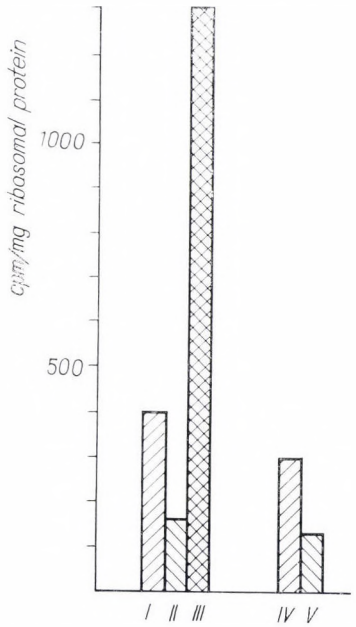

Fig. 1. Separation of two complementary "transfer factors" from E. coli B supernatant by protamine precipitation. Species specificity of the two factors. Fractionation procedure by protamine sulfate is described in the text. "Soluble enzyme fraction" was prepared from rabbit reticulocytes (see in "Methods") and was used in an amount of 0.35 mg per test tube. Transfer activities were determined by "assay method I". Blank value* (subtracted): 195 cpm per mg ribosomal protein. *Additions:* I: protamine supernatant 5 μl**; II: protamine precipitate 30 μg***; III: I and II combined: IV: protamine supernatant 8 μl** + "soluble enzyme fraction; V: protamine precipitate 30 μg*** + "soluble enzyme fraction"

On examining the heat resistance of these two factors it was observed that the factor precipitated by protamine was extremely heat labile: its activity was destroyed at 56° within 5 min. The component in the protamine supernatant proved to be surprisingly heat resistant, boiling for 10 min at 100° had apparently no influence on its activity (Fig. 2). Therefore we designated the protamine precipitated fraction as the "heat labile", and the protamine supernatant fraction as the "heat resistant" factor.

* Blank values refer to radioactivities counted in the samples without any transfer factor added. All values presented in the Figures were corrected by subtraction from these blanks.

** The amount of the "protamine supernatant" (heat resistant) factor in this and all the following figures is expressed in μl units. One such unit stands for that amount of the heat resistant factor which is contained in 1 μl of the original "40%—60% A. S. fraction".

*** The "protamine precipitate" fraction (heat labile factor) was used through out the experiments presented in the figures in amounts which saturated the assay system.

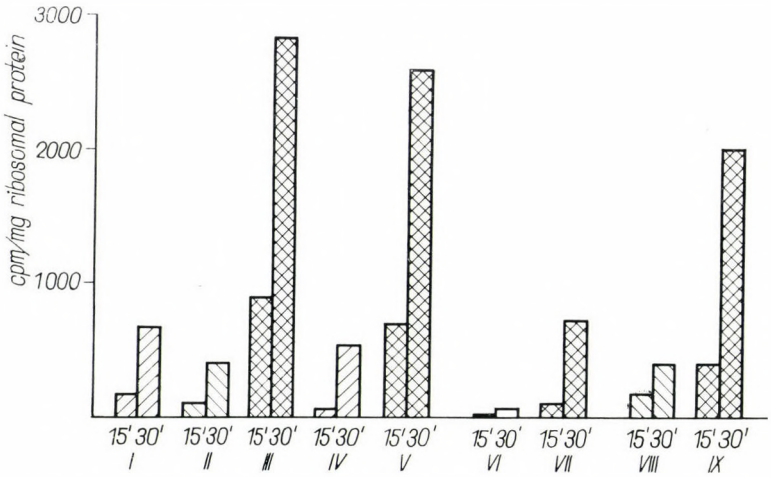

Fig. 2. Heat stability of "protamine supernatant" and "protamine precipitate" factors. Fractionation by protamine sulfate is described in the text. Aliquots of both factors were heated as indicated below. Heat denaturated proteins were removed by centrifugation. The heat treated samples were tested in the same dilution as the original fractions. Transfer activities were determined by "assay method II". Blank values* (subtracted): 155 cpm (15 min value) and 254 cpm per mg ribosomal protein (30 min value). Incubation periods are indicated in the Figure. *Additions:* I: protamine supernatant 5 μl**; II: protamine precipitate 30 μg***; III: I and II combined; IV: protamine supernatant heated at 56° for 5 min; V: II and IV combined; VI: protamine precipitate heated at 56° for 5 min; VII: I and VI combined; VIII: protamine supernatant heated at 100° for 10 min; IX: II and VIII combined

III. Purification of the heat resistant factor

The purification of the heat resistant factor was further facilitated by the observation that the material is precipitated from a neutral solution with Zn^{++} ions, acetone or by the addition of trichloroacetic acid (Fig. 3).

The following procedure was used for further purification: Protamine was precipitated from the "protamine supernatant" with an excess of polyvinyl sulfate. The solution was kept in a 100° water bath for 6 min (when working with larger quantities it was boiled for 10 min). Denatured protein was removed either by centrifugation at 3000 g for 10 min or by filtration (on a Buchner funnel). 1/10 vol. of 10% $ZnSO_4$ was added to the clear supernatant and the mixture was kept at 70° for 5 min. The precipitate contained the bulk of the active material and was sedimented by centrifugation (at 3000 g for 5 min). The precipitate was suspended in distilled water (half of the original volume of the "40–60% AS fraction") and was dissolved by dropwise addition of 0.2 M EDTA (adjusted to pH 7.5 with solid Tris). Undissolved material was centrifuged off and discarded.

* See legends to Fig. 1.
** See legends to Fig. 1.
*** See legends to Fig. 1.

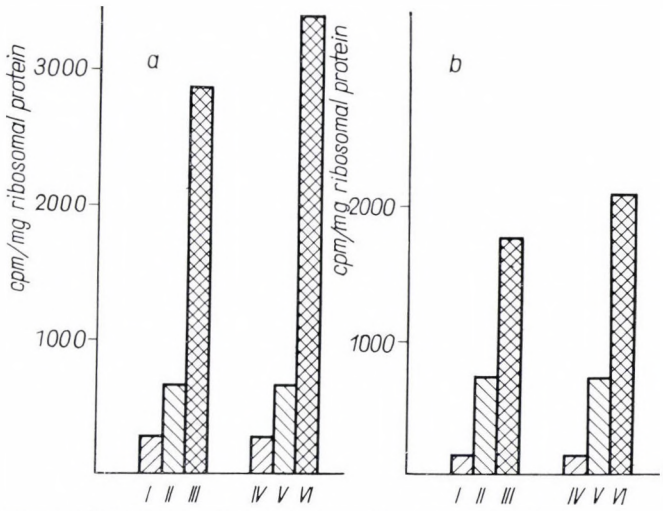

Fig. 3a Precipitation of the heat resistant factor with acetone. The heat resistant factor was precipitated from the protamine supernatant fraction (after heat denaturation of the proteins) with 3 volumes acetone. The precipitate was dissolved in 0.1 M Tris HCl buffer solution (pH 7.8) and tested. Transfer activities were determined by "assay method II". Blank value* (subtracted): 312 cpm per mg ribosomal protein. *Additions:* I: heat resistant factor 5 μl**; II: heat labile factor 35 μg***; III: I and II combined; IV: heat resistant factor precipitated with acetone 5 μl**; V: heat labile factor 35 μg***; VI: IV and V combined

Fig. 3b Precipitation of the heat resistant factor with Zn^{++} ions. The factor was precipitated from heat treated protamine supernatant with 1/10 volume of a 10% $ZnSO_4$ solution (details are given in the text). Transfer activities were measured by "assay method II". Blank value* (subtracted): 251 cpm per mg ribosomal protein. *Additions:* I: heat resistant factor 5 μl**; II: heat labile factor 30 μg***; III: I and II combined; IV: heat resistant factor precipitated with Zn^{++} ions 5 μl**; V: heat labile factor 30 μg***; VI: IV and V combined

The pH of the supernatant solution was adjusted to 7.8 by the addition of solid Tris and the active material was precipitated with 3 volumes of acetone. It was kept in an ice bath for 10 min. The precipitate was collected by centrefugation, the supernatant was discarded and the acetone remaining in the tube was evaporated in a vacuum desiccator. The dry material was dissolved in Tris buffer pH 7.8. The factor was found to retain its activity for several weeks in a refrigerator; in frozen state the activity was maintained for months.

The heat resistant factor was also precipitated with 5% trichloroacetic acid and the precipitate, after washing with acetone (to remove TCA) was dissolved in a neutral medium without any loss of activity. This precipitation was used routinely after DEAE cellulose column chromatography (to be described below) for the recovery of material from the eluate.

* See legend to Fig. 1.
** See legend to Fig. 1.
*** See legend to Fig. 1.

DEAE cellulose chromatography of the heat resistant factor

The DEAE cellulose column (1.2×20 cm) was equilibrated with 0.01 M Tris HCl buffer pH 7.5, at room temperature. Approximately 20 mg of the material obtained by the above-mentioned precipitation procedure was applied to the column in 5 ml of 0.01 M Tris HCl (pH 7.5). A KCl gradient was used for elution. The mixing chamber (120 ml) was filled with 0.01 M Tris HCl buffer pH 75. and the reservoir contained 1 M KCl in 0.01 M Tris HCl buffer (pH 7.5). The eluting solution was forced through the column by means of a mini-flow pump at a flow rate of 0.8 ml/min. Fractions of about 4 ml were collected. The position of the factor in the eluate was located by measuring the UV absorption of the

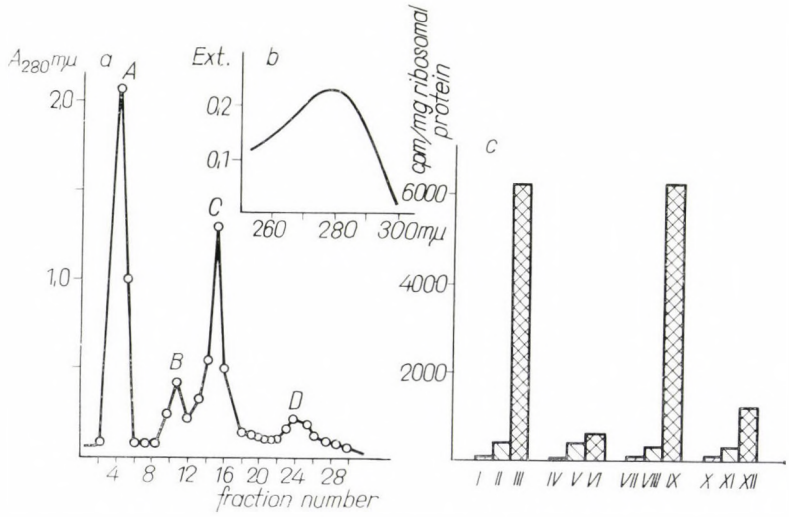

Fig. 4. DEAE cellulose column chromatography of the heat resistant factor. Fig. 4a. Elution profile of the heat resistant factor chromatographied on DEAE cellulose column. The procedure is described in the text. 4 ml fractions were collected. Fig. 4b. Absorption spectrum of the heat resistant factor recovered from peak C in Fig. 4a by TCA precipitation. Fig. 4c. Transfer activities of chromatographied fractions of the heat resistant factor (presented in the diagram of Fig. 4a). The procedure of the recovery of the materials from the eluate is described in the text. Transfer activities were determined by "assay method II". Blank value* (subtracted): 265 cpm per mg ribosomal protein. *Additions:* I: starting material (heat resistant factor applied to the column) 10 μl**; II: heat labile factor 30 μg***; III: I and II combined; IV: material recovered from peak B. 25 μl**; V: heat labile factor 30 μg***; VI: IV and V combined; VII: material recovered from peak C. 10 μl**; VIII: heat labile factor 30 μg***; IX: VII and VIII combined; X: material recovered form peak D. 25 μl**; XI: heat labile factor 30 μg***; XII: X and XI combined

* See legends to Fig. 1.
** See legends to Fig. 1.
*** See legends to Fig. 1.

fractions at 280 mμ (Fig. 4a). Fractions corresponding to each of the four peaks were combined and 1/10 vol. of ice cold 50% TCA was added. After standing for 10 min at 0° the precipitates were collected by centrifugation, washed with acetone, dried in vacuo. The samples were dissolved in 5 ml of 0.01 M Tris HCl buffer (pH 7.8) and this solution was used for activity measurements (Fig. 4c).

The collected fractions of peak A did not contain any TCA precipitable material. In another series of experiments when activity was determined directly in the elution fractions the same peak proved to be inactive. The bulk of the activity appeared in peak C.

The UV absorption spectrum of the heat resistant factor precipitated with TCA from peak C is shown in Fig. 4b.

IV. Some properties of the heat resistant factor

The heat treated and purified factor had no transferase activity and was effective only when combined with the heat sensitive protein fraction. Its effect was demonstrated not only in the polyphenylalanine synthesis directed by poly U, but also in the poly A directed polymerization of lysine (Fig. 5).

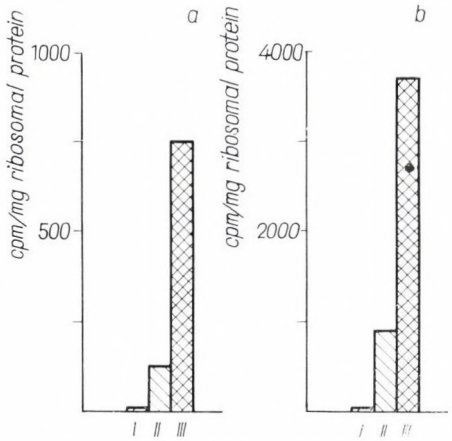

Fig. 5. Effect of the heat resistant factor on the polyadanylic acid directed lysine polymerization, measured by "assay method I" (Fig. 5a) and by "assay method II" (Fig. 5b). Separation of the two factors is given in the text. A partially purified heat resistant factor (fraction precipitated by acetone) was used. Blank values* (subtracted): 84 cpm (in Fig. 5a) and 48 cpm per mg ribosomal protein (in Fig. 5b). *Additions:* (in Fig. 5a and 5b) I: heat resistant factor 5 μl**; II: heat labile factor 30 μg***; III: I and II combined

* See legends to Fig. 1.
** See legends to Fig. 1.
*** See legends to Fig. 1.

The effect of the heat resistant factor on the amino acid polymerization was proportional to the amounts used (below a saturation level) when the heat labile factor was present in a saturating concentration.

The factor was characterized by its extreme heat stability, it was precipitated with Zn^{++} ions at slightly alkaline pH values, while no precipitate was formed with Mg^{++}, Ca^{++} and Mn^{++} ions. Furthermore it was precipitated with TCA or acetone in a reversible way. It was bound by DEAE cellulose under the conditions described above, and had an absorption spectrum characteristic of proteins or polypeptides containing aromatic amino acids.

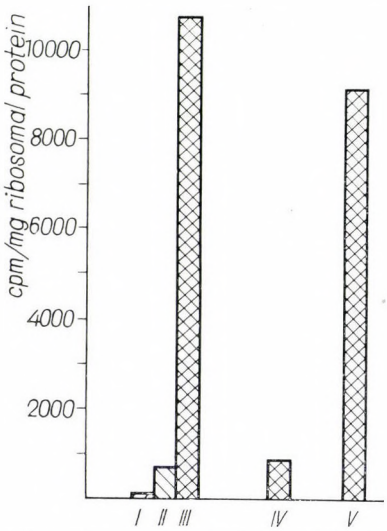

Fig. 6. Trypsin treatment of the heat resistant factor. 150 μg of partially purified heat resistant factor was incubated in the presence of 25 μg trypsin in 1 ml of 0.1 M Tris HCl buffer pH 8.0 at 30° for 45 min. The mixture was boiled for 30 min in order to destroy proteolytic activity. In the control tube the trypsin was boiled for 30 min before addition. All other conditions were the same. Transfer activities of the samples were measured by "assay method II". Blank value* (subtracted): 265 cpm per mg ribosomal protein. *Additions:* I: heat resistant factor (untreated) 10 μl**; II: heat labile factor 30 μg***; III: I and II combined; IV: heat resistant factor treated by trypsin 10 μl** + heat labile factor 30 μg***; V: heat resistant factor treated with previously boiled trypsin 10 μl** + heat labile factor 30 μg***

The factor retained its activity after boiling in 1N HCl for 10 min but was destroyed after hydrolysis in an azeotropic HCl solution for 48 hours. The material was found to be sensitive to proteolytic enzymes. As shown in Fig. 6 its activity was destroyed upon prolonged incubation in the presence of trypsin and was inactivated also by subtilisin (Fig. 7).

* See legend to Fig. 1.
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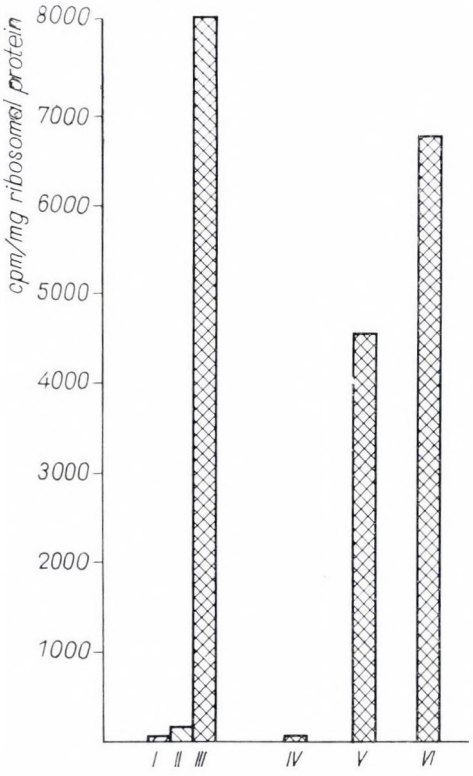

Fig. 7. Subtilisin treatment of the heat resistant factor. 130 mg partially purified (acetone precipitated) heat resistant factor was treated with 25 μg subtilisin in 1 ml of 0.1 M Tris HCl buffer pH 7.8, at 30° for 1 hour. The mixture was boiled for 30 min in order to destroy proteolytic activity. In the control tube the subtilisin was boiled for 30 min before addition. All other conditions were the same. Transfer activities of the samples were determined by "assay method II" (subtracted): 327 cpm per mg ribosomal protein. *Additions:* I: heat resistant factor (untreated) 10 μl**; II: heat labile factor 35 μg***; III: I and II combined; IV: subtilisin treated heat resistant factor 10 μl** + heat labile factor 35 mg***; V: heat resistant factor treated with previously boiled subtilisin 10 μl** + heat labile factor 35 μl***; VI: heat resistant factor (untreated) 10 μl** + subtilisin boiled for 30 min 2.5 μg + heat labile factor 35 μg***

The material isolated after the DEAE cellulose column chromatography was examined by paper electrophoresis. The active material moved in boratebuffer pH 9 towards the anode (the dislocation in an electric field of 10 volt/cm was 6 cm in 6 hours). There was no ninhydrin positive band on the electropherogram and staining with amidoblack resulted in the appearance of a single band. The active factor could be eluted after drying the paper. As shown in Fig. 8 the transfer activity moved together with the band stained with the protein indicating dye.

* See legend to Fig. 1.
** See legend to Fig. 1.
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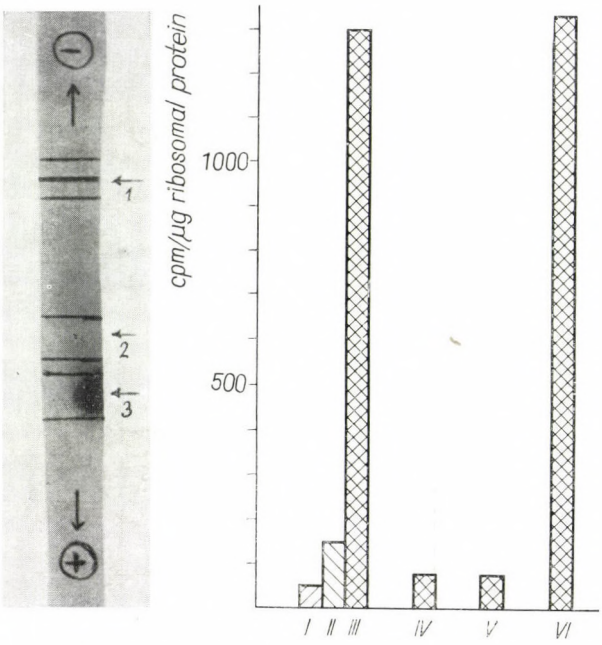

Fig. 8. Electrophoresis of the purified heat resistant factor. 30 μl** of the heat resistant factor recovered from the eluate after DEAE cellulose column chromatography (peak C in Fig. 4a) was electrophoretized on a 5×25 cm Schleicher Schull (20.43b) paper in 0.1 M borate buffer pH 9. Current intensity: 1 mA per paper strip. Duration of run: 6 hours (at room temperature). After drying an 1.5 cm strip was cut from the paper and stained with amidoblack. Three cross regions were cut from the unstained paper (indicated by 1 (start line), 2 ("tail"), 3 (stained band) on the left part of the figure) and these were eluted with 0.5 ml of 0.1 M Tris HCl buffer. The transfer activities of the starting material and the eluted fractions were tested by "assay method II". 0.05 ml borate buffer (pH 9) was added together with the starting material to the assay system. Blank value* (subtracted): 285 cpm per mg ribosomal protein. *Additions:* I: starting material (heat resistant factor) 5 μl**; II: heat labile factor 30 μg***; III: I and II combined + 0.05 ml of 0.1 M borate buffer pH 9; IV: eluted fraction from region 1 : 10 μl** + heat labile factor 30 μg***; V: eluted fraction from region 2 : 10μl** + heat labile factor 30 μg***; VI: eluted fraction from region 3 : 10 μl** + heat labile factor 30 μg***

Discussion

Two aminoacyl tRNA transfer factors were separated by protamine fractionation from the 105,000 g supernatant fluid of disrupted Escherichia coli B cells after p-MB treatment. A combination of these factors is necessary for the active

* See legend to Fig. 1.
** See legend to Fig. 1.
*** See legend to Fig. 1.

transfer process in an E. coli ribosomal system in which polyphenylalanine synthesis (directed by poly U) or polylysine synthesis (directed by poly A) is proceeding. One of the two factors is heat sensitive (the protamine precipitable fraction), the other one is extremely heat resistant. On the basis of its stability, precipitation characteristics, UV absorption spectrum and its sensitivity to proteolytic enzymes the latter factor is assumed to be a polypeptide having a relatively high molecular weight (or at least is firmly bound to such a substance).

It is difficult to evaluate the biological role of this factor. It seems to be specific for bacterial ribosomes because a similar material can be extracted from Micrococcus lysodeicticus cells which enhances amino acid polymerization in an E. coli ribosomal system complemented with the heat sensitive factor isolated from the E. coli cell sap. On the other hand, no such heat resistant factor was found in yeast or in animal cells. It could not be replaced by glutathione, oxidized glutathione, peptone or spermine. It has not yet been established whether this material is necessary for "true" protein synthesis taking place in the presence of natural messengers.

On the basis of our present knowledge of the mechanism of protein synthesis different steps have to be taken into consideration in which the heat resistant factor might play a definite role. Processes of this kind are: the binding of messenger RNA, the uptake of aminoacyl-tRNAs by the messenger-ribosomal complex or the translocation of aminoacyl-tRNAs. Its participation in the formation of peptide-bonds cannot be ruled out either (however, this assumption seems less probable). It cannot be excluded at present that this factor, as a member of an accessory system, may take part in chain initiation in the case of artificial messengers.

It is very difficult to ascertain the relationship between these factors and the "polymerization factors" isolated by Lukas-Lenard and Lipmann (1966) from the E. coli supernatant. The stability of our heat resistant factor undoubtedly excludes its identity with any of the factors reported by these authors. The heat resistant factor is most likely attached to one of the three factors in the course of fractionation by the method of Lucas-Lenard and Lipmann (1966). It is possible that the p-MB treatment applied in our procedure facilitated the separation of the heat resistant factor.

Acknowledgement

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Mechanism of Arginine Biosynthesis in Chlamydomonas Reinhardti

III. Purification and Properties of Acetylornithine-δ-transaminase

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1. Cell free extracts of C. reinhardti contain both acetylornithine-δ-transaminase (α-N-acetyl-L-ornithine: 2-oxoglutarate aminotransferase, EC 2.6.1.11) and ornithine-δ-transaminase (L-ornithine: 2-oxoacid aminotransferase, EC 2.6.1.13).

2. Acetylornithine-δ-transaminase was purified 75fold relative to the activity of the crude extract and was free from ornithine-δ-transaminase activity. The enzyme requires added pyridoxal phosphate for maximum activity. The apparent dissociation constant of the enzyme pyridoxal phosphate complex is 0.62×10^{-6} M.

3. At the optimal pH of 8.5, the apparent K_m for α-N-acetylornithine is 4.44 mM in the presence of 10 mM α-ketoglutarate, while the apparent K_m for α-ketoglutarate is 2.22 mM in the presence of 10 mM α-N-acetyl-L-ornithine. The activity of the enzyme is inhibited by high concentrations of α-ketoglutarate. The transaminase transfers the δ-amino group of α-N-propionyl-L-ornithine and α-N-butyryl-L-ornithine at the same rate as that of α-N-acetylornithine.

4. The activity of the enzyme is slightly inhibited by lysine, glutamic acid, glutamine and isoleucine of the fifteen amino acids tested.

Introduction

We have found that the arginine biosynthetic pathway in C. reinhardti involves α-N-acetylated intermediates between glutamic acid and ornithine (Staub, Dénes, 1966; Faragó, Dénes, 1967). In this respect the arginine pathway in the studied fresh water alga is very similar to the arginine pathway of different bacteria (Davis, Umbarger, 1962). It has already been described (Scher, Vogel, 1957) that cell- free extracts of C. reinhardti contain ornithine-δ-transaminase (L-ornithine: 2-oxoacid aminotransferase, EC 2.6.1.13). During our studies we have observed that cell-free extracts of this alga contain both ornithine-δ-transaminase and acetylornithine-δ-transaminase (α-N-acetyl-L-ornithine: 2-oxoglutarate aminotransferase, EC 2.6.1.11) with comparable specific activities. Since no cell type has been previously described in which both ornithine-δ-transaminase and acetylornithine-δ-transaminase would be present with comparable specific activities, we have studied these two enzyme activities of C. reinhardti cells in greater detail.

In this paper we present direct proof that the two δ-transaminase activities are due to two different enzymes and we describe the purification and properties of N-acetyl-ornithine-δ-transaminase.

Materials and Methods

Chemicals. The inorganic chemicals were of A. R. grade. L-ornithine, L-glutamic acid, α-ketoglutaric acid, and γ-aminobutyric acid were obtained from the firm "*Reanal*" (Budapest). Pyridoxal phosphate and o-aminobenzaldehyde were purchased from Fluka A. G. (Buchs). α-N-acetyl-L-ornithine, α-N-propionyl-L-ornithine and α-N-butyryl-L-ornithine were prepared chemically as described previously (Staub, Dénes, 1966).

Organisms and growth conditions. Wilde type plus and minus mating-type strains No 89 and 90 of Chlamydomonas reinhardti were kindly supplied by the Culture Collection of Algae, Department of Botany, Indiana University. Experimental and stock cultures of the minus mating-type strain were grown as described previously (Staub, Dénes, 1966). The centrifuged cells were stored at −20° before use.

Preparation of acetone-dry powder from alga cells. 250 g of the collected frozen cells were thawed and homogenized for 2 minutes at 0° in a Waring blendor. The homogenized cells were added to 5 liters of acetone at −10° and slowly stirred with a glass rod for 10 minutes. The precipitated cells were collected on a Büchner funnel and washed twice with 500 ml of cold acetone (−10°). The greenish-yellow filter cake was broken and dried at room temperature in a vacuum desiccator over calcium chloride with continuous suction until it was acetone free and then at 0° until it was completely dry. The resulting dry material was ground to a fine powder and stored at 2°. The yield is between 35 to 40 g of dry powder. Acetone powder can be stored for several months at 2° without significant loss of either acetylornithine-δ-transaminase or ornithine-δ-transaminase activity.

Acetylornithine-δ-transaminase assay. The determination of enzyme activity was based on the reaction of glutamic-γ-semialdehyde formed during acid hydrolysis of N-acetylglutamic-γ-semialdehyde by hydrochloric acid, with o-aminobenzaldehyde (Albrecht, Vogel, 1964). The standard reaction mixture contained, in a final volume of 0.5 ml, 30 μmoles of sodium pyrophosphate buffer (pH 8.5), 20 μmoles of pyridoxal phosphate, 5 μmoles of α-ketoglutarate, and 5 μmoles of α-N-acetyl-L-ornithine. The reaction was started by the addition of α-N-acetylornithine and the mixtures were incubated at 37°. The reaction was arrested by the addition of 0.5 ml of 2 N hydrochloric acid. The tubes were covered with marbles and placed into a boiling water bath for 90 minutes. After cooling to 25° the mixtures were neutralized by the addition of 2.0 ml of 0.5 N sodium hydroxide dissolved in 50 per cent ethanol. For the determination of glutamic-γ-semialdehyde formed during the acid hydrolysis, 1.0 ml of ethanol containing 1 per cent o-aminobenzaldehyde and 10 per cent trichloroacetic acid was added to the tubes. After standing for 15 min at 25°, the samples were centrifuged at 25°, and the absorbancy of the clear supernatant was determined at 435 mμ. A fresh solution of o-aminobenzaldehyde was used with each series of determinations. Under these conditions 1.0 μmole of the semialdehyde shows an absorbancy of 0.600 (1.0 cm light path).

One unit is defined as the amount of enzyme that catalyzes the formation

of 1.0 μmole of N-acetyl-L-glutamic-γ-semialdehyde in 60 min. at 37°. Specific activity is expressed as units per mg of protein.

Ornithine-δ-transaminase assay. For the determination of enzyme activity a standard reaction mixture was used containing in a final volume of 0.5 ml sodium pyrophosphate buffer (pH 8.5), 30 μmoles; pyridoxal phosphate, 0.02 μmole; α-ketoglutarate, 5 μmoles; L-ornithine, 10 μmoles. The reaction was started by the addition of L-ornithine and the mixtures were incubated at 25°. The reaction was arrested by the addition of 0.5 ml of 2 N hydrochloric acid. After neutralizing the mixtures by the addition of 2.0 ml of 0.5 N sodium hydroxide dissolved in 50 per cent ethanol, glutamic-γ-semialdehyde was determined as described under acetylornithine-δ-transaminase assay.

One unit is defined as the amount of enzyme which catalyzes the formation of 1.0 μmole of glutamic-γ-semialdehyde in 60 min at 25°. Specific activity is expressed as units per mg of protein.

Protein was determined with the phenol reagent using crystalline serum albumin as a standard (Lowry et al., 1951).

Results and Discussion

A comparison of the amount and some properties of the two δ-transaminases

Cell-free extracts prepared from C. reinhardti either by sonic treatment, or by freezing and grinding with dry-ice (Staub, Dénes, 1966), or by the acetone-dry powder method, will catalyze the transamination of both α-N-acetyl-ornithine and ornithine with comparable specific activities. The average enzyme content of the extracts of eight consecutive samples of acetone-dry powder obtained by extraction with 0.05 M sodium pyrophosphate buffer (pH 8.5) and subsequent centrifugation at 5000 g for 30 min for acetyl-ornithine-δ-transaminase was: 50.8 units per g of acetone-dry powder or 0.47 units per mg of protein; and for ornithine-δ-transaminase: 30.8 units per g of acetone-dry powder or 0.33 units per mg of protein. When comparing the specific activities of the two δ-transaminases in the extracts it should be mentioned that the activity of ornithine-δ-transaminase was determined at 25°, because of its instability at 37°. The activity of the enzyme determined at 25° was only about 50 per cent of the initial activity at 37° C.

As shown in Fig. 1, the thermal stability of the two enzymes is markedly different. In fact, in the finally adopted purification procedure for acetylornithine-δ-transaminase we have used a suitable heat treatment for the selective inactivation of ornithine-δ-transaminase. Another point which should be noted in Fig. 1a is the stabilizing effect of pyridoxal phosphate against thermal inactivation of acetylornithine-δ-transaminase. Fig. 1b shows that ornithine-δ-transaminase is not protected by pyridoxal phosphate at 55°C. The same observation was also made at 60 and 65°C.

The two enzymes also have a markedly different stability in the slightly acidic and slightly alkaline pH region. As shown in Fig. 2a, the incubation at

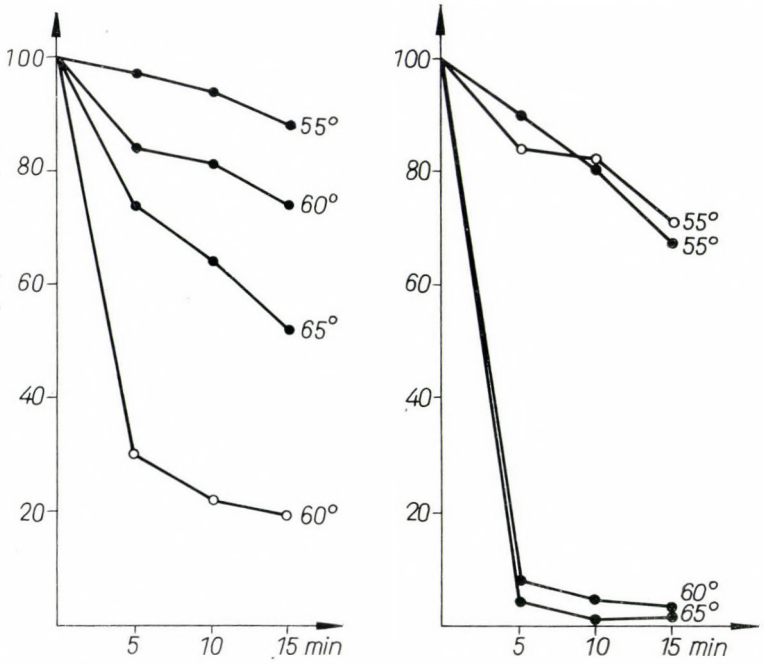

Fig. 1. Thermal inactivation of ornithine-δ-transaminase (A) and acetylornithine-δ-transaminase (B) in the presence and absence of pyridoxal phosphate. The mixture of the two transaminases obtained in Step 2 of the purification procedure was used. Samples containing 11.3 mg of protein per ml were incubated in 0.05 M sodium pyrophosphate buffer pH 8.5, at the temperatures indicated, either in the absence (○—○) or in the presence (●—●) of 0.1 mM of pyridoxal phosphate. Samples were taken at zero time and at other times as required and the residual activities were determined using the standard reaction mixtures as described under Methods

pH 3.5–3.8 of a crude preparation containing both enzymes completely inactivates acetylornithine-δ-transaminase without a significant effect on the activity of ornithine-δ-transaminase. This treatment could be used for preparing ornithine-δ-transaminase free of contamination with acetylornithine-δ-transaminase. At alkaline pH, acetylornithine-δ-transaminase is more stable than ornithine-δ-transaminase, as shown in Fig. 2b. Another interesting feature of the results presented in Fig. 2a and 2b is the stabilizing effect of added pyridoxal phosphate on acetylornithine-δ-transaminase both at acidic and at alkaline pH, and the absence of a similar stabilizing effect with ornithine-δ-transaminase.

Purification of acetylornithine-δ-transaminase

Step 1. Preparation of the crude extract. To 10 g of acetone dry powder of C. reinhardti cells 200 ml of ice cold 0.05 M pyrophosphate buffer (pH 8.5)

was added. Subsequent to the extraction for 60 min at 0° in a mortar, the extract was centrifuged at 5000 g for 30 min. The greenish-yellow supernatant was used for the purification of the enzyme.

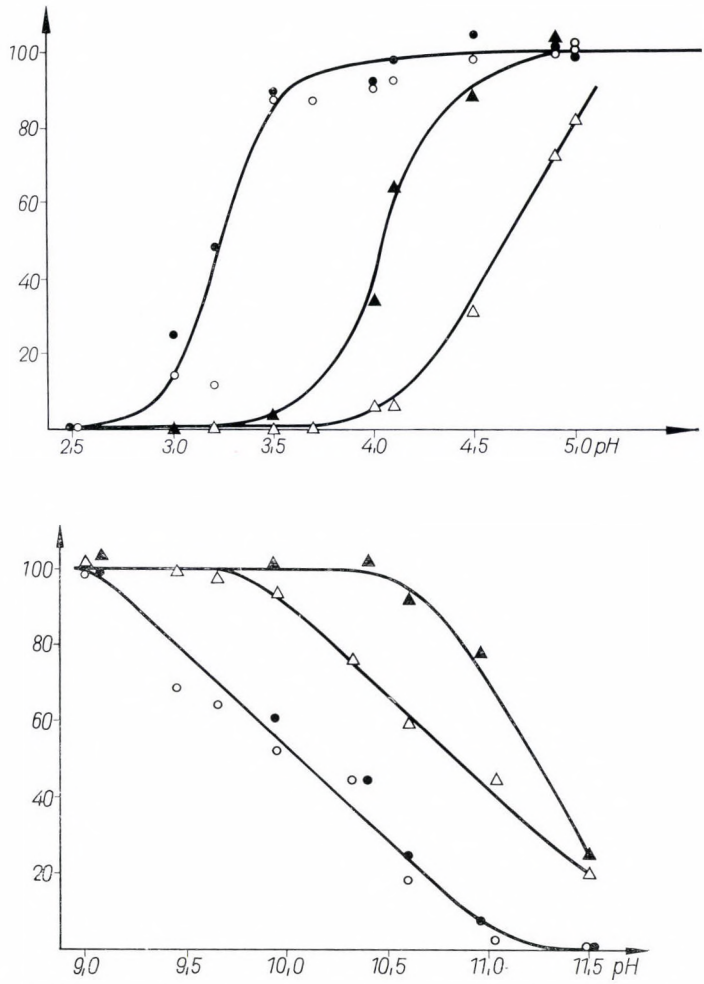

Fig. 2. Inactivation of ornithine-δ-transaminase and acetylornithine-δ-transaminase in the presence and absence of pyridoxal phosphate at acidic (a) and at alkaline (b) pH. The mixture of the two enzymes obtained in Step 2 of the purification procedure was used. The pH of the samples containing 12.2 mg protein per ml was adjusted to the value indicated with 0.1 M citric acid, and 0.1 N sodium hydroxyde, respectively. Samples were incubated for 60 min at 25°C at the pH indicated. The residual activity of ornithine-δ-transaminase after incubation in the presence (●) and in the absence (○) of pyridoxal phosphate, and the residual activity of acetylornithine-δ-transaminase after incubation in the absence (△) or in the presence (▲) of pyridoxal phosphate were determined as described under Methods

Step 2. Fractionation with ammonium sulfate. To each 100 ml of the crude extract 20 g ammonium sulfate was added. After centrifugation the precipitate was discarded and to the supernatant 12 g ammonium sulfate was added. The precipitate was sedimented at 8000 g for 30 min and was dissolved in 0.1 M sodium pyrophosphate buffer, pH 8.5.

Step 3. Heat treatment. To the enzyme solution obtained in Step 2 pyridoxal phosphate was added in a final concentration of 0.1 mM. The solution was heated rapidly to 60°–62° in a water bath and was kept at this temperature for 5 min. The heat treatment was terminated by transferring the enzyme to an ice water bath. A second ammonium sulfate fractionation was carried out as described in Step 2. The final precipitate was dissolved in distilled water and then dialyzed overnight against a 1000 fold volume of distilled water.

Step 4. DEAE-cellulose chromatography. The column (15 × 0.6 cm) was equilibrated with 0.01 M Tris-HCl buffer (pH 8.5) and loaded with the enzyme solution obtained in Step 3 diluted twofold with 0.02 M Tris-HCl buffer, pH 8.5. The enzyme solution contained 200 mg of protein. The column was washed with 0.075 M KCl in 0.01 M Tris-HCl buffer and the enzyme was eluted by applying a convex gradient of 0.075–0.3 M potassium chloride dissolved in 0.01 M Tris-HCl buffer. Elution was conducted at room temperature, at a rate of 50–60 ml per hour and the absorbance of the effluent was monitored by a recording ultraviolet flow analyzer (LKB 4701 A Uvicord ultraviolet absorptiometer). Fractions of 6.5 ml were collected. The fractions were further analyzed by measuring the enzyme activity and the absorbance at 280 mμ. Fig. 3 shows a representative chromatographic elution pattern. The enzyme was recovered as a single peak at

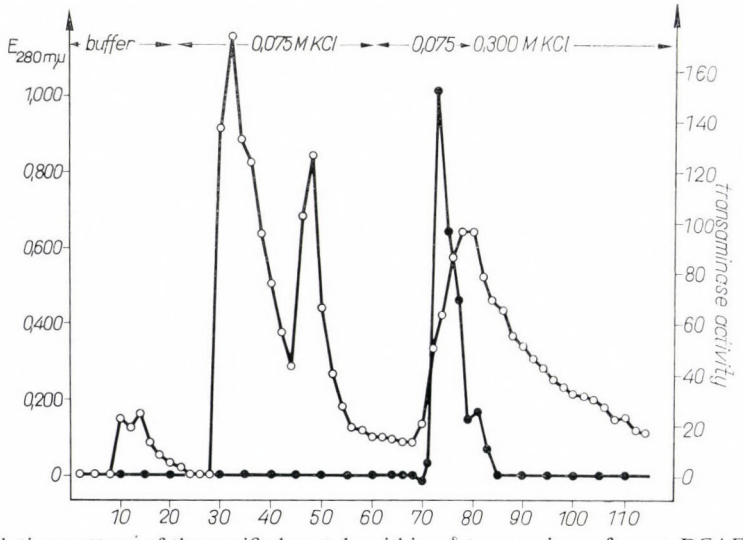

Fig. 3. Elution pattern of the purified acetylornithine-δ-transaminase from a DEAE-cellulose column. The adsorption and elution procedure as described in the text. o——o adsorbance at 280 mμ; ●——● transaminase activity

0.09–0.10 M KCl. The fractions were stored at 2° and the enzyme in the pooled active fractions was precipitated by the addition of 50 g solid ammonium sulfate per 100 ml of the solution.

The summary of a typical purification procedure is presented in Table 1.

Table 1

Summary of the purification procedure for acetylornithine-δ-transaminase

Step	Fraction	Volume ml	Total units	Total protein mg	Specific activity	Yield %
1	Crude extract	160	1660	1146	1.45	100
2	Ammonium sulfate I	34	1590	411	3.87	96
3	Heat treatment and ammonium sulfate II	20	757	57	13.3	46
4	DEAE-cellulose chromatography	63	551	12.7	43.8	33
4a	Peak fraction of DEAE-cellulose chromatography	5.7	97	0.89	109	6

Some properties of acetylornithine-δ-transaminase

pH optimum. As shown in Fig. 4, the pH optimum of the enzyme is between 8.5 and 9.0. The rate of the reaction does not change markedly when either Tris, phosphate, or borate buffer is substituted for pyrophosphate.

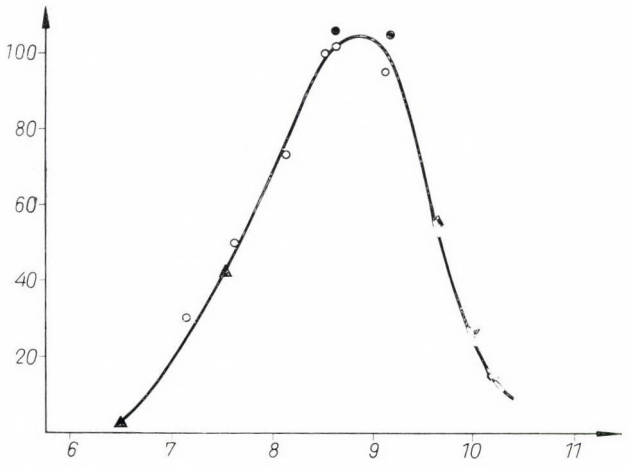

Fig. 4. Effect of pH on the activity of acetylornithine-δ-transaminase. With the exception of the buffer, the standard reaction mixture, as described under Methods, was used. The activity in 0.05 M pyrophosphate buffer, pH 8.5, was taken for 100 per cent. The buffers used were 0.12 M Tris (○); 0.15 M borate (●); and 0.10 M phosphate (▲)

Pyridoxal phosphate as coenzyme. Added pyridoxal phosphate was required for maximum activity of the enzyme. The presence of 0.04 mM pyridoxal phosphate proved to be optimal. The standard assay of enzyme activity was carried out with this concentration of the coenzyme. Although added pyridoxal phosphate was required for maximum activity of the enzyme, we could not completely resolve the coenzyme from the apoenzyme by any of the following methods:

a) Prolonged dialysis against one of the amino acid substrates (acetyl-ornithine and glutamate) at neutral pH;

b) Dialysis against cold 1×10^{-3} M hydroxylamine followed by dialysis against distilled water;

c) Treatment of the enzyme at room temperature with hydroxylamine, followed by Sephadex filtration.

All these treatments should result in the quantitative transformation of enzyme-bound pyridoxal phosphate to the amine and oxime forms, respectively. These results indicate that the binding of coenzyme to acetylornithine-δ-transaminase cannot to be greatly affected by the functional state of bound coenzyme. The pronounced stabilizing effect of pyridoxal phosphate on acetylornithine-δ-transaminase was not affected by the simultaneous presence of acetylornithine either. By analogy, this would indicate that the stabilizing effect of the coenzyme is also independent of the functional state of bound coenzyme.

From the double reciprocal plot of the effect of increasing concentrations of added pyridoxal phosphate on the activity of a partially coenzyme-free transaminase preparation, an apparent dissociation constant of 0.62×10^{-6} M was obtained.

Substrate specificity of the enzyme. In the standard acetylornithine-δ-transaminase assay we have used both acetyl-ornithine and α-ketoglutarate in a final concentration of 10 mM. Under the standard conditions and in the presence of 10 mM α-ketoglutarate, an apparent K_m of 4.44 mM was obtained for acetyl-ornithine. An apparent K_m of 2.22 mM was obtained for α-ketoglutarate in an analogous way. The dependence of reaction velocity on acetylornithine concentration follows normal Michaelis–Menten kinetics (Fig. 5), whereas 4–10 mM α-ketoglutarate is optimal and higher concentrations inhibit the reaction (Fig. 6).

The specificity of the colour reaction used for the determination of acetyl-glutamic-γ-semialdehyde enabled us to study more closely the substrate specificity of acetylornithine-δ-transaminase. None of the natural amino acids and only the two possible transamination products of lysine do react with o-amino-benzaldehyde (Vogel, Davis, 1952). Accordingly, lysine, ornithine, and α-N-substituted derivatives of ornithine could be directly tested as possible substrates of the enzyme. Neither L-ornithine nor L-lysine could be substituted for a α-N-acetyl-L-ornithine. On the other hand, α-N-propionyl-L-ornithine and α-N-butyryl-L-ornithine were good substrates for the enzyme. Indeed, under the standard conditions the relative transamination rates with the propionyl and butyryl analogues differed by less than 10 per cent from the transamination rate with acetylornithine.

Some further amino acids were also tested as possible inhibitors of acetylornithine-δ-transaminase when applied in a concentration ten times higher than acetylornithine. The results are shown in Table 2. It can be seen that of the fifteen amino acids tested only glutamic acid, glutamine, isoleucine, and lysine caused

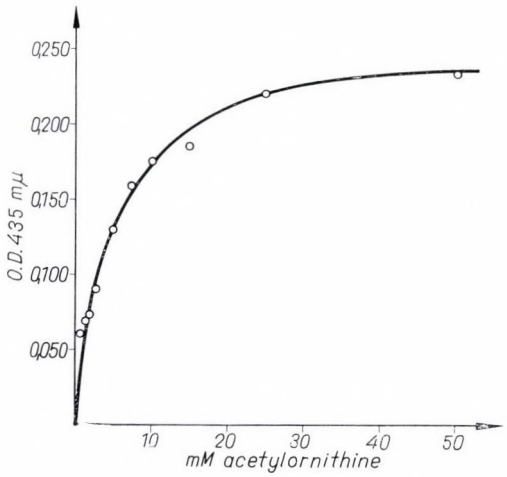

Fig. 5. Effect of N-acetyl-L-ornithine concentration on the activity of acetylornithine-δ-transaminase. The reaction mixture contained in 0.5 ml: 18 μg protein, 30 μmoles of sodium pyrophosphate buffer (pH 8.5), 0.02 μmoles of pyridoxal phosphate, 5 μmoles of α-ketoglutarate and various concentrations of α-N-acetyl-L-ornithine. Incubation at 37° for 10 minutes. Formation of N-acetylglutamic-γ-semialdehyde was measured as described under Methods

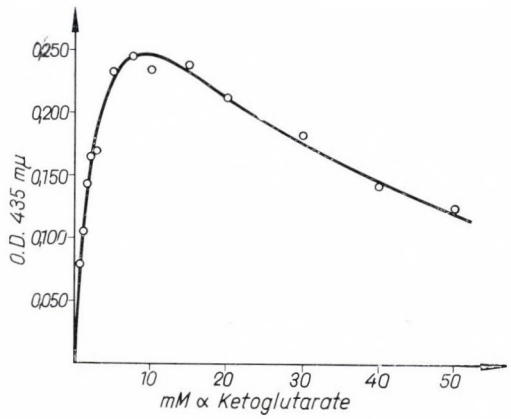

Fig. 6. Effect of α-ketoglutarate concentration on the activity of acetylornithine-δ-transaminase. Experimental conditions as in Fig. 5 except that 5 μmoles of α-N-acetyl-L-ornithine and various concentrations of α-ketoglutarate were used

Table 2

Effect of amino acids on the activity of acetylornithine-δ-transaminase

The standard reaction mixture was used as described under Methods. The reaction mixture contained in a final volume of 0.5 ml 100 μg ≥ protein and 50 μmoles of the amino acid tested

Amino acid	% activity
none	100
L-ornithine	100
L-lysine*	85
γ-aminobutyric acid	110
L-arginine	102
L-proline	91
L-glutamic acid	64
L-glutamine	62
L-aspartic acid	95
glycine	102
DL-alamine	98
L-isoleucine	86
L-leucine**	104
DL-norleucine***	105
L-valine	103
DL-norvaline	107

more than 10 per cent inhibition. Of these, glutamic acid is a product of the transamination reaction, and glutamine is a close structural analogue of it.

The relatively slight inhibition by lysine and by isoleucine might indicate a weak binding of these amino acids to the substrate binding site(s) of the enzyme. This point was further investigated with lysine. It was found that the inhibition by lysine was competitive with pyridoxal phosphate rather than with any of the substrates. Accordingly, lysine inhibited acetylornithine-δ-transaminase much more, when it was added to the enzyme prior to the addition of pyridoxal phosphate (and acetylornithine). The experiments summarized in Table 2 were carried out by adding the amino acid substrate plus inhibitor simultaneously to the enzyme which had been preincubated with pyridoxal phosphate. In fact, these conditions have been chosen to reduce the extent of an eventual "lysine-type inhibition" as characterized above, since this would have interfered with establishing the specificity of substrate binding.

Thus, the main conclusion which can be drawn from Table 2 is that acetylornithine-δ-transaminase is indeed a very specific transaminase. This is just what one would expect of a biosynthetic enzyme. We should like to point out that the results shown in Table 2 possibly support this claim even more, than it would

* 25 μmoles
** 46 μmoles
*** 36 μmoles

seem at first sight. It should be recalled, namely, that the reaction mechanism of all known transaminases is of the "ping-pong type" (Cleland 1963; Hammes, Fasella, 1963), with the keto analogue of an amino acid substrate being a possible substrate in the second half-reaction. Therefore, if an amino acid is not bound to the substrate site of a transaminase, the corresponding keto acid can not be a likely substrate of the enzyme either.

Based on this consideration we suggest that the keto acids corresponding to the non-inhibitory amino acids shown in Table 2 can neither be substrates of acetylornithine-δ-transaminase. Pyruvate and oxaloacetate are among the keto acids to which this consideration would apply. Pyruvate was tested directly and, indeed, it did not substitute for α-ketoglutarate.

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Mechanism of Arginine Biosynthesis in Chlamydomonas Reinhardti

IV. Purification and properties of ornithine-δ-transaminase

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1. Ornithine-δ-transaminase (L-ornithine: 2-oxoacid aminotransferase, EC 2.6.1.13) has been purified from cell-free extracts of the fresh-water alga Chlamydomonas reinhardti.

2. The purified enzyme has an absolute requirement for added pyridoxal phosphate. The apparent K_m of the transaminase for pyridoxal phosphate is 1.5×10^{-6}M. Quantitative removal of bound pyridoxal phosphate has been achieved by incubation at acidic pH.

3. The apparent K_m of the enzyme for ornithine is 4.45 mM in the presence of 10 mM α-ketoglutarate, and the apparent K_m for α-ketoglutarate is 2.55 mM in the presence of 20 mM ornithine. The activity of the enzyme is inhibited by high concentrations of α-ketoglutarate.

4. The activity of the enzyme is markedly inhibited by valine, norvaline, leucine, isoleucine, γ-aminobutyric acid, glutamic acid and glutamine. Lysine and α-N-acyl-L-ornithine derivatives do not affect the activity of the enzyme. Marked inhibition was also observed with glutaric and α-ketoisovaleric acids.

5. It is suggested, that the enzyme may have non-overlapping sites for the binding of ornithine and α-ketoglutarate. The inhibitors valine and α-ketoisovalerate would bind to the "ornithine site" while glutamate and glutarate would bind to the "α-ketoglutarate site".

Introduction

It has been found that between glutamic acid and ornithine the arginine biosynthetic pathway involves acetylated intermediates in Chlamydomonas reinhardti (Staub, Dénes, 1966; Faragó, Dénes, 1967; Südi, Dénes, 1967). Scher and Vogel (1957) have observed, that cell free extracts of C. reinhardti contain ornithine-δ-transaminase (L-ornithine: 2-oxoacid amino transferase, EC 2.6.1.13). Since the first detection of this enzyme in Neurospora (Fincham, 1953), it has been suggested from time to time that the presence of ornithine-δ-transaminase is characteristic of an arginine pathway, in which the intermediates are not acetylated. However, the argument that ornithine-δ-transaminase in Neurospora might have a role in arginine biosynthesis has been rejected by Davis (1955). We suggest, that the enzyme has a catabolic role in the alga cell. A similar enzyme has been purified from Neurospora (Vogel, Kopac, 1960) and from rat liver (Peraino, Pitot, 1963; Strecker, 1965).

The present paper describes the purification and properties of ornithine-δ-transaminase from cell-free extracts of C. reinhardti.

Materials and Methods

Chemicals. The inorganic chemicals were of analytical grade. L-ornithine, L-leucine, DL-norleucine, L-valine, DL-valine, DL-norvaline, DL-alanine, glycine, L-lysine, L-arginine, L-aspartic acid, L-glutamine, α-ketoglutaric acid, pyruvic acid were obtained from "REANAL" (Budapest). o-Aminobenzaldehyde and pyridoxal phosphate were purchased from Fluka A. G. (Buchs). α-N-acetyl-L-ornithine and α-N-propionyl-L-ornithine were prepared chemically (Staub, Dénes, 1966). α-Ketoisovaleric acid was prepared by the oxidation of DL-valine with pig kidney D-amino acid oxidase (Meister, 1953).

Organisms and growth conditions. Experimental and stock cultures of minus mating type strain of Chlamydomonas reinhardti No 90 were grown as described previously (Staub, Dénes, 1966). The centrifuged cells were stored at −20° before use. Acetone-dry powder was prepared from the frozen cells as described in our previous paper (Südi, Dénes, 1967).

Enzyme assay. For the determination of enzyme activity a standard reaction mixture was used containing 30 μmoles of sodium pyrophosphate buffer (pH 8.5) 0.02 μmoles of pyridoxal phosphate, 10 μmoles of L-ornithine and 5 μmoles of α-ketoglutarate in a final volume of 0.5 ml. The reaction mixtures were incubated at 25° and the reaction was arrested by the addition of 0.5 ml of 2 N hydrochloric acid. The amount of glutamic-γ-semialdehyde formed was determined by the o-aminobenzaldehyde method as described previously (Südi, Dénes 1967).

One unit is defined as that amount of enzyme which catalyzes the formation of 1 μmole of glutamic-γ-semialdehyde in 60 min at 25°C. Specific activity is expressed as units per mg of protein.

Protein was determined by the method of Lowry et al. with crystalline bovine serum albumin as a standard (Lowry et al., 1951).

Results and Discussion

Purification of the enzyme

Step 1. Preparation of crude extract. The acetone-dry powder of the cells of C. reinhardti was prepared as described previously (Südi, Dénes, 1967). 10 g of acetone-dry powder was extracted in a mortar with 200 ml of sodium pyrophosphate buffer (pH 8.5) at 0° for 60 min. The extract was sedimented at 5000 g for 30 min and the clear supernatant was used for the purification of the enzyme.

Step 2. Fractionation with $(NH_4)_2SO_4$. To 100 ml of the crude extract 20 g ammonium sulfate was added. The precipitate was sedimented by centrifugation

and discarded. To the supernatant 12 g ammonium sulfate was added. After centrifugation the supernatant was discarded and the precipitate was dissolved in distilled water.

Step 3. Fractionation at acidic pH. The pH of the enzyme solution obtained in Step 2 was adjusted to 3.7 by dropwise addition of 0.5 M citric acid. Subsequent to incubation for 20 min at room temperature, the precipitated protein was centrifuged at 2° and discarded. The pH of the supernatant solution was neutralized by dropwise addition of 0.1 N sodium hydroxide. Sodium pyrophosphate buffer (pH 8.5) was added to the neutralized solution to a final concentration of 0.3 M and the pH was adjusted to 8.5. To 100 ml of the enzyme solution 50 g ammonium sulfate was added. The precipitate was centrifuged and dissolved in 0.1 M sodium pyrophosphate buffer (pH 8.5).

Step 4. Fractionation with acetone. The enzyme solution obtained in Step 3 was chilled in an ice–alcohol mixture, and acetone (−15°) was added under vigorous stirring to a final concentration of 40 per cent (v/v). The precipitate was removed by centrifugation. To the supernatant solution more acetone (−15°) was added to a final concentration of 60 per cent (v/v). The precipitate was centrifuged and suspended in a small volume of 0.1 N pyrophosphate buffer (pH 8.5). After standing overnight at 0° the suspension was centrifuged and the clear supernatant was used as purified enzyme.

The purification procedure is summarized in Table 1.

Table 1

Summary of the procedure for purifying ornithine-δ-transaminase

Data from an experiment which was started with 28.5 g acetone dry powder

Fraction	Volume ml	Total units	Total protein mg	Spec. act. unit/mg	Yield %
1. Crude extract	403	1550	2750	0.56	100
2. Ammonium sulfate I	45	1170	945	1.24	75
3. pH 3.7 treatment and ammonium sulfate II	7.1	773	155	4.73	47
4. Acetone fraction	2.2	482	26	18.5*	31

Properties of the enzyme

Effect of pH on enzyme activity. The effect of pH was studied in the range of pH values from 6.5 to 10.5 as shown in Fig. 1. It is seen, that the rate of the reaction is not markedly affected by substituting phosphate, borate or Tris buffer for pyrophosphate. The optimal pH of the enzyme activity is 8.5.

* 33-fold purification in comparison to the crude extract.

Pyridoxal phosphate as coenzyme. For maximum activity of the enzyme added pyridoxal phosphate was required. The coenzyme could be completely resolved from the enzyme protein by incubation at pH 4.0 for a short time. The effect of increasing concentrations of pyridoxal phosphate on enzyme activity could be formally described by the Michaelis–Menten kinetics. The apparent K_m of the enzyme for pyridoxal phosphate was 1.54×10^{-6} M. The presence of

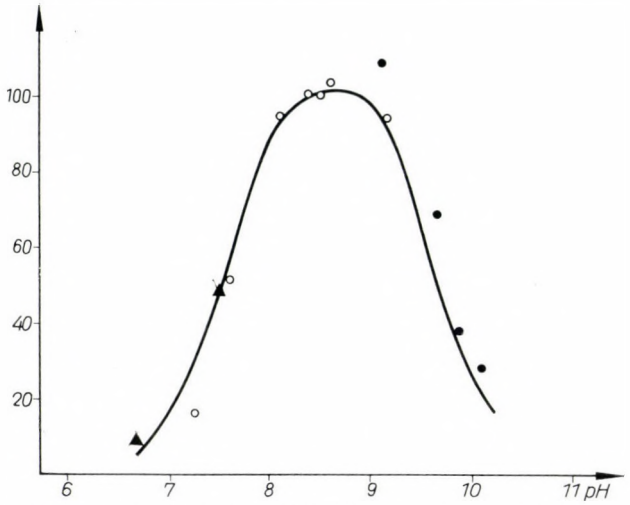

Fig. 1. Effect of pH on the activity of ornithine-δ-transaminase. With the exception of the buffer, the standard reaction mixture was used as described under Methods. The activity in 0.05 M pyrophosphate buffer (pH 8.5) was taken for 100 per cent. The buffers used were: 0.12 M Tris (○); 0.15 M borate (●) and 0.10 M phosphate (▲)

0.04 mM of added pyridoxal phosphate was found to be optimal in the reaction mixture and the standard assay of the enzyme was carried out with this concentration of coenzyme.

Substrate specificity of ornithine-δ-transaminase. In the standard ornithine-δ-transaminase assay 20 mM of ornithine and 10 mM of α-ketoglutarate were used as substrates. Under such conditions the apparent K_m values obtained were 4.45 mM and 2.55 mM for ornithine, and α-ketoglutarate, respectively. It can be seen, that these values do not differ markedly from the analogous substrate constants for acetylornithine-δ-transaminase described previously (Südi, Dénes, 1967). It is a further similarity between the two enzymes that high concentrations of α-ketoglutarate also inhibit ornithine-δ-transaminase (Fig 3). No substrate inhibition of ornithine-δ-transaminase was observed with ornithine (Fig. 2), just like acetylornithine-δ-transaminase was not inhibited by acetylornithine.

It is seen from Table 2 that α-N-substituted derivatives of ornithine, or lysine, did not markedly inhibit ornithine-δ-transaminase. This is a clear indication that the two enzymes have distinct specificities for substrate binding. It can be also seen

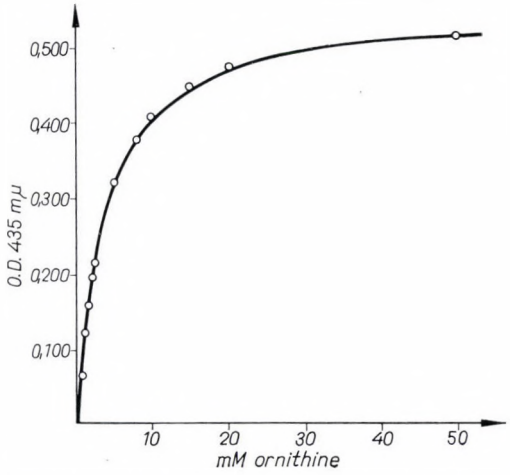

Fig. 2. Effect of L-ornithine concentration on the activity of transaminase. The reaction mixture contained in a final volume of 0.5 ml 30 μmoles of sodium pyrophosphate buffer (pH 8.5), 0.02 μmole of pyridoxal phosphate, 5 μmoles of α-ketoglutarate and increasing concentrations of L-ornithine. Incubation for 10 minutes at 25°C. Formation of glutamic-γ-semialdehyde was measured as described under Methods

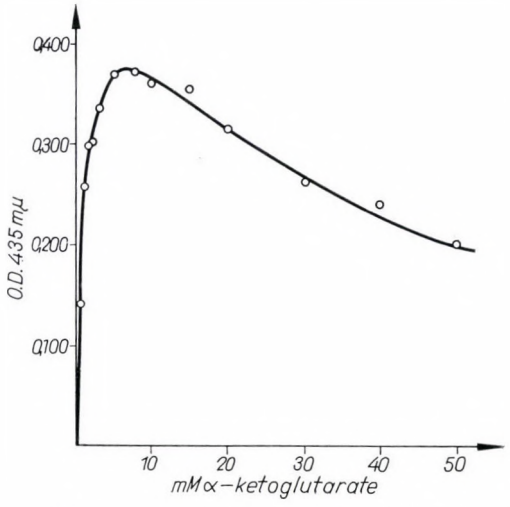

Fig. 3. Effect of α-ketoglutarate concentration on the activity of ornithine-δ-transaminase. Experimental conditions as in Fig. 2 except that 10 μmoles of L-ornithine and increasing concentrations of α-ketoglutarate were used

from Table 2 that ornithine-δ-transaminase must have (one or two) much less specific substrate binding site(s) than acetylornithine-δ-transaminase.

Table 2

Effect of amino acids and α-N-acylornithines on the activity of ornithine-δ-transaminase

The standard reaction mixture was used as described under Methods, containing 0.5 mg of protein and 50 μmoles of the amino acid tested. The reaction was started by the addition of a mixture of L-ornithine and the amino acid tested

Amino acid	% Activity
none	100
α-N-acetyl-L-ornithine	92
α-N-propionyl-L-ornithine	97
L-lysine	96
γ-aminobutyric acid	60
L-arginine	101
L-proline	101
L-glutamic acid	74
L-glutamine	70
L-aspartic acid	98
glycine	106
DL-alanine	86
L-isoleucine	25
L-leucine*	26
DL-norleucine**	49
L-valine	9
DL-norvaline	13

The marked inhibition of ornithine-δ-transaminase by γ-aminobutyric acid has not been investigated any closer. Glutamic acid is a product of the catalyzed reaction, and this explains the inhibition by glutamic acid and glutamine. However, the very pronounced inhibition which we observed with valine and leucine isomers (Table 2) seemed to require a special explanation. A similar finding has been reported by Strecker (1965) for the rat liver ornithine-δ-transaminase. Strecker, however, has made no attempt to explain the possible metabolic significance of these inhibitions.

Valine, leucine, and isoleucine are normal cell constituents. The inhibition studies (Table 2) indicated a specific binding of these amino acids to ornithine-δ-transaminase. Since it was further found that the inhibition by both valine (Fig. 4A) and isoleucine were competitive with ornithine we were led to think that these aliphatic amino acids might be substrates of the enzyme. The validity of this assumption was directly tested by substituting the keto analogue of valine, α-keto-isovaleric acid, for α-ketoglutarate in the standard ornithine-δ-transaminase assay. No production of glutamic-γ-semialdehyde could be demonstrated under these conditions, and so we had to conclude that α-ketoisovaleric acid was not a substrate for ornithine-δ-transaminase. This conclusion was further supported by the

* 46 μmoles
** 36 μmoles

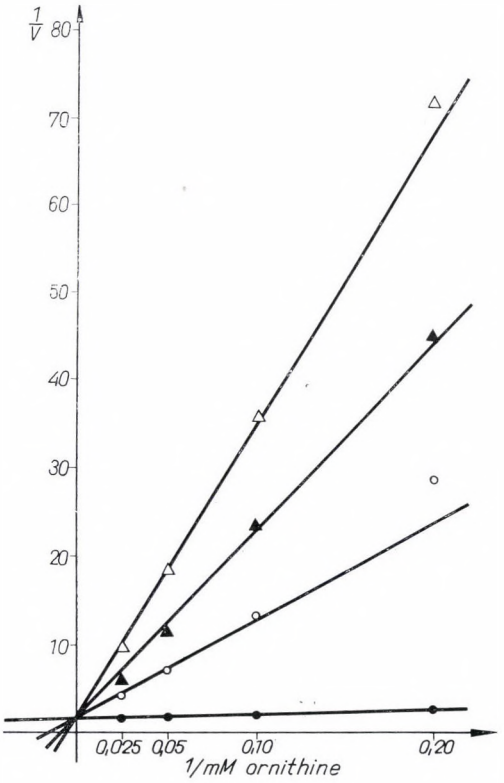

Fig. 4A

Fig. 4A. Lineweaver—Burk plot of the inhibition of ornithine-δ-transaminase by 0.0 mM (●), 25.0 mM (○), 50.0 mM (▲) and 100 mM (△) L-valine$^+$. The reciprocal of O. $D._{435\ m\mu}$ is given as 1/V. The constant α-ketoglutarate concentration was varied between 5 and 40 mM For Fig. 4B the constant concentration of ornithine was 20 mM and α-ketoglutarate concentration was varied between 0.63 and 5.0 mM. ($^+$Valine concentrations for Fig. 4B are lower by a factor of 0.4×)

observation that α-ketoglutarate did not disappear at a reasonable rate when a highly active and 25 times purified preparation of ornithine-δ-transaminase was incubated with valine plus α-ketoglutarate instead of ornithine plus α-ketoglutarate.

The above experiments provided unequivocal evidence that neither valine nor α-ketoisovaleric acid were substrates of ornithine-5-transaminase. Since both compounds were, nevertheless, potent inhibitors of the enzyme, we were interested to find out more about the way they inhibited the enzyme. The results show (Fig. 4 and Table 3) that the inhibition by both compounds is competitive with respect to ornithine (the amino acid substrate), and non-competitive with respect to α-ketoglutarate (the keto acid substrate). This obviously means that valine and α-ketoisovaleric acid interfere with the binding of ornithine, but not with the binding of α-ketoglutarate to the enzyme. Now we know from the general mecha-

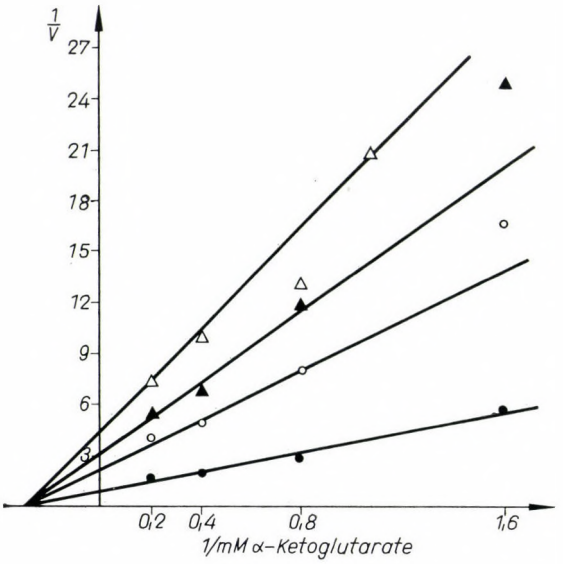

Fig. 4B

nism of transaminases that two alternate forms of the enzyme are generated in the course of the catalytic reaction: the pyridoxal enzyme and the pyridoxamine enzyme. In the case of the ornithine-δ-transaminase reaction:

enzyme-CHO + ornithine → enzyme-CH_2NH_2 + glutamic-γ-semialdehyde (Step 1)

enzyme-CH_2NH_2 + α-ketoglutarate → enzyme-CHO + glutamate (Step 2)

This type of reaction mechanisms has been denoted as "Ping-Pong Bi-Bi" by Cleland (Cleland 1963; Hammes, Fasella, 1963).

Table 3

Summary of a graphical analysis of Lineweaver—Burk plots of the inhibition of ornithine-δ-transaminase by some substrate analogues

Experimental design: The inhibitors were applied at 0, 25, 50 and 100 mM. When α-ketoglutarate was the substrate applied at a constant concentration this was 10 mM, and the concentration of ornithine was varied between 5 and 40 mM (column 2). Similarly (column 3), α-ketoglutarate concentration was varied between 0.63 and 5.0 mM at a constant concentration of 20 mM of ornithine

Substrate analogue	Type of inhibition	
	with ornithine varied	with α-ketoglutarate varied
glutaric acid	competitive	un-competitive
L-glutamic acid	partially competitive*	un-competitive
L-valine	competitive	non-competitive
α-ketoisovaleric acid	competitive	non-competitive

* No significant difference between inhibition by 25 and 50 mM glutamate.

We should like to point out that ornithine does not show substrate inhibition, while high concentrations of α-ketoglutarate markedly inhibit the enzyme. Velick and Vavra (1962) as well as Jenkins and Taylor (1965) have provided conclusive

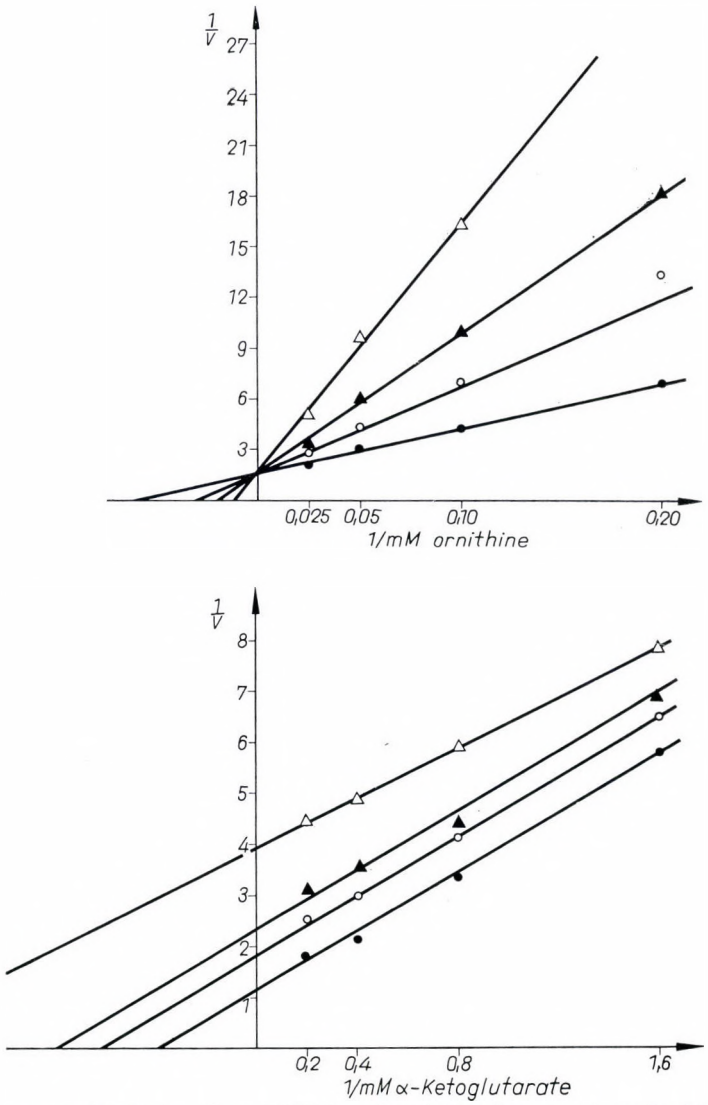

Fig. 5. Lineweaver—Burk plot of the inhibition of ornithine-δ-transaminase by 0.0 mM (●), 25.0 mM (○), 50.0 mM (▲) and 100 mM (△) glutaric acid. The reciprocal of O. $D._{435\ m\mu}$ is given as 1/V. The constant α-ketoglutarate concentration for Fig. 5A was 10 mM and L-ornithine concentration was varied between 5 and 40 mM. For Fig. 5B the constant concentration of L-ornithine was 20 mM and α-ketoglutarate concentration was varied between 0.63 and 5.0 mM

evidence that in the case of aspartate-glutamate transaminase substrate inhibition is due to the formation of an abortive complex between substrate and the wrong form of the enzyme (e.g. pyridoxalenzyme and α-ketoglutarate). We think that this mechanism of substrate inhibition is inherent in the "Ping-Pong" mechanism of all transaminases. Thus we suggest that absence of substrate inhibition of ornithine-δ-transaminase by ornithine indicates that ornithine is only bound to the pyridoxal enzyme, while from the substrate inhibition by α-ketoglutarate it follows that α-ketoglutarate is bound to both forms of the enzyme.

Similarly, the finding that the inhibition of the enzyme by both valine and α-ketoisovalerate is competitive with respect to ornithine and non-competitive with respect to α-ketoglutarate (Table 2, Fig. 4) would indicate that these two inhibitors are only bound to the pyridoxal enzyme. We should like to point out that this is a remarkable conclusion, because both inhibitors lack the terminal amino group of ornithine, the very group which reacts with the carbonyl of bound pyridoxal. In order to account for this type of inhibition we have to postulate that the site which binds valine and α-ketoisovalerate is indirectly affected by the functional state of bound coenzyme. We may even say that the "ornithine binding site" of the enzyme shows a high affinity towards ornithine, valine and α-ketoisovalerate when in the pyridoxal form and none when in the pyridoxamine form.

When looking for such inhibitors which might be the structural analogs of α-ketoglutarate rather than of ornithine, glutamate and glutarate have been our obvious choice. Glutamic acid is the reaction product formed from α-ketoglutarate, and glutaric acid is an almost universal inhibitor of transaminations involving α-ketoglutarate. The results obtained with these two compounds are shown in Fig. 5 and Table 3. It is seen, that inhibition by glutamate and glutarate is apparently un-competitive with respect to α-ketoglutarate, and competitive or partially so with respect to ornithine.

The qualitative interpretation of these results along the lines of our previous reasoning is quite easy. An important feature of these experiments is the agreement between the type of inhibition by glutamate and glutarate. Glutamate is a product of the measured reaction with a functional α-amino group, which can react with the pyridoxal enzyme. Glutarate, in turn, can only yield abortive complexes with either the pyridoxal or the pyridoxamine form of the enzyme. Nevertheless, the qualitative picture of the type of inhibition by these two α-ketoglutarate analogues is very similar. From this we may draw the same conclusion as in the case of ornithine analogues, namely, that the affinity of the binding site towards glutamate and glutarate cannot much depend on the functional state of bound coenzyme.

In our further discussion we have to start from the observation already stated that, in contrast to ornithine, α-ketoglutarate markedly inhibits the enzymatic reaction. For this reason, the Lineweaver–Burk analysis of Fig. 5B (and also Fig. 4B) was carried out with suboptimal concentrations of α-ketoglutarate; that is, the concentrations of α-ketoglutarate were such, that the formation of abortive complexes was negligible. However, the studied concentrations of gluta-

mate and glutarate (e.g. Fig. 5B) have been much higher and, indeed, their competition with ornithine indicates that they are bound to the pyridoxal enzyme.

Accordingly, the points to which the straight lines of Fig. 5B have been drawn and which, in turn, show an apparently uncompetitive interaction are presumably the result of two counteracting effects of increasing the concentration of α-ketoglutarate: (a) Increased velocity due to decreased competition of glutarate for the pyridoxamine enzyme; (b) Decreased velocity due to the increased formation of α-ketoglutarateo-pyridoxal enzyme complex. Surely, more precise data would be required to verify this qualitative suggestion in a quantitative manner.

We should like to discuss one further question. This concerns the suggestion that there exist two groups of inhibitors of ornithine-δ-transaminase: one group comprising the analogues of ornithine and the other those of α-ketoglutarate. We have shown above that these two groups of inhibitors can be characterized by different types of interference with the catalyzed reaction. We should like to make the further suggestion that this phenomenon is equally compatible with two very different types of a "Ping-Pong Bi-Bi" mechanism. The point we want to make is, that *the two substrates and the corresponding two groups of analogues may or may not be bound to the same (or even overlapping) site(s) of the enzyme in the two consecutive steps.* There may be two distinct binding sites for the two structurally different substrates within one active centre, it being understood that substrates bound to site I or site II should both be able to react with the same functional group of the bound coenzyme.

To our best knowledge, a similar suggestion of two non-overlapping substrate sites has not been made for the catalytic mechanism of any transaminase. However, even though our observations provide no conclusive evidence in favour of this hypothesis, some considerations clearly point to the existence of two substrate binding sites.

Firstly, bound pyridoxal (pyridoxamine) does react with a substituent on the δ-carbon atom of ornithine, (glutamic-γ-semialdehyde) and the α-carbon atom of glutamate (α-ketoglutarate). One can hardly imagine any template specifically designed so as to bind ornithine and glutamate, but not a great number of other amino acids (see Table 2), with a particular orientation in which the α-amino group of glutamate and the δ-amino group of ornithine would occupy the same position. We feel, that this argument is strongly supported by the observation, that α-ketoisovaleric acid and valine are not substrates of the enzyme. These inhibitors are structurally related to both ornithine (glutamic-γ-semialdehyde) and glutamate (α-ketoglutarate). Both inhibitors have prospectively functional α-substituents, and still the enzyme apparently recognizes them as structural analogues of ornithine (glutamic-γ-semialdehyde) and not of glutamate (α-ketoglutarate).

Secondly, there is the observation, that glutamate inhibits the reaction

ornithine + α-ketoglutarate → glutamic-δ-semialdehyde + glutamate

in a partially competitive manner with respect to ornithine. It is well known (Gutfreund, 1965) that competition for the same site cannot be a partial one. On the

other hand, it is equally clear that partially competitive inhibition can be obtained if (a) the binding of inhibitor and substrate occur at non-overlapping sites, and (b) if through some structural effect on the enzyme, the binding of any of these two ligands is mutually weakening the binding of the other.

Accordingly, the enzyme may be saturated with the inhibitor without losing its ability to catalyze the reaction, the only difference being that the dissociation constant of the substrate is higher for the ternary EIS complex than for the binary ES complex. Partial competition being necessarily an apparent competition where ternary complexes are formed, *it would follow that the pyridoxal form of ornithine-δ-transaminase can bind glutamate and ornithine at the same time.* Since it is a safe assumption that the inhibitory binding of glutamate occurs at the same site as that of α-ketoglutarate, the two compounds being substrate and product of the same reaction step, it would follow that in the two consecutive reaction steps of the "Ping-Pong" mechanism ornithine and α-ketoglutarate are bound to non-overlapping sites of the pyridoxal enzyme, and pyridoxamine enzyme, respectively.

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Role of Acetohydroxy Acid Synthetase in the Regulation of Valine and Isoleucine Biosynthesis in Pseudomonas aeruginosa

I. Characteristics and Stability of the Enzyme

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Cofactors of acetohydroxy acid synthetase from *Pseudomonas aeruginosa* are Mg^{++}, thiamine pyrophosphate (TPP) and flavine adenine dinucleotide (FAD). At 37°C the enzyme is reversibly inactivated in the absence of cofactors. TPP and FAD protect the enzyme against inactivation at 37°C. The inactivated enzyme can be reactivated by FAD. At 5°C FAD exerts a partial protection only, at 45°C it has no effect. In the course of inactivation the valine sensitivity of the enzyme temporarily increases. Valine a feedback inhibitor of the enzyme has no effect on inactivation but counteracts the inactivating effect of pyruvate, the substrate. The inactivation of the enzyme appeares to follow second order kinetics. The initial rate of inactivation is proportional to enzyme concentration. It is concluded that two forms of acetohydroxy acid synthetase are simultaneously inactivated.

Introduction

Acetohydroxy acid synthetase (AAS) which catalyses the synthesis of acetolactate and acetohydroxybutyrate is the first common enzyme of the valine and isoleucine pathways in *Escherichia coli* (Umbarger and Brown, 1958). Later on this enzyme was investigated in *Aerobacter aerogenes* (Halpern and Umbarger, 1959), in *Neurospora crassa* (Radhakrishnan and Snell, 1960), in *Salmonella typhimurium* (Bauerle et al., 1964) and in *Fraseolus radiatus* (Satyanarajana and Radhakrishnan, 1963). In accordance with the rule of end product inhibition as the first step of a biosynthetic sequence, the activity of AAS can be inhibited by L-valine one of the two end products of the combined pathway. However, the degree and characteristics of the inhibition were found to be different in various microorganisms. In crude extracts obtained from microorganisms AAS was unstable. This might be the reason why in spite of all efforts the purification of this enzyme has not been succesful. On the basis of the irregularities revealed by kinetic studies the presence of a complex enzyme system was suggested. AAS obtained from *E. coli* had a dilution effect, which could be abolished by a cofactor (Leavitt, 1964) identified as FAD (Stormer, Umbarger, 1964). FAD requirement of AAS has also been demonstrated in streptomycin-dependent *E. coli* (Desai, Polglase, 1965).

Growth inhibition of E. coli K12 by L-valine (Bonner, 1946; Tatum, 1946) can be explained on the basis of the valine sensitivity of AAS. This will result in isoleucine deficiency as a consequence of the operation of a combined pathway (Leawitt, Umbarger 1961, 1962). The presence of AAS in crude extracts of *P. aeruginosa* has been demonstrated in our laboratory (Horváth et al., 1964). The valine-sensitivity of AAS obtained from this microorganism was similar to that obtained from *E. coli* K12, nevertheless, valine failed to inhibit the growth of *P. aeruginosa*. In addition to characterizing AAS from *P. aeruginosa* our intention was to clarify this contradiction.

In this paper we report some characteristics and the stability of AAS from *P. aeruginosa*.

Materials and Methods

Enzyme assay. The activity of AAS was determined by the method of Umbarger and Brown (1958) with a slight modification. The reaction mixture consisted of 0.1 M potassium phosphate (pH 7.6); 0.05 M sodium pyruvate; TPP, 100 μg/ml; FAD, 2 μg/ml; 0.01 M $MgSO_4$, protein 0.8–1.0 mg/ml, in a total volume of 0.5 ml. Incubation was carried out for 15 minutes at 20°C in open glass tubes and the reaction was stopped by adding 0.1 volume of 10 N sulphuric acid. Deviations from this general method will be indicated. Acetolactate formed in the reaction was determined as acetoin after decarboxylation (Westerfeld 1945). Reaction velocity is given directly as the absorbancy at 520 mμ (ε_{520}). Protein was determined by the method of Lowry et al. (1951).

Cultivation of the microorganism. The experiments were carried out with *P. aeruginosa* strain 132. Culture conditions were described elsewhere (Horváth et al., 1964). *P. aeruginosa* was cultivated in 10 liter laboratory flasks containing 5 liters of medium at 28 °C, aerated at a rate of 5 liters of air per min., agitated with a speed of 300 r. p. m. The flasks were inoculated with 500 ml of flask cultures grown in reciprocal shakers for 12 hours. The cells were harvested after 2 hours of cultivation.

Preparation of the crude extract. The cells collected by centrifugation and washed twice with 0.1 M potassium phosphate, pH 7.6, were resuspended in 3 volumes (of wet weight) of a solution of 0.5 M potassium phosphate, pH 7.6, containing 0.001 M $MgSO_4$ and TPP, 100 μg/ml. High concentrations of potassium phosphate and cofactors served to stabilize the enzyme. Crude extracts prepared from cells suspended in distilled water were almost inactive. Disruption was carried out by a 20 Kc ultrasonic disintegrator. 5 ml cell suspensions were sonicated for 2 minutes. Disruption and all the following treatments were carried out at 0–4 °C. The supernatant obtained after centrifugation at 20,000 g for 10 minutes was used as crude extract. Cell debris washed with potassium phosphate had no significant activity. The crude extract was used immediately when indicated, the majority of the experiments were carried out with ammonium sulphate precipitates prepared from the crude extracts.

Ammonium sulphate precipitation. The crude extract was made 66 per cent with respect to ammonium sulphate by adding a solution of saturated ammonium sulphate in 0.5 M potassium phosphate (pH 7.2). The precipitate formed in 2 hours was centrifuged off at 25,000 g for 10 minutes. The supernatant was discarded. The activity of the precipitate obtained was 80–100 per cent of that of the crude extract. Ammonium sulphate precipitation served to get highly concentrated protein solutions and to increase in this way enzyme stability.

Treatment with Sephadex G 25. The ammonium sulphate precipitate was dissolved in 0.5 M potassium phosphate pH 7.6, containing 0.01 M $MgSO_4$ and 100 μg/ml TPP. Protein concentration was about 30 mg/ml. This was passed through a Sephadex G 25 column equilibrated with the same buffer-cofactor solution. Dissolution of the ammonium sulphate precipitate and gel filtration were carried out immediately before starting the experiments. Ammonium sulphate precipitates older than 4 hours have not been used.

All chemicals used were commercial products of analytical grade. Pyruvic acid was purified by fractionation in vacuo under N_2 atmosphere.

Results

Cofactor requirements

AAS of *P. aeruginosa* required TPP and Mg^{++} for its activity ($K_{mTPP} = 3.47 \times 10^{-5}$ M; $K_{mMg++} = 1.6 \times 10^{-3}$ M). In this respect it behaved similarly to AAS prepared from other microorganisms. Mg^{++} had a slight inhibitory effect when added in a concentration of 0.05 M or more. FAD did not enhance either enzyme activity or valine inhibition of AAS from *P. aeruginosa*, but protected the enzyme against heat inactivation in a very low concentration (see later). Thus FAD seems to be a cofactor of the enzyme. With other microorganisms FAD is needed both for enzyme activity and for valine inhibition (Stormer, Umbarger, 1964; Bauerle, et al., 1964; Desai, Polglase, 1965).

Effect of Sephadex G 25 treatment on the activity and valine inhibition of AAS

Treatment with Sephadex G 25 resulted in a fourfold increase in the enzyme activity of the crude extract, and valine-sensitivity of AAS simultaneously increased (Fig. 1).

Sephadex treated enzyme could be inhibited by valine in a concentration of 10^{-6} M. We concluded therefore, that the increase in enzyme activity and valine-sensitivity was a consequence of the removal of valine. Sephadex treated AAS from *P. aeruginosa* had a higher sensitivity to valine than that prepared from other microorganisms. In these latter cases the enzymes were investigated without Sephadex G 25 treatment, therefore, they might be in a valine inhibited state.

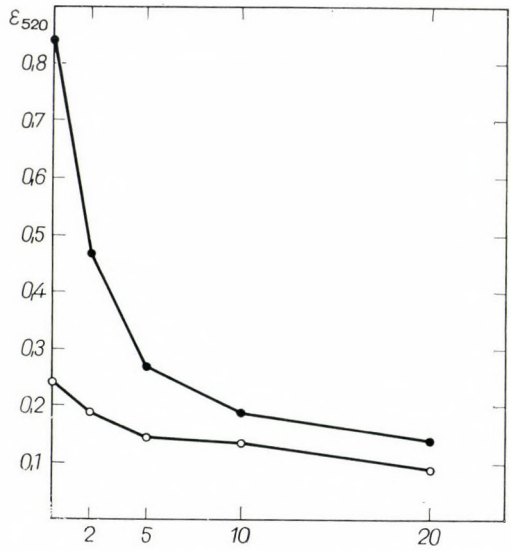

Fig. 1. The effect of molecular sieving on the activity and valine-sensitivity of AAS. 1.2 ml of crude extract was passed through a Sephadex G 25 column (14 cm × 1 cm diam.) equilibrated with the cofactor-containing potassium phosphate solution used for disruption. The effluent was collected in 1.2 ml fractions. The reaction mixture contained L-valine in the concentrations indicated in the figure; ● — ●, enzyme after fractionation on Sephadex G 25; ○ — ○ crude extract without treatment. (Ordinate = ε_{520}; abscissa = = L-valine cc. μM)

pH Optimum

Fig. 2 demonstrates the pH dependence of enzyme activity. Similarly to AAS enzymes extracted from other microorganisms its pH optimum was found to be about 8.

Effect of FAD and TPP on enzyme inactivation at 37°C

At 37°C AAS was rapidly inactivated in a solution of potassium phosphate. Inactivation could be abolished by TPP or by FAD added in very low concentrations (Fig. 3).

Inactivated enzyme could be reactivated by FAD but not by TPP (Fig. 4). The K_m value of reactivation by FAD was 1.17×10^{-7} M.

When FAD was added to the inactivated enzyme without preincubation, reactivation took place within a few minutes. In this case the time course of the reaction velocity had the characteristics of enzyme activation (Fig. 5).

Gel filtration of AAS from *P. aeruginosa* revealed two forms of enzyme which were in equilibrium (Varga, Horváth, 1965). Afterwards it seemed to be interesting

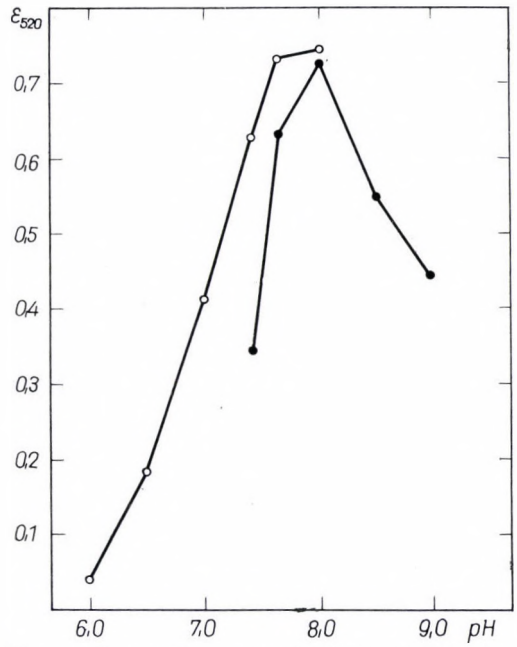

Fig. 2. pH Optimum. The reaction mixture contained: o — o 0.1 M potassium phosphate ● — ● 0.1 M TRIS-HCl pH as indicated in the figure. (Ordinate = ε_{520}; abscissa = pH)

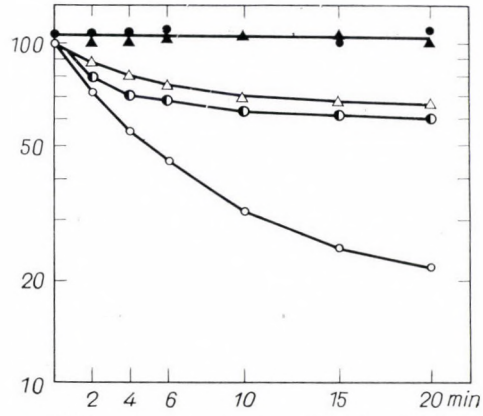

Fig. 3. Effect of FAD and TPP on the inactivation of AAS at 37°C. The ammonium sulphate precipitate was dissolved in 0.1 M potassium phosphate, pH 7.6 and applied to a Sephadex G 25 column equilibrated with the same buffer. The enzyme passed through the column was fourfold deluted in 0.1 M potassium phosphate, pH 7.6, incubated at 37°C; o—o without supplement; ● — ●, in the presence of FAD, 0.1 μg/ml; ◐ — ◐, in the presence of FAD, 10 μg/ml; △ — △, in the presence of TPP, 10 μg/ml; ▲ — ▲, in the presence of TPP, 100 μg/ml. 0.2 ml samples were taken in the time intervals indicated and the enzyme activity was assayed immediately in the reaction mixture without FAD. (Ordinate = remaining activity, per cent; abscissa = time of inactivation, minutes)

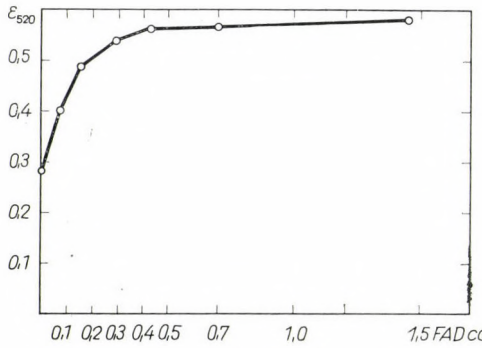

Fig. 4. Effect of FAD concentration on reactivation. The preparation of the enzyme solution and the conditions of inactivation were described in Fig. 3. After 10 minutes of inactivation the enzyme was preincubated at 20 °C for 5 minutes in the presence of FAD in the concentrations indicated. Enzyme activity was determined in the reaction mixture without FAD. (Ordinate = ε_{520}; abscissa = FAD cc., μM)

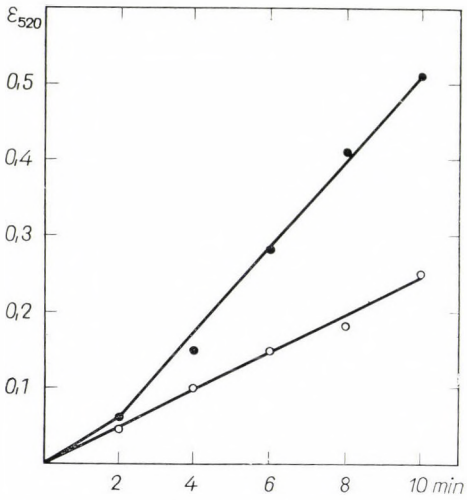

Fig. 5. Reactivating effect of FAD during the reaction. The time course of the reaction was investigated in a reaction mixture containing an enzyme solution inactivated according to Fig. 3; o — o, in the absence of FAD; ● — ●, in the presence of FAD, 2 μg/ml. (Ordinate = ε_{520}; abscissa = reaction time, minutes)

to investigate the behaviour of inactivated AAS on gel filtration. To decrease the time of gel filtration we used a small column of Sephadex G 200. This was not adequate for complete separation but was convenient to distinguish the two forms. When inactivated enzyme was gel filtrated FAD exerted a reactivating effect on the enzyme fraction within the gel particles of Sephadex G 200 only (Fig. 6). In agreement with our earlier observations (Varga, Horváth, 1965) we suppose that FAD reactivated the inactivated form of the valine insensitive (B) enzyme.

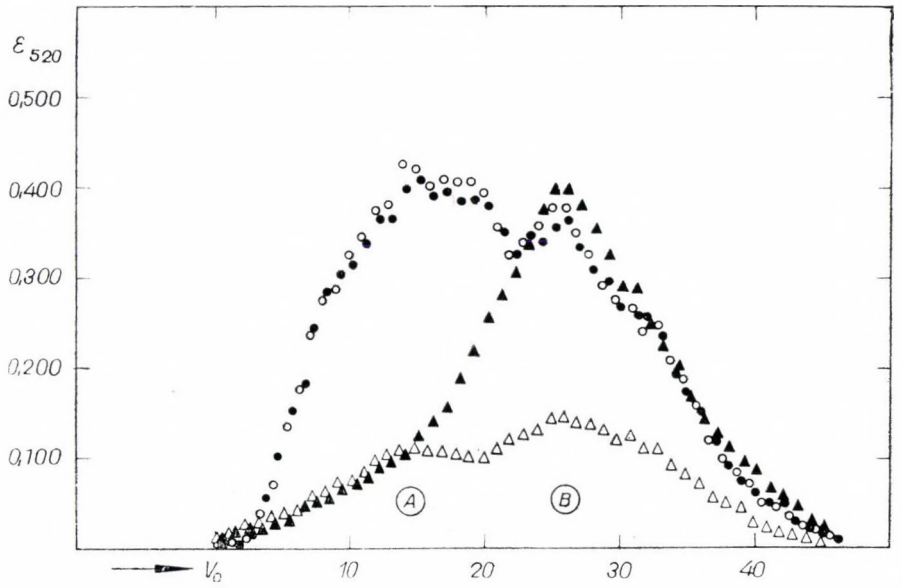

Fig. 6. Reactivating effect of FAD on the two forms of AAS. Gel filtration was carried out on Sephadex G 200 column (18 cm × 1 cm diam.) according to Varga and Horváth (1965). Every drop of effluent was assayed separately for enzyme activity. Drops were collected immediately into 0.45 ml of the reaction mixture. Gel filtration of the enzyme without inactivation was used as a control subsequent drops were collected in the reaction mixture ○ — ○, with FAD omitted; ● — ●, containing FAD, 2 μg/ml. An aliquot of the enzyme was inactivated at 37°C for 15 minutes according to Fig. 3, then applied to the same column of Sephadex G 200. Subsequent drops were collected into the reaction mixture △ — △, with FAD omitted; ▲ — ▲ containing FAD, 2 μg/ml. Exclusion volume (V_0) was derermined with blue dextran. (Ordinate = ε_{520}; abscissa = drop number)

Kinetics of enzyme inactivation at 37°C

Heat inactivation of AAS follows second order kinetics (Fig. 7A). The reason for this may be an aggregation of enzyme units associated with the enzyme denaturation. Alternatively, an apparent second order kinetics may result from the combined first order inactivation of two forms of an enzyme:

$$\begin{array}{ccc} E_A & \xrightarrow{k_1} & E_{inactive} \\ k_3 \uparrow\downarrow & & \\ E_B & \xrightarrow{k_2} & E_{inactive} \end{array}$$

Using appropriate values of k_1, k_2 and k_3 the curve obtained can be fitted to the curve of truly second order kinetics (Martin, 1963). These two alternatives can

be distinguished by measuring the initial rate of inactivation as a function of enzyme concentration: in the first case the initial rate of inactivation is proportional to the square of the enzyme concentration, whereas in the latter case linear proportionality is characteristic. This type of testing supported the latter alternative (Fig. 7B).

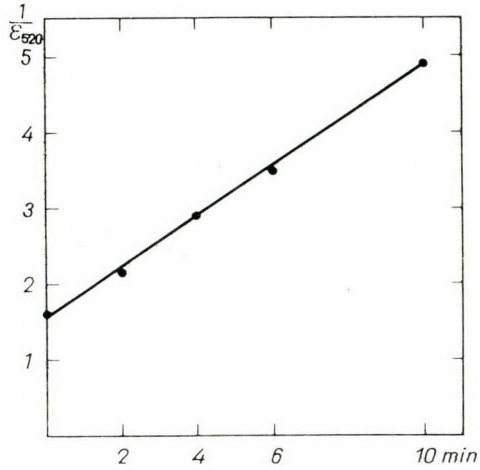

Fig. 7A. Kinetics of heat inactivation. A semilogarithmic plot of the inactivation according to Fig. 3 is demonstrated in Fig. 7A. (Ordinate = $1/\varepsilon_{520}$; abscissa = time of inactivation, minutes)

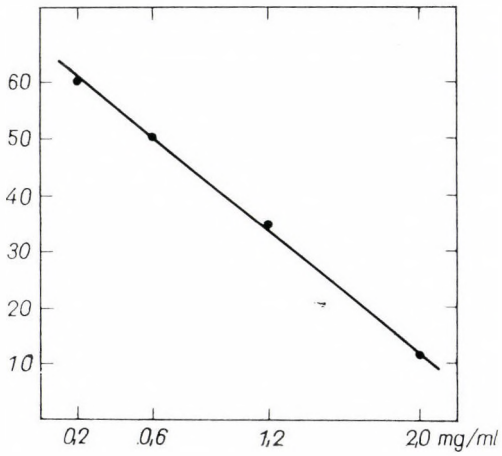

Fig. 7B. Enzyme was inactivated in 0.1 M potassium phosphate pH 7.6, at 37°C for 10 minutes at the protein concentrations indicated. Enzyme activity was assayed in the reaction mixture without FAD. (Ordinate = inactivation per cent; abscissa = protein cc. mg/ml)

Changes in the valine-sensitivity of AAS in the course of inactivation

According to the scheme mentioned above a significant difference in the rate of inactivation of the two enzyme forms (E_A and E_B) can be expected. It was reasonable to suppose that E_A and E_B postulated by kinetic studies were the same enzyme forms as demonstrated by gel filtration (Varga, Horváth, 1965). Supposing different rates of inactivation for the valine-sensitive E_A and insensitive E_B forms of AAS, an alteration of valine-sensitivity should be expected in the course of inactivation. This change could in fact be demonstrated: valine inhibition increased from 10 per cent to 45 per cent abruptly in the first minutes of inactivation and dropped again as inactivation proceeded further (Fig. 8). This means that the rate

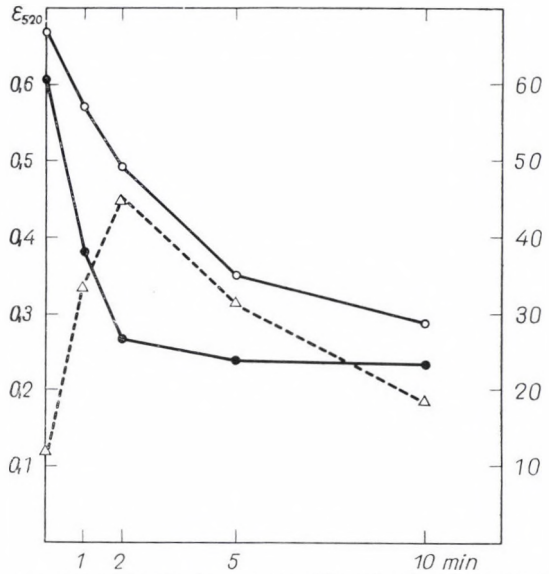

Fig. 8. Changes in valine sensitivity in the course of inactivation. Inactivation was carried out according to Fig. 3. Samples taken in the time intervals indicated were assayed in the reaction mixture o —o , without valine; • — • , containing 1 μM of L-valine. △ — △ Valine inhibition, per cent. In the reaction mixture FAD was omitted. (Ordinate left = ε_{520}; ordinate right = inhibition, per cent; abscissa = time of inactivation, minutes)

of inactivation was higher in the case of E_B (the valine-insensitive form) and the subsequent decrease of valine-sensitivity may be a consequence of an $E_A \rightarrow E_B$ transition. On the basis of these results the following scheme of enzyme inactivation at 37°C can be suggested:

$$E_A \xrightarrow{-\,FAD} E_B \longrightarrow E_{B\ \text{inactive}}$$

Effect of L-valine and pyruvate on enzyme inactivation at 37°C

At 37°C L-valine added in a low concentration inadequate for inhibition or in a concentration of inhibitor-saturation, had no effect on enzyme inactivation. Presence or absence of different cofactors (Mg^{++}, FAD, TPP) did not influence the ineffectiveness of valine. Pyruvate, the substrate of AAS enhanced the rate of inactivation. This effect of pyruvate could be counteracted by L-valine (Table 1).

Table 1

Effect of L-valine and pyruvate on inactivation at 37°C

The enzyme solution prepared according to Fig. 3, was inactivated at 37°C for 10 minutes in the presence of L-valine and pyruvate in the concentrations indicated. When inactivation was carried out in the presence of pyruvate, acetolactate formed during inactivation was determined separately and subtracted from the amounts of acetolactate formed in the reaction. FAD was omitted in the reaction mixture

L-valine cc. μM	Pyruvate cc. mM	Inactivation per cent
0	0	61
0.1	0	58
0.3	0	59
1.0	0	60
3.0	0	59
200.0	0	57*
0	5	78
0.1	5	75
0.3	5	67
1.0	5	60
3.0	5	44

Enzyme inactivation at 5°C and at 45°C

On inactivation at 5°C for a longer time FAD exerted a partial protection only (Table 2). At 45°C AAS was not protected against inactivation, not even in the presence of different cofactors and the feedback inhibitor of the enzyme.

Discussion

AAS obtained from *P. aeruginosa* has the same cofactor requirements and pH optimum as AAS prepared from other microorganisms. FAD plays a peculiar

* After inactivation the high concentration of valine was removed by Sephadex G 25 treatment. In the other cases valine was present in concentrations, wich had no inhibitory effect.

Table 2

Inactivation at 5°C and at 75°C

The enzyme solution prepared according to Fig. 3 was inactivated in 0.1 M potassium phosphate, pH 7.6, supplemented with the compounds indicated. Temperature and time of inactivation were indicated in the table. FAD was omitted in the reaction mixture

Supplement during inactivation	Time of inactivation	Temperature of inactivation °C	Inactivation per cent
none	18 hr	5	39
L-valine 1 μM	18	5	40
L-valine 200 μM	18	5	42*
FAD 10 μg/ml	18	5	24
FAD 10 μg/ml + L-valine 1 μM	18	5	21
FAD 10 μg/ml + L-valine 200 μM	18	5	22*
FAD 10 μg/ml + TPP 100 μg/ml + Mg^{++} 0.01 M	18	5	23
FAD 10 μg/ml + TPP 100 μg/ml + Mg^{++} 0.01 M L-valine 200 μM		5	25*
none	3 min	45	73
L-valine 200 μM	3	45	85*
FAD 10 μg/ml	3	45	9
FAD 10 μg/ml + L-valine 200 μM	3	45	17*
FAD 10 μg/ml	30	45	70
FAD 10 μg/ml + L-valine 200 μM	30	45	78*

role in this enzyme: it is not necessary either for enzyme activity or for valine inhibition but protects the enzyme against heat inactivation even in a very low concentration.

Furthermore, the inactivated enzyme can be reactivated by FAD. Therefore, we can assume that FAD is a constituent of AAS and the holoenzyme $\rightleftharpoons$ apoenzyme + FAD dissociation is responsible for the inactivation of the enzyme at 37 °C. This dissociation is favoured by rising the temperature and diluting the enzyme. Since TPP protects the enzyme even in the absence of FAD but it has no reactivating effect on the inactivated enzyme, we can assume that in the presence of TPP the dissociation of FAD is prevented. The apparent K_m for the reactivation of the enzyme by FAD is in the same order of magnitude as the K_m for the FAD requirement of AAS from various microorganisms (Stormer, Umbarger, 1964; Desai, Polglase, 1965).

Reactivating effect of FAD can only be demonstrated on the reversibly inactivated, valine insensitive (E_B) form of AAS. This involves that inactivation at 37 °C is a consequence of the dissociation E_B holoenzyme $\rightleftharpoons$ $E_{B\ apoenzyme}$ + FAD. Irreversible inactivation over 37 °C could result in a rapid irreversible denaturation of the unstable E_B apoenzyme.

* After inactivation the high concentration of valine was removed by Sephadex G 25 treatment.

The observed apparent second order kinetics of inactivation can be explained with two forms of AAS differing in the rate of inactivation. We suppose that the two forms of enzyme postulated by studies on inactivation kinetics correspond to the valine sensitive (E_A) and insensitive (E_B) forms of AAS as revealed by gel filtration. This is supported by the fact that valine inhibition increased abruptly in the first minutes of inactivation. The subsequent decrease in valine sensitivity can be explained on the basis of a $E_A \rightarrow E_B$ transition.

At lower temperature FAD can exert a partial protection only. Accordingly there is a FAD-independent inactivation, too. We suppose that the direct inactivation of the valine-sensitive (E_A) enzyme is responsible for this type of inactivation. The contribution of the inactivation of the two forms to the overall inactivation depends on several factors which have an influence on the $E_A \rightleftharpoons E_B$ equilibrium, such as temperature, pH, substrate and valine concentration etc. (Varga, Horváth, 1967a, b, c).

The following scheme represents the main postulated routes of enzyme inactivation:

$$\begin{array}{lll} & E_A \longrightarrow E_{1\ \text{inactive}} & \\ \text{valine} \uparrow\downarrow \text{pyruvate} & & \\ & E_B \underset{+\text{FAD}}{\overset{-\text{FAD}}{\rightleftharpoons}} E_{2\ \text{inactive}} \longrightarrow E_{3\ \text{inactive}} & \end{array}$$

At higher temperature with diluted enzyme the dissociation of a flavine E_B enzyme into apoenzyme and FAD may be responsible for the overall inactivation. The free E_B apoenzyme seems to be rather unstable and it is rapidly and irreversibly inactivated. At lower temperature with higher protein concentration direct inactivation of E_A results in a slow inactivation.

With a few exceptions allosteric inhibitors protect their allosteric enzyme against heat inactivation (Monod et al., 1965). Such a stabilizing effect of valine at different concentrations (10^{-7} to 10^{-3} M) on AAS at different temperatures in the presence or absence of various cofactors could not be demonstrated. The single positive effect of valine was that it counteracted the inactivating effect of pyruvate. This can be attributed to the opposite effect of substrate and feedback inhibitor on the $E_Y \rightleftarrows E_B$ equilibrium.

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Protein Distribution in Myofibrils of Different Sarcomere Lengths

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By using quantitative electron microscopy it has been shown that during contraction in the striated muscle of vertebrates in addition to the sliding of filaments there is a definit movement of protein towards the "I" band and the "Z" membranes. These changes may be explained on the basis of a change in the localization of myosin within the thick filaments. The thick filaments do not seem to be rigid rods.

Introduction

As a consequence of the rapid progress in biology the interpretation of the mechanism of the functioning striated muscle at a molecular level has become a real possibility.

According to such a concept during contraction the shortening of the sarcomere is brought about by the sliding of rigid filaments built up of well-ordered protein molecules (Huxley, 1960). Nevertheless one can find other explanations for the contraction (which are more complicated but perhaps more adequate for a living system) e.g. that the function is based on the changes in the state of aggregation and distribution of the protein system (Guba et al., 1965; Hodge, 1956; Szent-Györgyi, 1945; Szent-Györgyi, Johnson, 1964; Vajda et al., 1965).

The interference microscope which is a reliable tool in quantitative work has already been employed in muscle research (Bennett, 1955; Garamvölgyi et al., 1964; Huxley, Hanson, 1957). The protein distribution in isolated myofibrils in relaxed or stretched states has been investigated. Unfortunately the resolution power of light microscopes sets a limit to the investigation of shortened sarcomeres.

The electron microscope, owing to its greater resolution power may help to overcome these difficulties. It is known that the electron microscope can be used for quantitative determination of the total dry mass and that of the distribution of dry mass in isolated objects.

Mass determination by electron microscopy is based on the fact that the transmission of an electron micrograph, taken at appropriate conditions, is proportional to the mass per area of the object producing this transmission.

Photometric procedures are available to evaluate electron micrographs in terms of mass per area, or mass.

The working range of the method is of 10^{-11} g to 10^{-18} g total mass, or mass thickness of $34-60$ $\mu g/cm^2$ (at $50-100$ k. e. v. accelerating voltage) (Zeitler, Bahr, 1965; Bahr, Zeitler, 1965; these publications also contain the detailed literature on quantitative electron microscopy).

In this paper quantitative data on the distribution of dry mass in isolated myofibrils are reported and correlated with the contraction mechanism.

It should be mentioned that some measurements have already been reported (Bennett, Porter, 1953) on sectioned material without correlating them with function.

Materials and Methods

Preparation of separated myofibrils

Rabbit m. psoas was used in the experiments. The cooled muscle was passed through a prechilled meat grinder. The minced muscle was then blended in a medium containing 10^{-3} M $Na_4P_2O_7$ and 10^{-4} M EDTA, at pH 7.0. The temperature of the solution was 0 °C. The time of blending in a home made homogenizer was three minutes (the homogenization was interrupted every half-minute to allow cooling). This time proved to be sufficient to break up the fibres into separated myofibrils. When checked in the phase contrast light microscope the suspension contained isolated myofibrils in various physiological states. The preparation was then slowly spun down at about 300 g for 5 minutes. The sediment was resuspended in the same solution and the sedimentation process was repeated twice. In this way isolated myofibrils free of sarcoplasmic proteins were obtained.

Preparation for electron microscopy

A single drop of the suspension to be examined was applied to a carbon-filmed specimen grid. A few minutes of standing was required for the attachment of an appropriate quantity of myofibrils to the grid. Thereafter the excess fluid was blotted. The salts adhering to the membrane were removed by rinsing with distilled water and the preparation was allowed to dry.

Electron microscopy

The preparations were examined in a Hitachi HU-10 electron microscope at an accelerating voltage of 75 k. e. v., beam current $30-50$ μA, double condensor illumination, a 150 μ condensor aperture, and a 20 μ aperture in objective. The same electron optical magnification (5000 times) was used in all investigations. The magnification was calibrated by polystyrene standards 1880 Å(SD = $\pm$ 0.12

per cent) in diameter. The expositions were taken on Agfa Diapositive Hart plates. The plates were developed in Agfa 80 solution for 3 minutes. All plates had a background density of 0.5–0.7. According to published data (Zeitler, Bahr, 1965) the slope of the calibration curve in this density range is rather insensitive to variations in the background density of the plate.

Mass determination

The transmissions of the electron micrographs were measured by an autorecording Schnell-photometer. Slit size was 0.012×40 mm. This proved to be small enough compared to the size of the object. The sensitivity was 100, $v/n = 100$ (v = speed of the paper drive of the recorder, n = speed of the object).

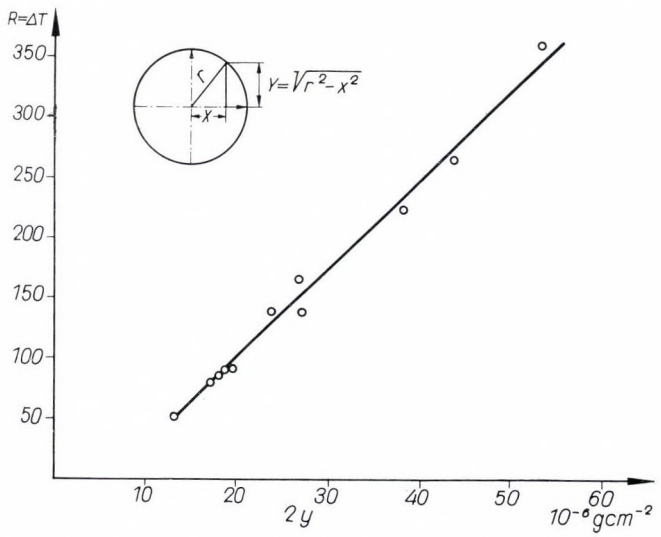

Fig. 1. Calibration curve for quantitative mass determination. Polystyrene spheres were scanned in the "Schnell" photometer with a small aperture. The spheres represent standards with a continuously changing mass profile

The difference in transmission between a chosen part of the object and the background near the object is called photometric reading (R, i.e. the transmission caused by the object).

Polystyrene latex spheres of three different diameters were used as weight standards. The diameters of the spheres were 0.188 μ, SD = $\pm$0.12 per cent; 0.264 μ, SD = $\pm$0.09 per cent; 1.171 μ, SD = 0.06 per cent, respectively.

By scanning the image of the polystyrene spheres and the background one can obtain the transmission data of various masses. When the photometric reading is plotted versus mass per unit area, one gets the calibration curve (Fig. 1). In accordance with Bahr and Zeitler a linear relationship was found between these parameters (Bahr, Zeitler, 1965).

Measurements on the myofibrils

The transmission of the different parts of a myofibril on electron micrographs was measured as mentioned above. The myofibrils and the background near the fibrils were scanned parallel to their longitudinal axes.

As the photometric readings of the different parts of a myofibril lie in the linear part of the calibration curve, each recorded point on the photometer diagram represents the real mass thickness of myofibrils at the given spot.

By simple planimetrical measurements of such recordings the *relative mass distribution* in the sarcomere can be determined. Owing to the variability in scanning speed the area of the scans could be adjusted to keep the planimeter errors within ± 2 per cent. As the dry mass of myofibrils consists in 99 per cent of proteins, the relative mass distribution corresponds to the distribution of the proteins in the sarcomere. Since the given myofibrils were free of sarcoplasmic proteins, the protein distribution obtained by measuring sarcomeres of various lengths represents the distribution of structural proteins (i.e. there is a possibility to detect the distribution of structural proteins during the contraction relaxation cycle).

The conclusions were drown from the data obtained with about 100 myofibrils of various sarcomere lengths.

Results and Discussion

The results obtained by the above measurements on rabbit myofibrils are shown in Fig. 2. In the Figure the relative amount of proteins in the different bands is plotted against sarcomere length. It can be seen (upper curve) that as

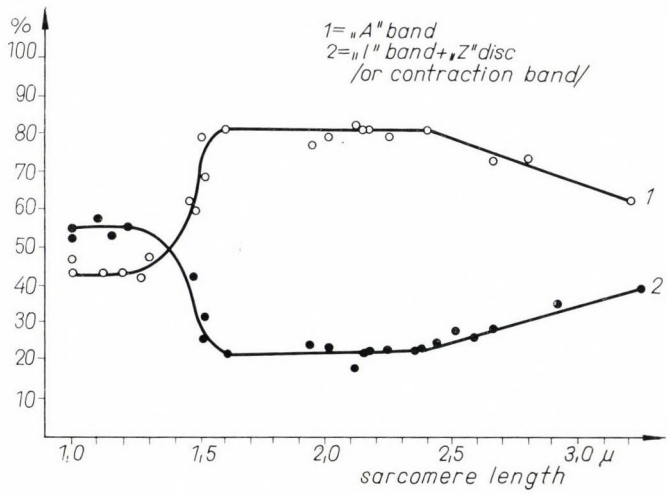

Fig. 2. The protein content of the different bands of vertebrate striated muscle as a function of sarcomere length

long as the protein content of the anisotropic ("A") band in stretched myofibril (sarcomere length > 2.3 μ) diminishes proportionally with stretching, in resting condition and in physiologically contracted states (sarcomere length from 2.3 to 1.5 μ) it doesn't change. At further shortening the protein content of the "A" band diminishes rapidly and at about 1 μ sarcomere length becomes constant. In Fig. 2 the change of the sum of proteins present in the "Z" membrane and in the isotropic ("I") band also can be seen (lower curve), which is the inverse of the former mentioned graph. Here the protein content increases proportionally

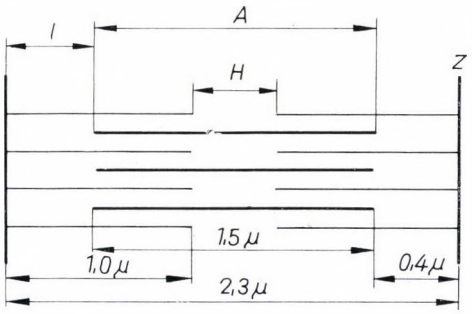

Fig. 3. Schematic drawing of the vertebrate's sarcomere in the resting state

with stretching, it is constant between resting and physiologically contracted states, while with shorter sarcomeres it shows a rapid increase. We did not find any myofibrils with sarcomere lengths shorter than 1 μ.

To explain the results described above let us recall some generally accepted facts.

The ultrastructural feature of a sarcomere (the functional unit) is shown in Fig. 3. In the ultrastructural organization of the sarcomere two sets of filaments are to be found: a set of thick filaments (~ 1.5 μ long and ~ 150 Å in diameter) and a set of interdigitating thin filaments (~ 2 μ long and ~ 70 Å in diameter). (The "Z" membranes are located in the middle of the set of thin filaments.)

Let us accept furthermore the data of Huxley and Hanson (1957), according to which in the striated muscle of vertebrates, the thick filaments (then called "A" substance) account for about 55 per cent, the thin filaments (called "I" substance) for 36 per cent, the "Z" membranes for an additional 6 per cent of the total protein content of myofibrils. The "H"-zone contains the remaining 3 per cent

The experimental facts show, that while the fiber is contracting the isotropic band diminishes and the "H"-zone disappears and in the middle of the sarcomere a protein accumulation takes place. During an additional shortening the isotropic band will also disappear, and around the "Z" membrane contraction bands will be formed. In the final stage of contraction the contraction bands can be seen (at the "Z" membranes "C_z") and in between a uniformly distributed longitudinal

structure. The protein accumulation formerly seen in the middle of the sarcomere has disappeared. In the case of stretching the lighter region in the center of the "A" band becomes longer.

All these observations are in full qualitative agreement with the sliding filament theory. If the shortening–lengthening process is accompanied indeed by the side-by-side movement of the two sets of filaments, the first sign of the contraction is expected to be the disappearance of the "H"-zone, the shortening and later the vanishing of the "I" band. In the course of contraction there are some sarcomere lengths which seem to be of particular interest. These are the sarcomere lengths of 2 μ, 1.5 μ and 1 μ. At a sarcomere length of 2 μ the opposite sets of thin filaments meet in the middle of the sarcomere (i.e. the "H"-zone will vanish), while during further shortening the thin filaments overlap at the same place. As a consequence of an additional sliding of myofibrils with a sarcomere length of 1.5 μ the overlapping zone of the thin filaments is lengthened, the thick filaments touch the "Z" membranes, so the "I" band disappears. During the subsequent contraction, the length of the overlapping zone of thin filaments continues to increase and a portion of the thick filaments gets crushed on the "Z" membranes (thus taking part in the production of the contraction bands). At a sarcomere length of 1 μ the thin filaments also touch with their two ends the opposite "Z" membranes (i.e. the contraction bands).

As a consequence of the sliding of filaments discussed above the shortening is completed by a simultaneous change in the protein content of the individual bands or of some of their parts. Fig. 4 shows the sliding of the filaments and the concomitant change in protein content.

According to the sliding mechanism during stretch the filaments move in the opposite direction: the thin filaments are drawn out of the "A" band causing the lengthening of the latter.

As it has been mentioned, this was a qualitative description of the events which take place in a shortening–lengthening sarcomere. Nevertheless, there is a possibility for the quantitative characterization of the sliding concept. According to the data obtained by interference microscopy in relaxed myofibrils of vertebrates (sarcomere length 2.3 μ, length of "I" band 2×0.4 μ, and that of "A" band 1.5 μ) 40 per cent of the proteins, represented by the thin filaments $\left(\frac{36 \times 40}{100} = \right.$ $= 14.4$ per cent of the total fibrillar protein) is accumulated in the "I" bands. The rest (i.e. 60 per cent) is in the "A" band in addition to the amount of protein coming from the thick filaments and the "H"-zone material $\left(\frac{36 \times 60}{100} + 55 + 3 = \right.$ $= 79.6$ per cent of the fibrillar protein). During contraction, as the amount of protein in the "I" band diminishes, it ought to increase the protein content of the "A" band. At a sarcomere length of 1.5 μ the "I" band contains no protein and the "A" band contains 94 per cent of the total myofibrillar proteins. Below this sarcomere length the protein content of "C_z" increases on account of the

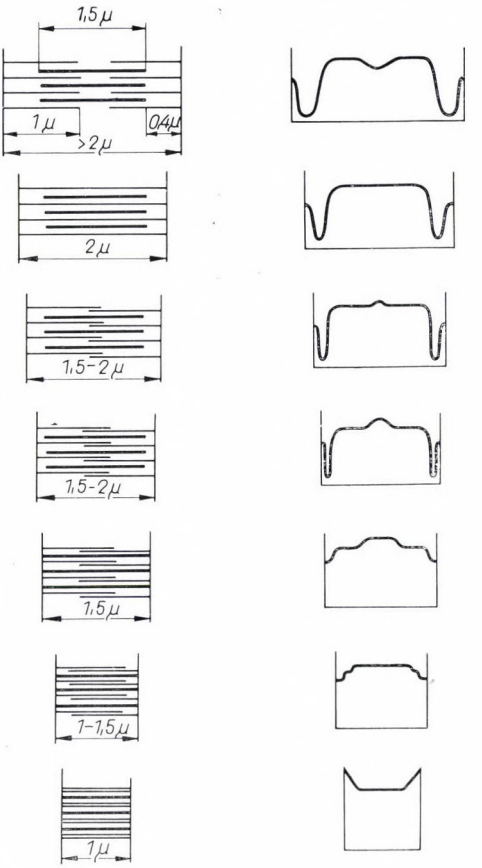

Fig. 4. Different contraction phases and the corresponding changes in the protein distribution of a sarcomere according to the sliding mechanism

thick filaments: i.e. at a sarcomere length of 1 μ, the thick filaments are shortened to 2/3 of their initial length; the amount of protein in the "C_z" band will be $6 + \frac{55}{3} = 24.3$ per cent, while at the same time the zone between the contraction bands contains the protein of the thin filaments in addition to the amount which is in the "H"-zone and the 2/3 of the thick filaments: $36 + 3 + \frac{55 \times 2}{3} = 75.6$ per cent.

In the case of stretch there is a possibility for the protein content to diminish in the "A" band concomitantly with the increase in the protein content of the "I" band. At a sarcomere length of 3.5 μ where the overlapping of the two sets of filaments has completely disappeared, according to the previous explanation the "I" band should contain 42 per cent, while the "A" band 58 per cent of the fibril-

lar proteins. In Fig. 5 the relative protein content of the "I" + "Z" and the "A" bands is plotted against the sarcomere length in compliance with the distribution described above.

By comparing Fig. 2 and Fig. 5 it can be seen that the relative distribution of protein in the different bands calculated on the basis of the sliding hypothesis using the data obtained by interference microscope and measured by our technique is only partly consistent. During a stretch there is an agreement between the two respective data. With myofibrils with sarcomere lengths from 2.3 μ to 1.5 μ plotted in Fig. 2, as compared to Fig. 5, in the protein content of the "I" + "Z"

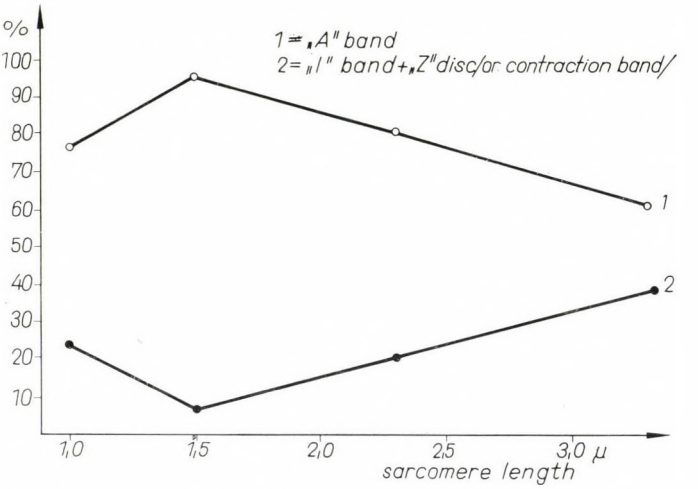

Fig. 5. The relative distribution of proteins in the vertebrate striated muscle at various sarcomere lengths according to the sliding mechanism

bands there is an increasing, while in the "A" band a decreasing tendency: e.g. at a sarcomere length of 1.5 μ this discrepancy has a value of 14 per cent (of the total fibrillar proteins) in both cases. Upon further shortening there is a larger discrepancy. At a sarcomere length of 1 μ the contraction bands contain 55 per cent (instead of 24.3 per cent), whereas the substance between the bands represents 45 per cent (i.e. a 30 per cent decrease) of the total fibrillar proteins.

These observations support the view that during contraction, in addition to the sliding of the filaments, some other processes must also take part, during which there is a protein migration from the "A" band into the "I" band and finally toward the contraction bands. This migration of proteins, a discrepancy from the sliding concept is not surprising when one examines carefully the electron micrographs or the corresponding graphs taken with the densitometer (Fig. 6). For instance at a sarcomere length of 1.6 μ (the fifth sarcomere in Fig. 6) there is an accumulation of protein at the "A"—"I" junction. This is inconsistent with the simple sliding conception.

The change in protein distribution in the above-mentioned sense is in agreement with the early qualitative findings (Hodge, 1956), namely that during contraction protein moves from the "A" band toward the "Z" membranes and accumulates in the contraction band.

It is worth-while to mention that on muscle fibers treated with salt solution of high ionic strength, that is, during the extraction of proteins a similar migration of dry mass was reported and interpreted as a possible analogy to the formation of contraction band (Hasselbach, 1953; Tigyi-Sebes, 1964; Garamvölgyi, Kerner, 1966).

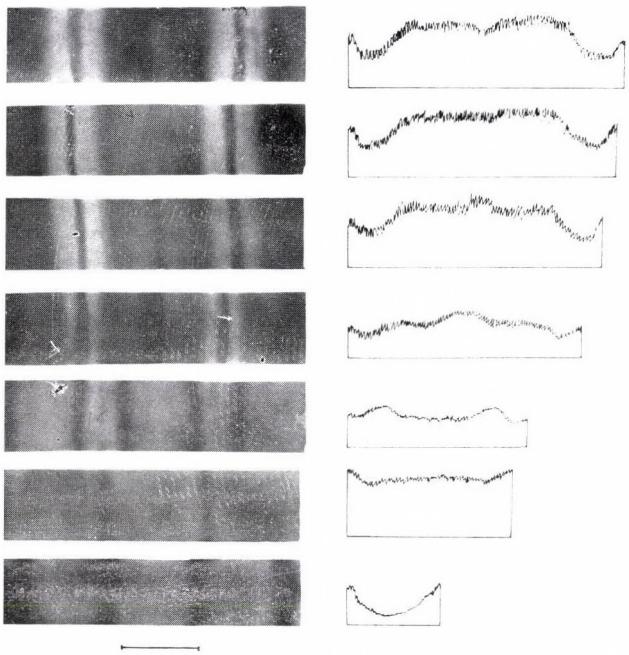

Fig. 6. Myofibrils of different sarcomere lengths and their densitometric graphs

Investigations made on isolated structural proteins and on transversal sections of relaxed and contracted muscle (Guba et al., 1965; Harsányi et al., 1965) have led us to the conclusion that myosin containing thick filaments are more hydrated, have a looser structure, hence they are less rigid, than actin containing thin filaments. This conclusion is consistent with the fact that in the case of vertebrates the length of thick filaments (1.5 μ) does not hinder the contraction below a sarcomere length of 1.5 μ, while the length of thin filaments (1 μ) seems to be a real barrier for additional shortening. Thus, thick filaments might be responsible for the migration of proteins during contraction.

These experiments do not give any information as to the mechanism of the migration mentioned above. Nevertheless they offer a piece of evidence that the

observed movement is a consequence of the change in the gel structure and of the state of aggregation of the two types of filaments during the contraction as it was stated by the authors in a previous work (Guba et al., 1965).

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Effect of Ultrasonic Treatment on the Iron Binding Capacity of Human Blood Serum

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Human blood serum was treated with ultrasound (800 kHz, max. 3 $W \cdot cm^{-2}$) and the change in the total iron binding capacity was investigated as a function of time and intensity. The total iron binding capacity was found to decrease upon ultrasonication in both cases compared to the control. When this decrease was plotted versus time it was found that the change took place in a very short time. By increasing the intensity the total iron binding capacity showed a regular (in a square ratio) decrease.

Introduction

In the human organism iron transport between cells and organs is known to take place by the mediation of the intercellular fluid and blood. In this process transferrin is of major importance from the point of view of the intermediary iron metabolism. Under normal conditions one liter serum contains 2 to 3 g transferrin which carries about 3 to 4 mg iron in a concentration of 120 μg per 100 ml. The unsaturated iron binding capacity is 230 μg per 100 ml. Thus, the mean value of saturated iron binding capacity is 350 μg per 100 ml (Laurell, 1959). In the last two decades several authors have been investigating the factors on which the iron content of the blood serum and its total iron binding capacity depend. Significant changes have been found in the serum iron and in the total iron binding capacity. These were age dependent (Hagberg, 1953). With animals the total iron binding capacity increases upon bleeding in spits of protein loss (Dezső and Bot, 1962). With both tumorous patients and animals (rabbits) X-ray irradiation of 100 to 900 r resulted in a significant decrease of iron binding capacity (Schmitt, 1960).

Methods

Transferrin is known to be a β_1 globulin. Those influences which produce some kind of change in the serum protein are very likely to affect the iron binding capacity, too. The question was therefore, whether ultrasonic treatment is able to produce such changes in transferrin which would be reflected in a change of the total iron binding capacity of the serum. To answer this question human blood serum was treated in the following ways:

a) First, serum samples were treated with a constant dose of 1.5 $W \cdot cm^{-2}$ at a constant temperature of 38 °C but for different periods (a "Sonotherm" ultrasound apparatus was used).

b) Secondly, the time of irradiation was kept constant (5 minutes) but the intensity of ultrasound was varied from 0.5 $W \cdot cm^{-2}$ to 3 $W \cdot cm^{-2}$. At intensities higher than 1 $W \cdot cm^{-2}$ the irradiation head unit was wrapped up in wet cotton-wool and was cooled with an electric fan.

After treatment the total iron binding capacity was determined by a combination of the method suggested by Ramsay (1957) and of that reported by Dezső and Fülöp (1960) for the estimation of iron.

Results

The results obtained with the method described under *a)* are summarized in Table 1. It may be seen that compared to the control, the total iron binding capacity of the serum samples decreased upon ultrasonic treatment in every case.

Table 1

Time of ultrasonic treatment (sec)	Iron binding capacity		
	μgFe per 100 ml	Decrease	
		μgFe per 100 ml	per cent
15	385	—	—
30	350	35	9
45	355	30	7.8
60	287	98	25.4
90	300	85	22.1
120	295	90	23.4
Control	385	—	—

This decrease took place in a rather short time and reached a maximum value in 1 minute. When the time of the treatment was further increased this maximum value did not change significantly. There was no unambiguous relation between the increase of time and decrease of the total iron binding capacity. Therefore another experiment, as described under *b)* was performed. The results of this experiment are shown in Table 2.

Before each measurement the intensity scale of the ultrasound generator was standardized by the Tarnóczy's (1954) sound pressure phonometer.

The data in Table 2 show that the total iron binding capacity decreases with increasing intensities. The objection that the decrease observed was due to the fact that ultrasonic treatment resulted in the solubilization of some substance of the wall of the plexi glass vessel used and that this substance reacted with transferrin was excluded by a few control experiments.

Table 2

Intensity ($W \cdot cm^{-2}$)	Iron binding capacity		
	μgFe per 100 ml	Decrease	
		μgFe per 100 ml	per cent
0.5	377	3	0.8
1.0	373	7	1.8
1.5	369	11	2.9
2.0	358	22	5.5
2.5	340	40	10.5
3.0	323	57	15.0
Control	380	—	—

Discussion

In our opinion the effect of ultrasonic treatment to decrease the total iron binding capacity cannot be attributed to any substance dissolved from the wall of the vessel, but rather to the physico-chemical influence of ultrasonic treatment on the serum. Even if it is supposed that a certain degree of splitting of transferrin and other serum protein molecules also take part in the decrease of the total iron binding capacity, this is still not what is really decisive. It can be much rather supposed, that certain groups situated on the surface of the transferrin molecules change, or get split from the molecules. Experiments along these lines are in progress.

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Repressibility by Puromycin of the Preloading Effect in Amino Acid Absorption

(Short communication)

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While studying the methionine uptake by a strain of brewer's yeast (Saccharomyces carlsbergensis, strain No. 2 "Schwechat") we found (Balogh et al., 1966) that after cultivation on wort supplemented with DL-methionine the ^{35}S DL-methionine influx was remarkably reduced in short experiments, that followed the pretreatment. Earlier Vogel (1960), Inui and Akedo (1965) reported the repressive effect of preloading on further absorption in experiments on amino acid uptake by Escherichia coli. In higher plants bromide influx could be considerably reduced by growing them for a few days on bromide solution containing $CaSO_4$ (Böszörményi, 1966), or even after a pretreatment for a few hours (unpublished).

In order to obtain information about the characteristics of the preloading effect detected in the methionine absorption by brewer's yeast we have examined the time course of repression and the effects of different antibiotics on the repression.

The yeast was grown in wort in 100 ml shaken liquid cultures at 20° for two or three days. The cells were then centrifuged and washed three times with 0.05 M phosphate buffer (pH 4.5). The suspension was diluted to 10^8 cells ml and was used within one and a half hour for the actual absorption experiment.

In the pretreatment an aliquot of the yeast suspension was centrifuged and the cells were resuspended in phosphate buffer which, in some variants included 4 mM inactive DL-methionine and/or antibiotics. At certain intervals 1 ml aliquots were filtered through MF 50 membrane filters (Membranfilter Ges., Göttingen) and washed with 10 ml distilled water.

An aliquot of 10^8 cells was then placed in 50 ml 0.1 mM ^{35}S DL-methionine solution together with the membrane filter for a 20-minute absorption period. During this period the suspension was shaken at 22°. At the end of this period the suspension was filtered through a new membrane filter and rinsed twice with 10 ml distilled water. The yeast layer was then removed from the filter, hydrolyzed with 6 N HCl, and the radioactivity of the hydrolysate was measured with liquid scintillation technique. No quenching effect was experienced. Each treatment was run in duplicates, and the averages are given in Figure 1 and in Tables 1 and 2.

Under the influence of 4 mM DL-methionine added to the phosphate buffer a remarkable inhibition of methionine influx developed in the first hour

of the pretreatment, and maximum inhibition could be achieved within two hours (Fig. 1). If, instead of phosphate buffer wort was used for incubation, the inhibition took a longer time to develop and it failed to reach the level experienced with the phosphate buffer even at the end of the three-hour experiment.

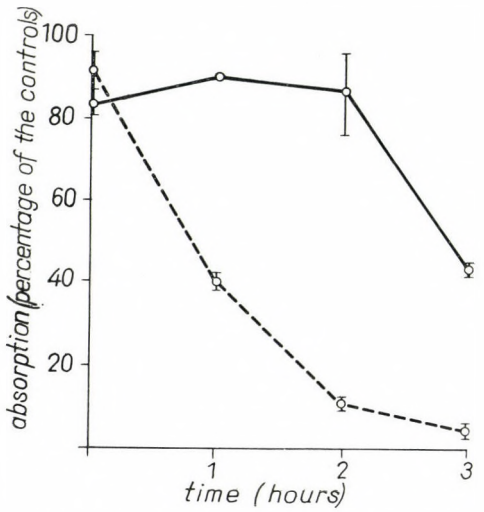

Fig. 1. Time-course of the repression of methonine influx by preloading with 4 mM inactive DL-methionine in 0.05 M phosphate buffer (pH 4.5) ---------, and in wort ———. Absorption from 0.1 mM ^{35}S DL-methionine, 20 minutes, at 22°

Table 1

Effect of antibiotics on the repression of methionine influx by preloading

Preloading for 1 1/2 hour in phosphate buffer (pH 4.5) supplemented with 4 mM DL-methionine. Absorption from 0.1mM ^{35}S DL-methionine, 20 minutes. Absorption by antibiotic-pretreated variants as percentage of the respective controls

	Experiment 1	Experiment 2
phosphate buffer	68	43
5×10^{-5}M actinomycin D	71	—
10^{-4}M puromycin	94	—
10^{-3}M chloramphenicol	61	—
2 μg/ml nystatin	—	70
10^{-3}M p-fluorophenylalanine	—	72

Of the antibiotics employed, such as actinomycin D, puromycin, chloramphenicol, nystatin and the amino acid analogue p-fluorophenylalanine only puro-

mycin was capable of preventing the repressive effect of methionine preloading (Table 1).

The effect experienced with puromycin treatment prompted us to examine whether or not it was due directly to the inhibition of methionine absorption. To this end the effect of 10^{-4} M puromycin, given simultaneously, on the uptake and incorporation of ^{35}S DL-methionine (Table 2) was determined under conditions identical with those of the pretreatment (the only difference was that the

Table 2

Effect of simultaneously given puromycin on methionine absorption

90 minutes absorption from 10 ml aerated phosphate buffer (pH 4.5) supplemented with ^{35}S DL-methione

	counts/min	μmole/5 × × 10^7 cell
0.1 mM methionine	14 139	0.0145
0.1 mM methionine + 10^{-4}M puromycin	14 578	0.0150
4.0 mM methionine	1 119	0.0475
4.0 mM methionine	1 510	0.0640

number of cells was reduced to 10^7 cells/ml). Under these conditions puromycin did not affect methionine absorption.

In the amino acid uptake by Escherichia coli Inui and Akedo (1965) were able to inhibit the preloading effect by 100 μg/ml chloramphenicol. In our experiments cloramphenicol had no influence whatever, but a comparable inhibition could be detected with puromycin. In experiments with intact cells there can be significant differences between the relative rates of absorption (or permeation) of certain antibiotics, and a different concentration or period of treatment might be necessary for the formation of the effect. Therefore no far reaching conclusions can be drawn from this difference.

If we accept the general opinion (cf. Hahn, 1966) that in a cell-free system puromycin inhibits protein synthesis at the ribosome level and supposing that this is the only influence exerted by puromycin in the case of intact yeast cells, we must draw the conclusion that the preloading effect is connected with protein synthesis. Concerning the function of the synthetized protein, at the moment only assumptions can be made, such as 1. the protein is a specific "transport inhibitor protein", the synthesis of which has been induced by the preloading and which reacts directly with a transport protein (permease) or 2. the protein is a repressor inhibiting the transcription of a specific transport protein at the nuclear level.

It should be mentioned that according to our experiments (Balogh et al. 1966) the amino acid uptake by yeast is a dual process as indicated by the concentration curve: the system operating at lower concentrations becomes saturated at about

0.3 mM. (The data of 0.1 mM preloading refer to this system.) According to our data, and those by others (Halvorson et al., 1961; Sorsoli et al., 1964; Surdin et al., 1965) the amino acid absorption by yeast is less specific than that by bacteria. (E.g. Data about competitions suggest the operation of a common amino acid carrier system.) This opinion is supported by experiments in which the preloading effect in the methionine influx could be brought about by supplementing wort with glycine, valine or alanine. On the 20 mM concentration level glycine and alanine were found to have had an even greater effect than methionine.

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Feedback Effect on the Inactivation of Phosphorylase

(Preliminary Report)

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It is generally assumed that hormonal control of the enzymatic activation and inactivation of phosphorylase plays the dominant role in the regulation of glycogenolysis (Posner et al., 1962; Merlevede, Riley, 1966). Active phosphorylase is inactivated by phosphorylase phosphatase (EC 3.13.17). Though phosphorylase phosphatase is always present, tissues do contain a certain amount of active phosphorylase. This fact leads to the postulation of a control mechanism by which the enzymatic inactivation of active phosphorylase is reversibly inhibited. In this paper we show that the postulated mechanism can be found in the muscle.

AMP*, G-6-P and G-1-P have an allosteric effect on phosphorylase. AMP has a double effect: it increases the activity of phosphorylase and, at the same time inhibits the inactivation of phosphorylase by phosphatase. Nolan et al. (1964) suggested that inhibition of the inactivation of phosphorylase by AMP was due to an allosteric effect on phosphorylase *a* and not to any effect on phosphorylase phosphatase. This was demonstrated by the observation that AMP had no effect on the cleavage by phosphorylase phosphatase of phosphopeptides obtained from phosphorylase *a* (Nolan et al., 1964). The allosteric nature of the effect of G-1-P is shown by the finding (Cori, Cori, 1945) that G-1-P inhibits the inactivation of phosphorilase *a* by trypsin. The physiological level of AMP in tissues is sufficiently high to inhibit permanently the inactivation of phosphorylase. However, the allosteric effect of G-6-P is known to be the opposite of that of AMP since G-6-P inhibits the activity both of phosphorylase *a* (Bot et al., 1966) and phosphorylase *b* (Morgan and Parmeggiani, 1964). This indicates that G-6-P might also effect the inactivation of phosphorylase.

In this paper we report studies on the effect of G-6-P on the inactivation of phosphorylase by phosphorylase phosphatase. We have used a rabbit muscle extract which contained both phosphorylase *a* and phosphorylase phosphatase. Nucleotides were removed from the extract with Norit. The Norit-treated extracts were incubated at 30 °C and the inactivation of phosphorylase was determined by measuring the decrease in phosphorylase *a* activity.

The experimental results show that inactivation of phosphorylase *a* is inhibited in the presence of AMP and also that G-6-P relieves this inhibition. When

* Abbreviations: AMP = adenosine 5'-monophosphate; G-6-P = glucose 6-phosphate; G-1-P = glucose 1-phosphate; F-6-P = fructose 6-phosphate; FDP = fructose diphosphate.

G-6-P is also added, the enzyme is inactivated even in the presence of AMP. The time course of the transformation of phosphorylase *a* is shown in Fig. 1 in the absence and presence of AMP and G-6-P.

It is seen from the figure that 0.1 mM AMP almost completely inhibits the inactivation of phosphorylase *a* by phosphorylase phosphatase. The inhibitory effect of AMP is abolished by the addition of 10 mM G-6-P. The rate of inactivation is markedly enhanced by G-6-P even in the absence of AMP.

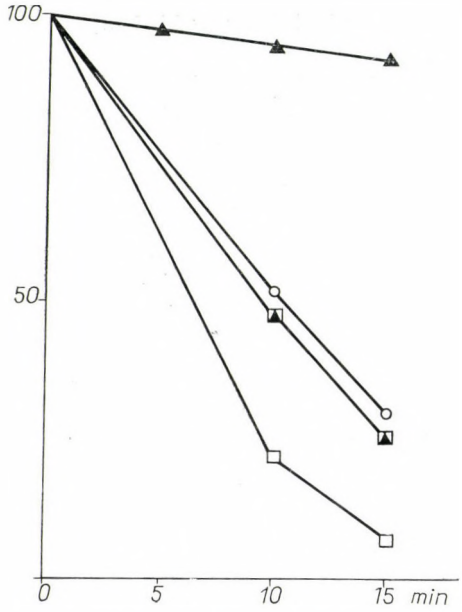

Fig. 1. The effect of AMP and G-6-P on the inactivation of phosphorylase *a* in a muscle extract. Rabbit muscle was extracted with 4 volumes of 0.02 M Tris buffer and treated with Norit for the removal of nucleotides. The extract was incubated at 30°C, in 0.02 M Tris, pH 6.8, in the presence of 0.002 M EDTA. No inhibitor ○—○; 0.1 mM AMP; ▲—▲ 10 mM G-6-P □—□; 0.1 mM AMP + 10 mM G-6-P ▲—▲. Aliquots were withdrawn from the samples as indicated and diluted with Tris/EDTA buffer also containing 0.1 M NaF. Further inactivation was stopped by NaF, and in the resulting low concentrations the inhibitors did not interfere with the activity assay. Residual phosphorylase *a* activity was determined according to Cori et al. (1955) by measuring the phosphate liberated from G-1-P. Phosphate was determined according to Taussky and Shorr (1953). Residual phosphorylase *a* activity was expressed as per cent of the activity at 0 min. When phosphorylase *a* is inactivated, phosphorylase *b* is produced. Inactivation of phosphorylase *a* was checked by determining phosphorylase *b* activity in the presence of 0.001 M AMP

In agreement with literary data (Cori, Cori, 1945; Hurd et al., 1966) we have observed that G-1-P inhibits the transformation of phosphorylase *a* to phosphorylase *b* similarly to AMP. The inhibitory effect of G-1-P is also abolished by G-6-P, since phosphorylase phosphatase is apparently active in the presence of G-1-P plus G-6-P (Fig. 2).

It is seen from the Figure that the inhibitory effect of G-1-P is markedly decreased in the presence of 5 mM G-6-P, and completely abolished by the addition of 10 mM G-6-P.

We have further found that, in contrast to G-6-P, neither F-6-P nor FDP counteracts the inhibitory effect of AMP or G-1-P.

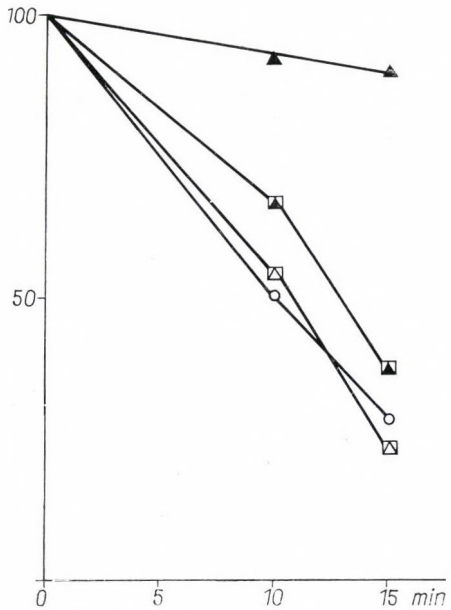

Fig. 2. The effect of G-1-P and G-6-P on the inactivation of phosphorylase *a*. Rabbit muscle extract was incubated at 30°C in 0.02 M Tris, pH 6.8, and 0.002 M EDTA, in the absence of inhibitors ○— ○; with 1 mM G-1-P ▲ — ■; with 1 mM G-1-P + 5 mM G-6-P ▲—▲; and with 1 mMG-1-P + 10 mM G-6-P △—△. Experimental methods as in the legend to Fig. 1

From these findings it is concluded that active phosphorylase can be rapidly inactivated even in the presence of AMP or G-1-P when the level of G-6-P is sufficiently high. We suggest that this is another important regulatory action of G-6-P, in addition to those already known. During the course of glycogen mobilization in the cell the concentration of G-6-P is maintained at a low level consequently AMP is inhibiting the inactivation of phosphorylase and in this way the enzymatic activity of phosphorylase is maintained. When, on the other hand, glycogen is being synthesized the inhibition of inactivation by AMP is relieved as the concentration of G-6-P is raised and this results in the transformation of phosphorylase *a* to phosphorylase *b*. This, in turn, will result in a lowering of the rate of mobilization of glycogen and in an increased rate of glycogen synthesis (negative feedback).

The allosteric nature of the action of G-6-P is indicated by the finding that it counteracts the inhibitory effect of AMP and G-1-P and also, that G-6-P by

itself increases the rate of inactivation of phosphorylase *a* by phosphorylase phosphatase (Fig. 1).

Therefore, glycogen mobilization is regulated by a double action of G-6-P: direct inhibition of phosphorylase activity, and enhancement of the rate of enzymatic inactivation of active phosphorylase.

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Book Review

Richard BACKHAUSZ: *Immunodiffusion und Immunoelektrophorese*. Akadémiai Kiadó, Budapest, 1967. 516 pages, 76 figures and 26 tables

The use of immunochemical methods has already got beyond the field of immunology, as interpreted in its narrow sense, and, due mainly to the immunodiffusion methods, has become one of the most profitable and versatile tools of both fundamental and applied research in medicine, biochemistry, microbiology, zoology, and botanics. Although the immunodiffusion methods were introduced not long ago, several books have already been published surveying these methods. Dr Backhausz's book still fills a gap, since it not only helps in the theoretical understanding and practical performance of immunodiffusion methods, but also gives a survey of the possible applications of these methods and of the results thus obtained in several branches of the biological sciences. A great merit of the book is the fact that, in addition to the theoretical and practical problems of the methods, it gives a brief summary of the latest results in immunology, and within this frame, of immunochemistry. The presentation of nearly two thousand references also contributes to the book's becoming a useful and popular handbook for those who are interested in the various branches of immunochemistry. The value of the book is greatly enhanced by the illustrations most of which were taken from the author's own experimental material.

After the first, introductory chapter, the author gives a brief account of the immunological examination methods. The in vitro and in vivo procedures are dealt with separately. Almost every method used in practice is listed in this chapter.

In the third chapter the theoretical questions of immunodiffusion methods are discussed, the gels used in the diffusion methods, the principles of methods, and the factors influencing immunodiffusion are set forth.

In chapters 4 and 5 the theoretical questions of simple and double immunodiffusion methods are dealt with, while chapter 6 presents the problems of immunoelectrophoresis. In this latter chapter not only the generally used techniques are described, but attention is paid to the quantitative methods, to the well-established procedures of antigen, identification as well as to the very promising, but still not so widespread, electrosyneresis.

Chapter 7, which deals with practical techniques is of great value. Several immunization procedures are presented in detail to help the investigators to prepare specific immune sera for their experiments. Practical work is also facilitated by the enclosure of the composition of several buffers, dye-solutions, etc. The evaluation of results obtained with immunodiffusion is also treated in this chapter. The author's many-sided methodical experience is reflected in this chapter, rendering it one of the most profitable part of the book.

In addition to the merits mentioned above, the subsequent two chapters make the book prominent among the other compilations of the same kind. In these chapters some particular questions are treated from a point of view which cannot be found elsewhere.

Chapter 8 deals with the applicability of immunodiffusion methods in the study of microorganisms (viruses, Rickettsiae, bac-

teria, fungi, protozoa), while chapteu 9 deals with the study of the antigens of multicellular organisms. Plant antigens, as well, as the antigens of vertebrates and invertebrates are dealt with. The section concerned with the study of human serum proteins might claim great interest. From these chapters the readar can get a thorough survey not only of the methodical problems, but also of the most important results.

The last chapter is again of great practical help in the planning of immunodiffusion examinations.

Immunochemistry has amazingly developed during the course of the last ten years, and in this process the development of the modern immunodiffusion and biochemical methods played an important role. This progress led to the formulation of the concept of immunoglobulin, and to the understanding of the structure of immunoglobulins in more detail. It is a great merit of the book that it reflects the view of this modern immunochemistry.

The usefulness of the book is supported by its good construction. The fine quality is due to the careful work of the Hungarian Academic Press.

Dr J. GERGELY

Printed in Hungary

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The Enzymic Preparation of Glucose-1-phosphate and its Separation from Glucose Diphosphate

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(Received 1 February, 1967)

It has been shown that in the course of enzymic preparation of G-1-P, the GDP contamination of the preparations originates from the potato extract and starch. Charcoal treatment of the potato extract and starch prior to the preparation makes it possible to obtain G-1-P free of GDP. Pure G-1-P can be obtained also with crystalline muscle phosphorylase.

G-1-P free of GDP can be obtained if the inorganic phosphate is removed by Ba-acetate during preparation.

The subsequent removal of GDP from G-1-P preparations has also been marked out by means of the adsorption of GDP on the Ba-phosphate precipitate.

Introduction

There are several methods reported in the literature for the enzymic preparation of G-1-P* (Cori et al., 1937; Sumner, Somers, 1944; McCready, Hassid, 1955). The method is based on the phosphorylation of starch by potato phosphorylase in the presence of inorganic phosphate, which yields G-1-P. In an earlier procedure (Sumner, Somers, 1944) G-1-P was obtained in the form of Ba-salt and the latter was converted into crystalline potassium salt. However, the product often contained sulphate contaminations. In a more recent method (McCready, Hassid, 1955) ion exchange resin is applied for the separation of G-1-P; the product obtained in this way is considerably purer than that prepared *via* the Ba-salt. However, the products of both procedures still contain GDP contaminations, even after several recrystallizations (Sutherland et al., 1949), and often small amounts of starch as well (Abdullah et al., 1965). For the study of the mechanism of action of PGM, however, the G-1-P preparations used should be free of both GDP and sulphate contaminations. In addition, in studies on the mechanism of action of phosphorylase in certain cases starch-free G-1-P preparations are to be used (Abdullah et al., 1965).

In the present paper we describe the origin of GDP contamination in G-1-P preparations and the way of its removal during the preparative procedure or from the final preparation. A survey of the utilizability for the preparation of G-1-P of ion exchange resins manufactured in Hungary is also presented.

* *Abbreviations:* G-1-P = glucose-1-phosphate G-6-P = glucose-6-phosphate
GDP = glucose-1.6-diphosphate PGM = phosphoglucomutase

Experimental

Application of ion exchange resins manufactured in Hungary for the preparation of G-1-P

The enzymic part of the preparation of G-1-P was carried out according to the method of McCready and Hassid (1955).

500 ml of 0.8 M KH_2PO_4 solution (0.4 mole) was adjusted to pH 7.4 with KOH, then 500 ml of 4 per cent starch solution and 500 ml of potato extract ([1]) were added. To the mixture 25 ml of toluene was added and the mixture was kept at room temperature for 24 hours. The formation of G-1-P was controlled from time to time by measuring the amount of hydrolyzable phosphate within 10 minutes after the removal of inorganic phosphate (McCready, Hassid 1955). Phosphate was determined by the method of Taussky, and Shorr (1953). After 24 hours, i.e. after the formation of sufficient amount of G-1-P, the mixture was heated to 95°C to denature the proteins. After cooling 0.4 mole of Mg acetate was added to the mixture and the pH was adjusted to 8.5 with NH_4OH diluted 1 : 1. The precipitated $MgNH_4PO_4$ and the coagulated proteins were then filtered off. The filtrate containing the G-1-P was applied to a cation exchange colunm.[2] The flow rate was 15 ml per minute. The pH of the eluate was about 3.5, and from time to time the eluate was checked for NH_4^+ and Mg^{++} cations. The effluent was collected in four fractions, immediately cooled and combined, then it was let through an anion exchange column[3] at a flow rate of 10 ml per minute. The anion exchange resin retains G-1-P, while the contaminating substances (starch, acetic acid) pass through the column. The column was then washed with 4 to 5 liters of distilled water until no starch could be detected with anthron reagent in the effluent (flow rate 20 to 25 ml per minute).

The G-1-P bound to the column was then eluted with 1 M KCl.[4] The eluate was collected in 50 ml fractions. Fractions 2—10 contained about 90—95 per cent of the G-1-P. The pooled fraction were then brought to pH 8.5 with 1 N KOH and treated with 2—3 g of Norit—A to remove any traces of starch. After filtration 1 to 1,5 volumes of ethanol were added to the clear solution with constant stirring. G-1-P crystallized while standing at 0°C within 12 to 24 hours. The crystals were filtered and after drying by suction they were dissolved in 130—160 ml of distilled water. The solution was filtered until it became clear and the G-1-P was recrystallized by the addition of 1 to 1.5 volumes of ethanol.

[1] Preparation of potato extract: 300 g of peeled potatoes are homogenized in 500 ml of 0.001 M thioglycolic acid. The pulp is forced through cheese cloth, the fluid is allowed to stand for 10 minutes then decanted.

[2] About 900 ml of wet Varion KS resin (20—50 mesh) in the H form, in a 60×4.6 cm column.

[3] About 100 ml of Varion AD (20—50 mesh) anion exchange resin in the OH form, in a 14×3 cm column.

[4] The reason for the elution with KCL is the fact that Varion AD is a strongly basic exchange resin to which Cl ions are more firmly adsorbed than OH ions, and thus G-1-P can be eluted in a smaller volume with KCl than with KOH.

The product was filtered with suction and washed first with about 50 per cent, then about 95 per cent ethanol, and at last with dry acetone. The crystals were dried in air and weighed. The yield was 13 to 16 g, about 80 to 90 per cent of the enzymatically formed G-1-P.

Origin of the GDP-content of G-1-P preparations

The enzymatically prepared G-1-P preparations contain more or less GDP (Sutherland et al., 1949). To be able to prepare G-1-P free of GDP, first we studied the source of GDP contamination of G-1-P preparations. The question was, whether it was present already in the ingredients used, or it was formed during incubation. As a preliminary experiment, 0.5 g of GDP-free G-1-P was treated with 5 ml of potato extract (1) for half an hour at room temperature, then the solution was boiled the proteins removed and the G-1-P was recrystallized with ethanol. After filtration and drying by suction 0.3 g of dry crystalline substance was regained.

The GDP-contents of G-1-P preparations were compared in the following way. From the G-1-P preparations substrate solutions were prepared for PGM activity assay and incubated with an appropriate solution of crystalline PGM; the amount of G-6-P formed was measured. The composition of the reaction mixture was the following: 0.004 M G-1-P (untreated or treated with potato extract); 0.0012 M $MgCl_2$; 0.035 units for PGM. Incubation was carried out at 30°C for 10 minutes at pH 7.5. The amount of G-6-P formed was measured by the method of Somogyi and Nelson (Nelson, 1944). The results are shown in Table 1.

Table 1

Substrate	G-6-P formed in 10 minutes
G-1-P treated with potato extract	1.05 μmoles
G-1-P, untreated	0.19 μmoles

It can be seen from Table 1 that from G-1-P substrate treated with potato extract, more G-6-P was formed by PGM, than from the untreated substrate. This finding indicates that a considerable amount of GDP got into the G-1-P preparation from the potato extract. Thus it can be assumed that also in the preparation of G-1-P, the GDP found in the preparation comes from the potato extract and appears as contamination during the course of further purification and recrystallizations. However, GDP may get into the G-1-P preparations also from the starch used for the preparation. It has been known (Abdullah et al., 1965) and we observed it as well that starch contains GDP. To exclude both sources of contamination, the potato extract as well as the starch solution were treated with Norit-A before the preparation of G-1-P. Norit-A is known to adsorb hexose phosphates (Crane, 1958). The G-1-P thus prepared by the method of

McCready and Hassid contained considerably less GDP than the product obtained from ingredients not treated with Norit. The GDP contents of the preparations thus obtained are shown in Table 2. The presence of GDP was detected by measuring the amount of G-6-P formed by PGM[5].

It can be established on the basis of the above experiments that the GDP contamination of G-1-P preparations originates from the potato extract and from the starch and can be markedly reduced by charcoal treatment.

Table 2

Substrate	G-6-P formed in 10 minutes, μmoles	
	—	+ GDP (2×10^{-6}M)
G-1-P prepared from starch and potato extract, both treated with Norit	0.22	1.72
G-1-P prepared from starch and potato extract, both untreated	1.72	1.90

Preparation of G-1-P with crystalline phosphorylase

It could be inferred from our previous experiments that if crystalline phosphorylase is used instead of potato extract and purified glycogen instead of starch, GDP-free G-1-P could be prepared. To test this assumption, Norit-treated glycogen and inorganic phosphate were incubated with crystalline rabbit muscle phosphorylase b in the presence of AMP. Phosphorylase b was prepared according to Fischer and Krebs' (1955) method.

100 ml of 4 per cent Norit-treated glycogen solution and 100 ml of 0.4 M KH_2PO_4 solution (pH 7.4) were incubated with 100 ml of twice recrystallized

Table 3

Substrate	G-6-P formed in 10 minutes μmoles	
	—	+ GDP (2×10^{-6} M)
G-1-P prepared with crystalline phosphorylase	0.00	1.85
G-1-P prepared with untreated potato extract	1.81	1.82

[5] That the decreased formation of G-6-P was really caused by the lack of GDP, was verified by the addition of GDP to the incubation mixture (second column). It is known that 2×10^{-6} M GDP is required for the full activity of PGM (Sutherland et al., 1949). It can be seen that in this case nearly the same amount of G-6-P is formed from both G-1-P substrates.

rabbit muscle phosphorylase b solution (about 800 IU) for 24 hours in the presence of 160 mg AMP. Further details of the procedure were identical with those of the ion exchange method described above. The amount of G-1-P obtained was relatively small (1.1 g), but contained no GDP at all. The purity of the product is demonstrated in Table 3, where it is compared to that of G-1-P, prepared by the method of McCready and Hassid from untreated potato extract and starch.

Table 3 shows that G-1-P obtained with crystalline phosphorylase contains no GDP, at least less than the amount required for the activation of PGM. The pure G-1-P is naturally a completely effective substrate after the addition of GDP. In G-1-P preparations obtained with potato extract, however, there is so much GDP contamination present that it ensures the full activity of PGM and there is no increase in PGM activity on the addition of GDP.

Preparation of GDP-free G-1-P according to the modification of McCready and Hassid's method

We have shown in our previous experiments that the GDP contamination of G-1-P preparations originates from the potato extract and starch. The removal of GDP from these substances with Norit-treatment is, however, difficult and not always sufficient. Therefore we attempted to modify the preparation procedure, to be able to remove GDP contamination in another way during preparation. This kind of modification was made possible by our observation, according to which those preparations from which inorganic phosphate was removed with Ba-acetate, during the procedure, contained less GDP than those, from which phosphate was removed with Mg mixture. Studying the conditions of the precipitation of phosphate we have observed that if Ba-acetate is applied in excess, the precipitating Ba-phosphate adsorbs the Ba-salt of GDP at pH 10 and 100°C. We made use of this observation in the enzymic preparation of G-1-P and thus the method of McCready and Hassid has been modified as follows:

500 ml of 0.8 M KH_2PO_4 solution (0.4 mole) and 500 ml of 4 per cent starch solution were incubated with 500 ml of potato extract for 24 hours. The mixture was then boiled and the precipitated proteins were removed by filtration. To the hot filtrate 125 g of solid Ba-acetate 4 H_2O (0.45 mole) was added stepwise. After the addition of each portion, the pH of the solution was adjusted to 10 (pink colour with alizarin yellow) with saturated KOH. After the addition of the total amount of Ba-acetate the hot precipitate was filtered off. The filtrate was cooled down and then mixed with about 1300 ml of Varion KS cation exchange resin, in the H form, until all the Ba was bound. The solution, which became acidic after this procedure, was decanted and about 100 ml of Varion AD anion exchange resin in the OH form, was added to it. The resin adsorbed about 90 per cent of the G-1-P from the solution after 10–20 minutes of stirring. The supernatant was decanted and the resin was washed with water until starch reaction became negative. (If the starch could not be removed completely by washing with water, the eluate containing the G-1-P had to be treated with Norit, which removed all traces of starch.) The washed resin was then washed with water into a glass tube, thus a column was prepared, from which G-1-P was eluted with

1 N KCL. The effluent was collected in 50 ml fractions and those fractions which contained G-1-P (fractions 2 to 10) were pooled. The total volume was about 450 ml. The solution was made alkaline with a small amount of KOH and 1.5 volumes of ethanol were added to it under constant stirring. The crystallization of the dipotassium salt of G-1-P started immediately. After standing for 12–24 hours at 0°C, the crystals were filtered and dried. The yield was 12 g.

The G-1-P obtained contained almost no GDP. Table 4 shows the behaviour in the presence of PGM of the substrates, obtained from preparations from which phosphate had been removed by two different methods.

Table 4

Way of the removal of phosphate during the preparation of G-1-P	G-6-P formed in 10 minutes μmoles	
	–	+ GDP (2×10^{-6} M)
Ba-acetate, pH 10, 100°C	0.24	1.79
Mg mixture	1.81	1.82

The G-6-P formation indicated in Table 4 shows that when phosphate was removed with Ba-acetate, the resulting G-1-P preparation contained only very little amount of GDP (0.005 per cent), in contrast to the 0.1 per cent GDP content of G-1-P preparations obtained with the original method. The G-1-P thus prepared, when applied as the substrate of PGM, exhibits in itself only negligible PGM activity and in this way it makes possible the study of the effect of GDP.

Removal of GDP from G-1-P preparations

The observation that the precipitation of inorganic phosphate in the form of Ba-phosphate results in the removal of GDP, could be made use of not only in the preparation of G-1-P but also in the purification of G-1-P preparations contamined with GDP. The principle of the purification procedure is following. To the potassium salt of G-1-P Ba-acetate is added and then a small amount of KH_2PO_4. At pH 10 and 100°C GDP co-precipitates with the precipitating Ba-phosphate and the G-1-P free of GDP can be crystallized from the solution. It is important that the KH_2PO_4 should be less than the amount of Ba ions present in the solution, i.e. the precipitation of phosphate and the removal of GDP must take place in the presence of excess Ba ions. After the removal of the precipitate, the Ba still present in the solution is removed by a slight excess of K_2CO_3.

The application of the method is visualized by the following example: 26.1 g (0.07 mole) of G-1-P is dissolved in 20 volumes of water (about 520 ml) and the solution is filtered through filter paper. To the filtrate 5.4 g (0.02 mole) of Ba-acetate is added in the form of a concentrated solution, then the pH of the solution is adjusted to 10 with concentrated KOH (pink colour with alizarin yellow). A concentrated solution of 1.36 g (0.01 mole) of KH_2PO_4 adjusted pre-

viously to pH 10, is then added to the mixture dropwise under constant stirring. The mixture is boiled for some minutes and the precipitated Ba-phosphate is filtered or centrifuged off while hot. Prolonged boiling is unfavourable due to caramellization. To the clear filtrate 12 ml of 1 M K_2CO_3 is added to remove the Ba still present in the solution and to convert G-1-P to the potassium salt. K_2CO_3 should be only in a slight excess (0.012 mole). The hot mixture is filtered and the clear filtrate is cooled. The solution must not contain either Ba ions or inorganic phosphate. On the addition of 1.5 volumes of ethanol, the crystallization of G-1-P begins. The yield will be about 21 g (80 per cent of the starting material).

The G-1-P obtained is then recrystallized. The product contains no, or considerably less, GDP than before treatment. The GDP contamination and its removal is checked by applying the preparation as substrate in the PGM reaction (Table 5).

Table 5

Substrate	G-6-P formed in 10 minutes, μmoles	
	–	+ GDP (2×10^{-6} M)
G-l-P, untreated	1.81	1.82
G-l-P, purified	0.24	1.84

Table 5 shows that the original G-1-P preparation contains sufficient amounts of GDP for the full activity of PGM (about 0.1 per cent). As a result of the above procedure, the GDP content of G-1-P preparation decreases to such an extent (to about 0.005 per cent), that PGM activity will only slightly be affected. The example shows that the method is suitable for the removal of GDP contaminations from G-1-P preparations.

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The Effect of Mg^{++}-Concentration and pH on the Activity of Phosphoglucomutase

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In order to study the mechanism of action of phosphoglucomutase, crystalline PGM* was prepared. GDP, the coenzyme of PGM was obtained from erythrocyte hemolysate in the form of Ba-salt. Its purification was carried out by adsorbing on G-1-P and crystallizing.

The pH-optimum of PGM is 7.5 only if the GDP concentration is less than 1 μM. In the presence of 1—2 μM GDP the enzyme activity shows a maximum (plateau) in the range from pH 5.5 to 8.5.

The enzyme activity can be increased at low pH-values not only by increasing the concentration of GDP, but also by that of the Mg^{++}-level. Excess of GDP counteracts the effect of Mg^{++} in the inhibitory concentration range.

Based on the above observations the role of the GDP-Mg-enzyme complex is stressed and a modified scheme of the mode of action of PGM is given.

Introduction

Phosphoglucomutase (D-glucose-1,6-diphosphate: D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) catalyses the G-1-P $\rightleftharpoons$ G-6-P conversion. Mg^{++} and GDP are needed for the enzyme activity. The optimal Mg^{++}-concentration for PGM activity in rat liver homogenate is 1 mM (Goodlad and Mills, 1957), for PGM extracted from yeast 1 – 3 mM (McCoy and Najjar, 1959) and for PGM from muscle 1 mM (Robinson and Najjar, 1961).

The determination of the optimal GDP concentration is difficult, as the G-1-P used as substrate generally contains GDP. Moreover, the optimal concentration of GDP also depends upon the concentration of the substrate used (Sutherland et al. 1949; Najjar and McCoy, 1958). For maximal activity approximately 2 μM GDP is necessary in the presence of 4 mM G-1-P; for 50% activity 0.5 μM GDP is needed (Sutherland et al. 1949).

The estimation of Mg^{++} and GDP concentration needed for maximal activity was carried out at the pH-optimum of PGM. The pH-optimum was 7.5 for the crystalline enzyme from muscle (Najjar, 1948), 7.2 for rat liver homogenate (Goodlad and Mills, 1957), and 7.5 for an enzyme preparation from yeast (McCoy and Najjar, 1959).

* *Abbreviations:* G-1-P = D-glucose-1-phosphate; G-6-P = D-glucose-6-phosphate; GDP = glucose-1.6-diphosphate; PGM = phosphoglucomutase

The activity of PGM decreases at lower pH values and at pH 6.0 the enzyme is almost entirely inactive. This decrease in activity is not the result of irreversible denaturation of the enzyme, because PGM is most stable at pH 5.0, and remains active after heat treatment at 65°C (Najjar, 1948).

The correlation between resistance against low pH-values and the simultaneous decrease in activity has not been studied as yet.

In this paper we present data to show that PGM is significantly, or even maximally active at low pH-values at suitable Mg^{++} and GDP concentrations. It should be noted that these concentrations differ considerably from those considered to be optimal so far. We have established that the pH-optimum of the enzyme changes with the Mg^{++} and GDP concentration.

The present-day ideas on the mechanism of action of PGM was developed by taking into account the correlation between the change of enzyme activity, the pH-value, as well as the Mg^{++} and GDP concentration.

Methods

Crystalline PGM was prepared from rabbit muscle by the method of Najjar (Najjar, 1948).

Preparation of GDP. Human erythrocytes were hemolysed with distilled water of same volume for 4 hours, then 50% TCA was added to a final concentration of 10% TCA in the solution. The precipitated protein was centrifuged and the supernatant was treated with Norit in order to remove the nucleotides. After filtration, the solution was adjusted to pH 8.0 with ammoniumhydroxyde diluted 1 : 1. The proteins were boiled for 2 minutes under continuous addition of $BaCl_2$ solution, until the precipitate aggregated.

After centrifugation the precipitate, which contained also GDP, was suspended in a small amount of water and treated with cation-exchange resin until the Ba^{++} ions were removed. The solution was adjusted to pH 7.6 with 1 N KOH. This solution was used as a crude GDP-preparation.

In order to achieve further purification GDP was crystallized by adsorption on G-1-P. 2 g crystalline G-1-P was added to the crude solution; after the G-1-P was dissolved it was crystallized by adding the same volume of alcohol. Crystals were filtrated and dried. G-1-P thus obtained contained significant amounts of GDP. The GDP content of G-1-P was not decreased by crystallization.

Assay of the activity of PGM. The crystalline enzyme was diluted with 0.1 M acetate buffer, pH 5, and freed of ammonium sulfate by passing through a Sephadex G-50 column previously equilibrated with 0.01 M acetate buffer, pH 5. The enzyme solution was eluted by the same buffer from the column (flow rate: 30 ml/h). The enzyme purified by the above method was stored in the frozen state.

The assay of PGM activity was made in an incubation mixture of the following composition: G-1-P 4×10^{-3} M; $MgCl_2$ 1.2×10^{-3} M; 8 hydroxyquinoline 2×10^{-3} M; Tris 4×10^{-2} M; PGM 0.1 – 0.3 unit/ml. Time of incubation was 10 minutes at pH 7.5 and 30°C.

The enzyme was diluted with 4×10^{-2} M Tris buffer, containing 1.2×10^{-3} $MgCl_2$.

The activity was expressed by the quantity of G-6-P formed in 5 minutes. The amount of G-6-P formed was determined by the method of Somogy and Nelson (Nelson, 1944). The unit of the enzyme activity is defined as the amount of enzyme which catalyzed the formation of 1 mg acid-stable P, i.e. 32 μmoles G-6-P in 5 minutes at 30°C (Najjar, 1948).

The enzymatically formed G-1-P contains GDP in sufficient amount for the PGM-reaction. However, in the investigation the effect of pH and Mg^{++}-concentration, GDP-free G-1-P preparation was used (Bot and Dósa, 1967) and GDP was added separately to the incubation medium. 1 and 0.1 μM GDP used in these experiments allowed 50 and 20 per cent, respectively, of the maximal activity of the mutase (Sutherland et al. 1949).

The determination of GDP was carried out by using Horecker's method, which consists in the determination of G-6-P with G-6-P dehydrogenase, after hydrolysis with hydrochloric acid for 10 minutes (Horecker et al. 1955).

Results

Effect of pH on the activity of PGM

In the first part of our experiments we studied the effect of pH on the activity of PGM at various GDP concentrations. The results can be seen in Fig. 1.

It can be seen from Fig. 1 that the activity of PGM decreases with the decrease of pH if the GDP concentration is less than 1 μM. By increasing the

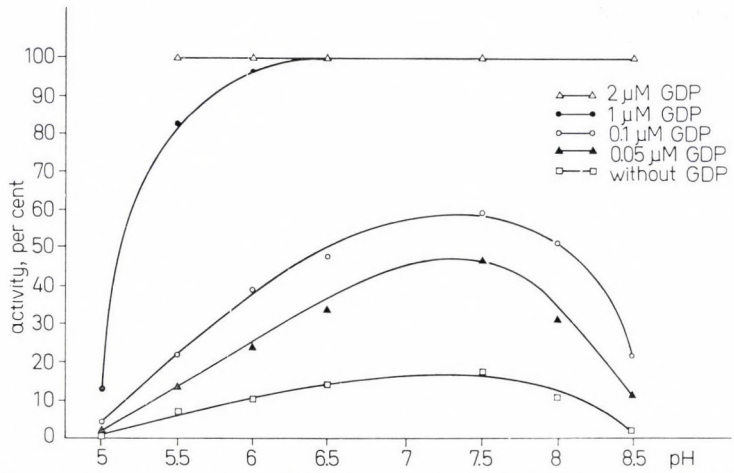

Fig. 1. Effect of pH on the activity of PGM in the presence of various GDP-concentrations. Composition of 1 ml incubation medium: G-1-P 4 μmoles; $Mg\ Cl_2$ 1.2 μmoles; 8-hydroxy-quinoline 2 μmoles; Tris 40 μmoles; GDP in the indicated concentrations; PGM 0.25 unit. Time of incubation: 5 minutes at 30°C. pH-adjustement with HCl and Tris-buffer. The determination of G-6-P formed was carried out on the basis of its reducing property. Activity is expressed in the per cent of the activity measured in the presence of 1 μM GDP at pH 7.5

concentration of GDP the effect of pH can be counteracted. In the presence of 1 μM GDP the activity of PGM slightly changes, with 2 μM GDP it does not change between pH 5.5 and 8.5. It is seen that in the presence of excess GDP, PGM has a maximal activity in a wide pH-range. To explain this phenomenon it can be assumed that in the presence of low concentrations of GDP, at low pH-values the dissociation of the enzyme-GDP complex (or possibly of the enzyme-Mg-GDP complex) is more intense and this leads to a decrease of activity. In the case of higher GDP concentrations, the dissociation of the enzyme-GDP complex does not occur even at low pH-values.

Effect of Mg^{++}-concentration and pH on the activity of PGM

The effect of various Mg^{++}-concentrations and various pH-values was studied. The Mg^{++}-concentration was varied between 0.4 and 12 mM at different pH-values. The GDP-concentration was 0.1 μM. The results are presented in Fig. 2.

It is seen from Fig. 2 that the pH-optimum of PGM is 7.5 at a Mg^{++}-concentration of 1.2 mM. At this pH-value both higher and lower Mg^{++}-concentrations than 1.2 mM result in a decrease of activity. At higher pH-values, the

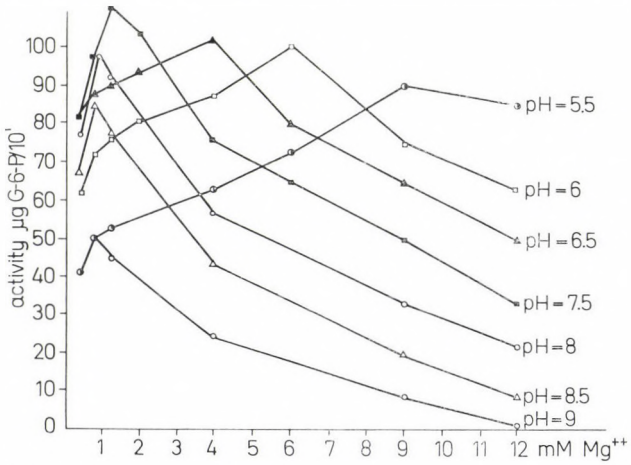

Fig. 2. Effect of Mg^{++}-concentration and pH upon the activity of PGM

maximal activity can be observed at lower Mg^{++}-concentration (0.8 mM), higher Mg^{++}-concentrations inhibit the activity. When the pH-value of the medium is higher, the inhibition caused by the excess of Mg^{++} is greater. On the other hand, under pH 7.5 PGM exhibits maximal activity only at higher Mg^{++}-concentration. Thus at pH 6.5 4 mM, at pH 6.0 6 mM and, at pH 5.5 9 mM Mg^{++} is needed for maximal activity. Therefore, at a given pH-value the activity depends upon the Mg^{++}-concentration. On the other hand, at various Mg^{++}-concentrations the pH-optima of PGM are different. Thus the pH is the function of Mg^{++}-concentration.

Our further experiments were carried out in order to show the correlation between the effect of Mg^{++} upon the activity of PGM and the concentration of GDP. In our previous experiments the concentration of GDP was 0.1 μM. In Table 1 the activity of PGM is presented at a 10 times higher GDP-concentration (1 μM) in the presence of various Mg^{++}-contents and pH-values.

Table 1

Effect of excess GDP on the activity of PGM at various Mg^{++}-concentrations and pH-values

The composition of incubation mixture see in the text of Fig. 1.
Mg^{++} 0.4—12 mM, GDP 1 μM.

Mg^{++}	5.5	6.0	6.5	pH 7.5	8.0	8.5	9.0
	G-6-P μg formed in 10 minutes						
0.4 mM	184	184	190	184	184	182	126
0.8 mM	184	184	190	184	184	184	126
1.2 mM	184	184	190	186	184	184	126
2.0 mM	—	186	190	188	—	—	—
4.0 mM	184	184	190	188	184	184	96
6.0 mM	184	184	190	186	190	188	—
9.0 mM	184	188	190	186	184	184	61
12.0 mM	180	188	190	186	184	184	44

It can be seen from Table 1 that the activity of PGM is maximal at all pH-values studied in the presence of 1.2 mM Mg^{++}-concentration, if GDP is used in an excess concentration (1 μM). It can be seen, too, that GDP used in such a high concentration counteracts the inhibitory effect of the Mg^{++}-excess. Due to the excess of GDP the activity of PGM remains maximal at lower pH-values, even in the absence of Mg^{++}-excess. The activity is stable between pH 8.5 and 9.0, too, if GDP is in an excess concentration.

By comparing the data of Table 1 with the highest values of Fig. 2 (values measured at pH 7.5 and 1.2 mM Mg^{++}), it can be seen that almost the double amount of G-6-P was formed. Consequently, in the presence of 1 μM GDP PGM is twice as active as in the presence of 0.1 μM GDP.

Discussion

According to the literature pH 7.5 is the pH-optimum of PGM and 1.2 mM the optimal Mg^{++}-concentration. Generally, in earlier studies the G-1-P preparation used as substrate contained GDP. This resulted in a variable concentration of GDP in the incubation medium. It seems probable that the amount of GDP present in the reaction mixture was not sufficient for the maximal activity of PGM neither in acidic nor in alcaline media.

In our experiments G-1-P was considerably freed of GDP contamination. Thus a more accurate dosage of GDP became possible and we were able to determine the pH-optimum at various GDP and Mg^{++} concentrations. It was

found that the pH-optimum 7.5 is valid only at low GDP and Mg^{++}- concentrations. In the presence of $1-2\ \mu M$ GDP the activity of PGM is maximal between pH 5.5 and 8.5. The optimal Mg^{++}-concentration also depends on the GDP-concentration.

It was found that 1.2 mM Mg^{++} is optimal *only* at pH 7.5. At lower pH higher Mg^{++}-concentration is needed for maximal activity and, lower Mg^{++}-concentration is necessary at higher pH-values. However, the effect of Mg^{++}-concentration on the enzyme activity is evident only at low GDP-concentration. Therefore, in the presence of 1 μM GDP the Mg^{++}-concentration can be varied in a wide range without influence on the activity of PGM.

On the basis of the observation that at lower pH-values more GDP or Mg^{++} is necessary for the maximal activity, we conclude that at low pH-values the GDP or Mg^{++} dissociates easily from the enzyme complex. Thus, the scheme introduced by Najjar and Pullman (1954) can be modified. The following equation describes the mechanism of PGM-action better:

$$\text{G-1-P} + \text{P-enzyme} \rightleftharpoons \text{GDP} + \text{enzyme} \rightleftharpoons \text{G-6-P} + \text{P-enzyme}$$

(Najjar, Pullman)

$$\begin{array}{ccccc} \text{G-1-P} + \text{Mg-P-enzyme} & \rightleftharpoons & \text{GDP-Mg-enzyme} & \rightleftharpoons & \text{G-6-P} + \text{Mg-P-enzyme} \\ & & \uparrow\downarrow & & \\ & & \text{GDP} + \text{Mg} + \text{enzyme} & & \end{array}$$

According to this scheme during the transformation of G-1-P into G-6-P free GDP is not formed as an intermediate. However, GDP may be split off in a side reaction due to the dissociation of the P-enzyme-Mg-substrate complex. This dissociation is more pronounced at low pH-values than at higher ones. Consequently, a dephospho-enzyme is formed and the velocity of the main reaction decreases. By increasing the GDP or Mg^{++} concentration the dissociation can be suppressed and the velocity of the reaction can be re-established. For this reason the GDP and Mg^{++}- concentration, necessary for the maximal activity, depends on the extent of the dissociation of the P-enzyme-Mg-substrate complex. At lower pH-values higher GDP or Mg^{++}-concentration is needed; at pH 7.5 lower concentrations are necessary.

Our results are in agreement with the findings of Ray and Roscelli (1964) as well as with those of Gounaris et al. (1967) and confirm the idea that GDP and the dephospho-enzyme are "abortive" products of the P-enzyme-substrate complex and are not necessarily intermediates. Moreover, our results point to the fact that the extent of this "abortive" process depends upon the pH and the Mg^{++}- concentration in addition to GDP- concentration. The P-enzyme-substrate may be practically rather P-enzyme-Mg-substrate complex and its "abortive" dissociation involves not only the splitting off of dephospho-enzyme and GDP, but of Mg^{++}, too.

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Role of Acetohydroxy Acid Synthetase in the Regulation of Valine and Isoleucine Biosynthesis in Pseudomonas aeruginosa

II. Studies on Enzyme Kinetics

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Increasing the substrate concentration the pH-optimum of acetohydroxy acid synthetase* is shifted towards acidic pH-values. At pH 7.6 there is a break in the Arrhenius plot of enzyme activity in the range of 20—25°C. Valine inhibition of AAS changes parallel with the irregularities of the temperature dependence of enzyme activity. Substrate saturation shows normal first order kinetics. The values calculated for K_m and V_{max} considerably differ at different temperatures and pH values.

At lower valine concentrations valine inhibits the activity of AAS according to an apparent uncompetitive type of kinetics. Higher valine concentrations result in a nonlinear function in the reciproc plot. There is a substrate concentration-optimum for valine inhibition, which depends on the pH. Increasing the pH results in an exponential decrease of valine sensitivity. At higher pyruvate concentrations there is a substrate inhibition which depends on the pH and enzyme concentration. At inhibitory substrate concentrations, valine added in low concentrations seems to have a stimulatory effect on AAS. This depends on the pH and enzyme concentration.

Valine inhibition is highly specific for L-valine.

Introduction

The behaviour of AAS on gel filtration and studies of enzyme inactivation revealed two forms of AAS obtained from *Pseudomonas aeruginosa* (Varga and Horváth, 1965, 1967 a, b). An equilibrium of a valine sensitive and insensitive form of AAS may play an essential role in the physiological function of this regulatory enzyme (Varga, Horváth, 1967c).

In this paper some results of the kinetic studies carried out on AAS of *Ps. aeruginosa* will be reported.

Materials and methods

Cultivation of *Ps. aeruginosa*, preparation of the crude extract and of the ammonium sulphate precipitate as well as the assay of AAS enzyme activity were described earlier (Horváth et al., 1964, Varga, Horváth, 1967a). Throughout

* *Abbreviations:* AAS = acetohydroxy acid synthetase; TPP = thiamine pyrophosphate; FAD = flavine adenine dinucleotide; GHS = reduced glutathione; $(VAL)_{50}$ = valine concentration necessary for 50 per cent inhibition; V = reaction velocity is expressed as micromoles of acetolactate formed in 1 hour.

the experiments the ammonium sulphate precipitate was used after treatment with Sephadex G 25. The enzyme reactions, if not stated otherwise were carried out with pyruvate as substrate (50 mM), at pH 7.6, at 20°C, in the presence of 1 mg/ml protein. Reaction velocity is expressed either directly as the absorbancy (E_{520}) obtained in the acetoin determination according to Westerfeld (1945) or as micromoles of acetolactate formed in one hour (V). Where indicated, the reaction mixture contained a mixture of potassium phosphate (0.05 M) and TRIS-HCl (0.1 M) (PT-buffer), instead of potassium phosphate.

The chemicals used were commercial products of analytical grade. α-Acetolactate and α-aceto-β-hydroxybutyrate were synthetized according to the method of Krampitz (1948), α,β-dihydroxyisovalerate and α,β-dihydroxy-β-methylvalerate according to Sjolander et al. (1954). Thanks are due to Dr. Sándor Bajusz for the derivatives of L-valine and L-isoleucine.

Results

Time course of the reaction and the dependence of reaction velocity on enzyme concentration

In the presence of fresh enzyme, acetolactate formed in the reaction was proportional to the time and to the enzyme concentration (Fig. 1 and Fig 4). With an old enzyme preparation stored for a day or longer irregularities could be demonstrated in the time course and in the dependence of the reaction velocity on enzyme concentration. In a few experiments a negative dilution effect similar to that of *Escherichia coli* K 12 (Umbarger, Brown, 1958) appeared, but an opposite dilution effect was more frequent when enzyme activity increased with dilution

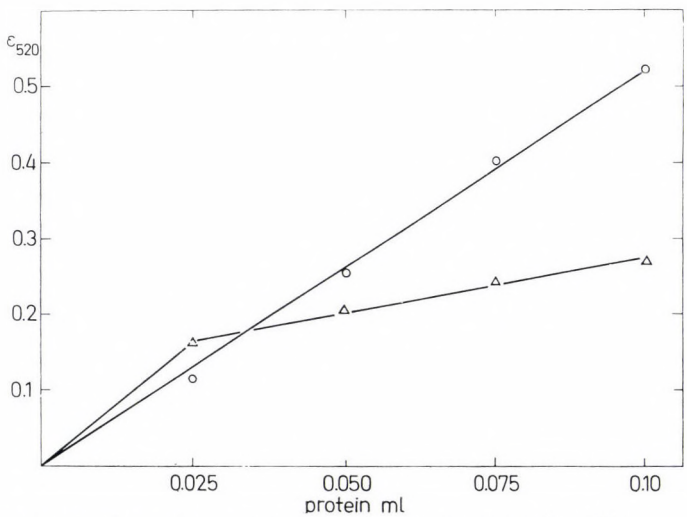

Fig. 1. Dependence of reaction velocity on enzyme concentration. The experiment was carried out ○—○, with fresh enzyme; Δ—Δ, with an ammonium sulphate precipitate stored for three days at 0°C

(Fig. 1). The slope of the curves differed strongly under different conditions e.g. upon changing the substrate concentration or the pH, etc., but these alterations were not reproducible. Consequently we used fresh enzyme for the following experiments.

Dependence of pH optimum on substrate concentrations

One of the common features of AAS obtained from different microorganisms is its pH optimum of about pH 8.0. This was also found valid for the AAS of *Ps.*

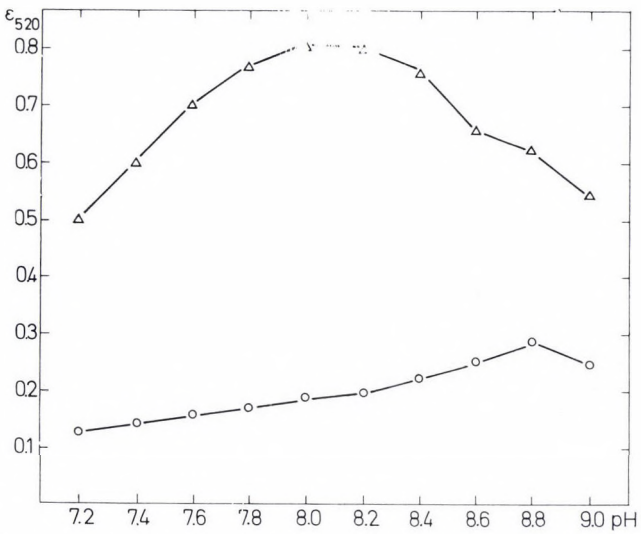

Fig. 2. Dependence of pH optimum on substrate concentration. PT-buffer was used. Pyruvate concentrations: ○—○, 2 mM; △—△ 20 mM

aeruginosa (Varga, Horváth, 1967a), but lowering the substrate concentration the pH optimum increased and in the presence of 2 mM pyruvate it was about 8.8–9.0 (Fig. 2).

Effect of temperature on reaction velocity and valine sensitivity

At pH 7.6 there was a break in the Arrhenius plot of enzyme activity around 20–25°C. Above pH 8.0 this break could not be demonstrated (Fig. 3). Valine inhibition of AAS changed parallel with the irregularities in the temperature-dependence of enzyme activity. Namely, an increase in valine sensitivity was found to occur concomitantly with the irregular decrease of enzyme activity below 25°C.

At 37° C in the presence of pyruvate (Fig. 4) the diluted enzyme was reversibly desensitized. At 20°C or using the enzyme in higher concentrations this phenomenon could not be demonstrated.

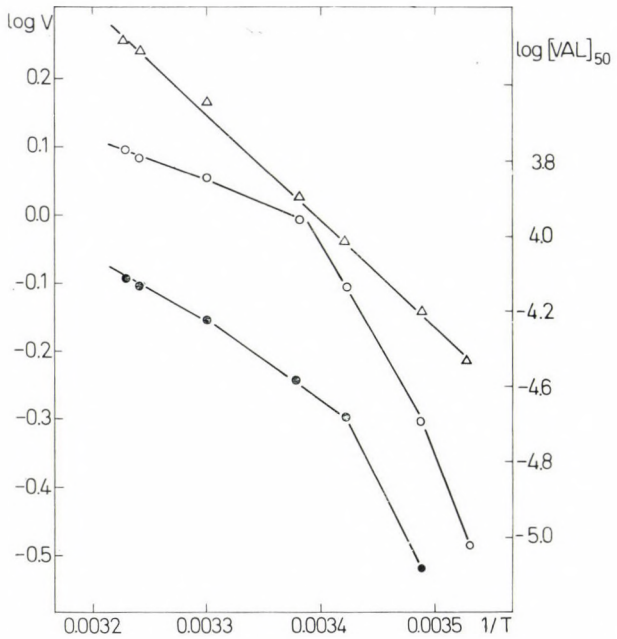

Fig. 3. Effect of temperature on enzyme activity and valine inhibition. For the determination of $(VAL)_{50}$ the reaction velocity was measured in the presence of different L-valine concentrations: 0—5—10—20—40—60—80—100 μM and from the curves obtained the valine concentration necessary for a 50 per cent inhibition was determined. ○—○, log V determined at pH 7.6; Δ—Δ, Log V determined at pH 8.2; ●—●, log $(VAL)_{50}$ determined at pH 7.6

Table 1

The apparent K_m and V_{max} values determined at different temperature and pH values

pH		Temperature °C			
		15	20	25	30
7.4	K_m mM	10.0	10.0	12.5	14.3
	V_{max}	0.37	0.55	0.99	1.25
7.8	K_m mM	8.60	8.80	11.1	13.3
	V_{max}	0.38	0 59	1.0	1.43
8.2	K_m mM	7.15	8.00	10.0	12.5
	V_{max}	0.40	0.66	1.02	1.66

PT-buffer was used

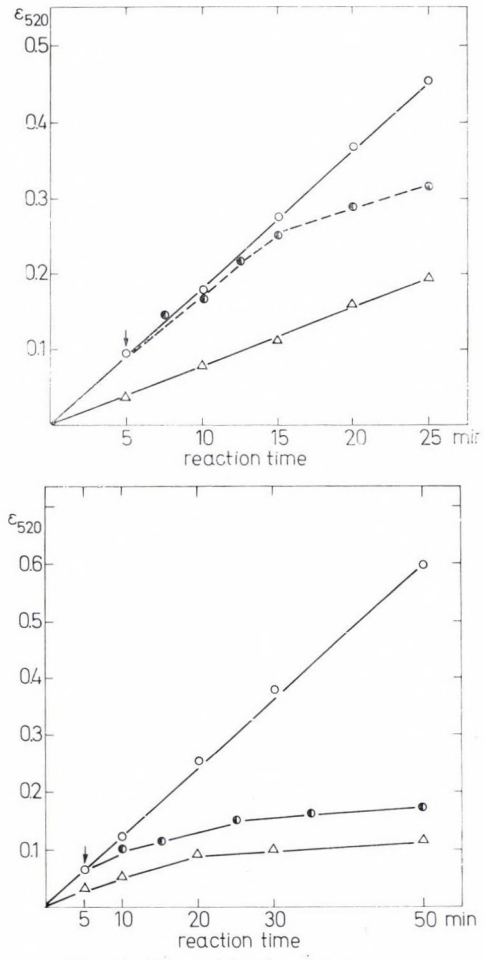

Fig. 4. Desensitization by pyruvate

Fig. 4A. The time course of the reaction was investigated. ○—○, without valine; △—△, in the presence of 50 μM L-valine, added before starting the reaction with the substrate; ◐—◐, 50 μM L-valine added 5 minutes after the reaction started

Fig. 4B. The time course of the reaction was investigated. ○—○, without valine; △—△, in the presence of 10 μM L-valine, added at zero time; ◐—◐, 10 μM L-valine added 5 minutes after the reaction started. Enzyme concentration: 0.1 mg/ml

Effect of substrate concentration on AAS activity

Substrate saturation of a number of regulatory enzymes is known not to follow the first order kinetics of Michaelis-Menten (Monod et al., 1965). In the case of AAS obtained from *Ps. aeruginosa* the velocity functions of the substrate concentration investigated at different pH values and different temperatures gave rectangular hyperbolic curves.

Table 1 demonstrated the apparent K_m and V_{max} values obtained at different pH values and at different temperatures. Increasing the pH resulted in a moderate

decrease of K_m and V_{max}. Rising the temperature from 15°C to 30°C resulted in about a 50 per cent increase in K_m and a 3–4 fold increase in V_{max} (V_{max} calculated differed from the actual values because of the substrate inhibition).

End product inhibition by valine

Different types of inhibition kinetics have been found for valine inhibition of AAS obtained from various microorganisms. In *E. coli* an apparent competitive

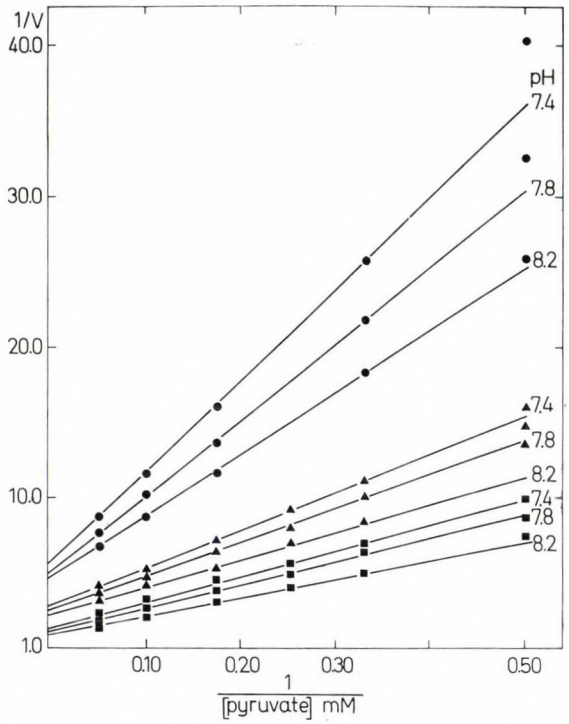

Fig. 5. Effect of substrate concentration on reaction velocity. Reaction velocity was determined in the presence of substrate concentrations and pH values indicated in the figure. PT-buffer was used. Conditions: ●—●, temperature 15°C, time of the reaction 30 min. ▲—▲, temperature 25°C, time of the reaction 10 min., ■—■, temperature 37°C, time of the reaction 5 min.

inhibition (Umbarger, Brown, 1958), in *Salmonella typhimurium* non-competitive inhibition (Bauerle et al., 1964) could be demonstrated. In the case of *Ps. aeruginosa*, valine in lower concentrations inhibited the activity of AAS according to an apparent uncompetitive type of kinetics, suggesting different substrate and inhibitor-binding sites. At higher valine concentrations (but lower than that of inhibitor-saturation) nonlinear functions could be demonstrated in the reciprocal plot (Fig. 6).

Allosteric inhibitions frequently result in a nonlinear function of the Lineweaver-Burk plot, but deviation from normal kinetics is usually opposite to that

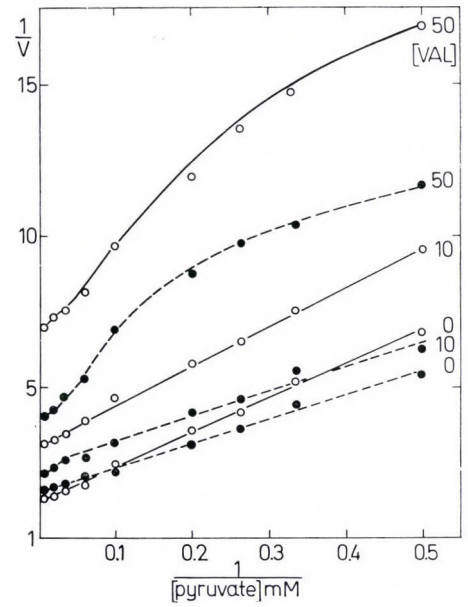

Fig. 6. Kinetics of valine inhibition. Reaction velocity was measured in the presence of pyruvate, concentrations indicated, PT-buffer, ○—○, at pH 7.4, ●—●, at pH 8.2

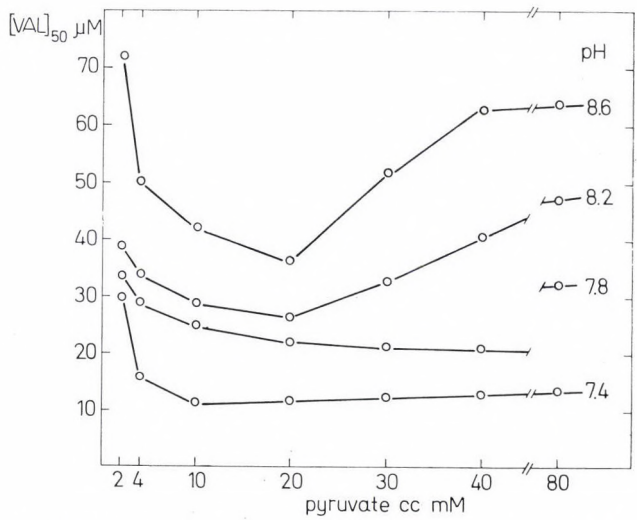

Fig. 7. Influence of pyruvate concentrations on valine inhibition. Determination of $(VAL)_{50}$ is is described in Fig. 3. pH is indicated in the figure, PT-buffer was used

observed with the AAS of *Ps. aeruginosa*. In the other cases the degree of inhibition generally increases with decreasing substrate concentrations. This can be explained by supposing an increased cooperativity of substrate saturation in the presence of the allosteric inhibitor (Monod et al., 1965). A detailed investigation of this

phenomenon is presented in Fig. 7. Valine concentration necessary for a 50 per cent inhibition temporarily decreased with increasing substrate concentrations, but above an optimal substrate concentration $(VAL)_{50}$ increased again. The substrate optimum for valine inhibition depended on the pH: there was no decrease in valine sensitivity with increasing substrate concentrations when investigated below pH 7.5, but above pH 8.0 there was.

The pH-functions of valine inhibition depended on the substrate concentration (Fig. 8). At lower substrate concentrations valine sensitivity was con-

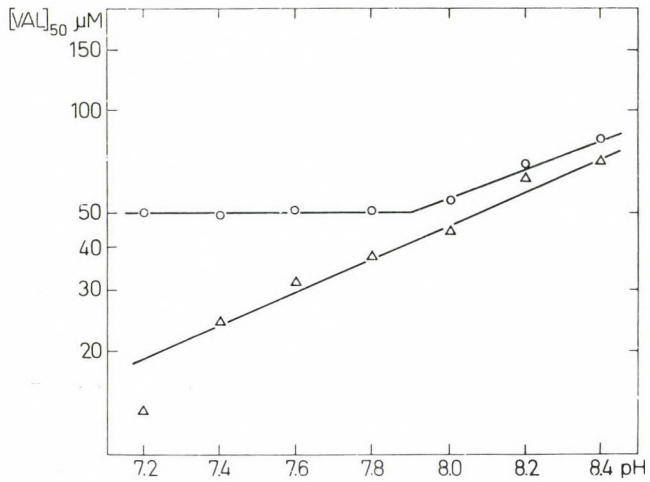

Fig. 8. Influence of the pH on valine inhibition. Determination of $(VAL)_{50}$ is described in Fig. 3., pH values are indicated in the figure. PT-buffer was used. Pyruvate concentrations: ○—○, 2 mM; △—△, 20 mM

stant between pH 7.2 and 7.8, above pH 8.0 it decreased. In the presence of 20 mM pyruvate, valine sensitivity exponentially decreased with increasing pH in the whole range of the pH studied. pH-functions of enzyme inhibition can be interpreted on the basis of the ionization of the nonenzyme components of the system and an alteration of protein structure (Webb, 1963).

From the foregoing it is clear that the inhibitor, the substrate-concentration and the pH exert an elaborate effect on the reaction velocity catalysed by AAS. An investigation of substrate saturation at different pH values and inhibitor concentrations revealed that at lower pH values the substrate saturation curves obtained in the presence of L-valine ran nearly parallel to the control curve without valine (Fig. 9a). Higher concentrations of pyruvate resulted in substrate inhibition which was more pronounced at higher pH values and appeared at lower pyruvate concentrations (Fig. 9b). Valine added in lower concentrations seems to compensate substrate inhibition. Thus, at pyruvate concentrations higher than 100 mM, L-valine added in a concentration of 1 μM actually increased the reaction velocity. According to the saturation curves valine-stimulation can be effective at higher valine concentrations, too, and this may contribute to the substrate optimum of valine sensitivity (Fig. 7). Stimulation by valine under the circumstances of

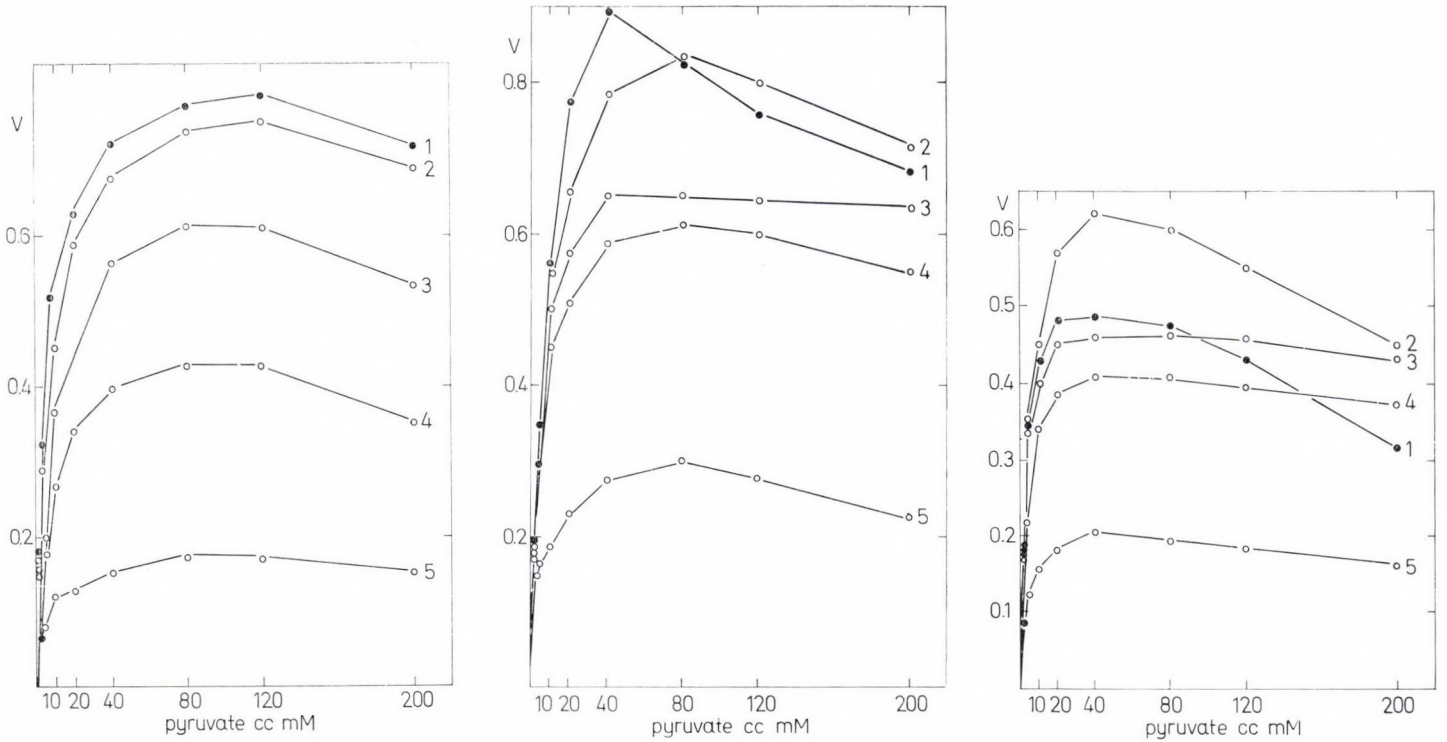

Fig. 9. Substrate saturation in the presence of different valine concentrations. The reaction was carried out in the presence of PT-buffer. Pyruvate and valine concentration are indicated in the figure

Fig. 9A. pH 7.4 total volume of the reaction mixture 2 ml

Fig. 9B. pH 7.8 total volume of the reaction mixture 2 ml

Fig. 9C. pH 7.8, total volume of the reaction mixture, 8 ml

The amount of protein was 0.3 mg in all experiments. Valine concentrations: 1. 0,0 μM; 2. 1.0 μM; 3. 4.0 μM; 4. 10.0 μM; 5. 50.0 μM

Table 2

Effect of amino acids, amino acid derivatives and other compounds on enzyme activity

Addition				Relative activity
none				100
L-valine	2 μM			62
D-valine	1 mM			100
L-isoleucine	2 μM			113
L-isoleucine	20 μM			100
L-isoleucine	200 μM			24
L-leucine	2 μM			100
L-leucine	20 μM			100
L-leucine	200 μM			75
L-valine	2 μM + L-isoleucine	2 μM + L-leucine	2 μM	70
L-valine	2 μM + L-isoleucine	200 μM + L-leucine	200 μM	24
DL-norvaline	1 mM			100
DL-norleucine	1 mM			100
L-glutamic acid	1 mM			59
L-phenylalanine	1 mM			63
L-proline	1 mM			70
L-serine	1 mM			72
L-tyrosine	1 mM			74
L-threonine	0.2 mM			50
L-cysteine	1 mM			87
L-methionine	1 mM			82
L-alanine	1 mM			44
L-valine methylester	20 μM			100
L-valine methylester	200 μM			45
L-valineamide	200 μM			100
N-acetyl-L-valine	200 μM			100
N-formyl-L-isoleucine	200 μM			110
α-ketobutyric acid		2 mM		66
α-ketobutyric acid		20 mM		45
α-ketoisovaleric acid		5 mM		85
α-acetolactic acid		1 mM		100
α-aceto-2-oxybutyric acid		1 mM		100
α-, β-dioxyisovaleric acid		10 mM		100
α, β-dioxy-β-methylvaleric acid		10 mM		100
Thioethanol		0,32 mM		77
Thioethanol 0.32 mM + L-valine		2 μM		50
GSH		0.33 mM		83
GSH		1 mM		36
GSH	0.33 mM + L-valine	2 μM		55
GSH	1 mM + L-valine	2 mM		23
Hg^{++}		3 μM		111
Hg^{++}		12 μM		45
Hg^{++}	3 μM + L-valine	2 μM		70
Hg^{++}	12 μM + L-valine	2 μM		35

Determination of the rate of reaction velocity according to the method described in "Materials and methods" in the presence of the additions indicated in the Table.

substrate inhibition was more pronounced when the enzyme diluted in the reaction mixture (Fig. 9c). In this case valine increased the reaction velocity even at lower substrate concentrations.

Specificity of valine inhibition

The inhibition was definitely specific for L-valine (Table 2). D-valine had no effect. L-isoleucine and L-leucine inhibited AAS in concentrations two orders of magnitude higher than the inhibitory concentration of L-valine. For binding the enzyme, L-valine needs both its dissociable carboxyl and amino groups. Substitutions of these groups of L-valine decreased the inhibition to a great extent.

α-Ketobutyric acid had a significant inhibitory effect on AAS. We suppose it acts as structural analogue of pyruvate in the formation of hydroxy-ethyl-TPP. In a separate paper we report a detailed study of ketobutyrate inhibition and its physiological significance (Varga, Horváth, 1967d). α-Ketoisovaleric acid also had an inhibitory effect. This keto acid is an analogue of pyruvate, therefore inhibition based on structural similarity may be expected. The reaction products (α-acetolactate and α-aceto-β-hydroxybutyrate) had no effect on enzyme activity. Accordingly, there is no product inhibition and other intermediates of the pathway of valine-isoleucine biosynthesis do not act as modifiers of AAS. In lower concentrations, Hg^{++} had a stimulatory effect and at the same time decreased valine inhibition. At higher concentrations (10^{-5} M or higher) it had a strong inhibitory effect. Desensitization could not be achieved by mercuric ions. The enzyme was also sensitive to thiol-compounds.

Discussion

Irregularities in the pH and temperature-dependence of enzyme activity and valine inhibition, and a rather complicated effect of substrate and feedback inhibitor; these are the most striking characteristics of acetohydroxy acid synthetase revealed by kinetic studies. Studies on regulatory enzymes carried out since the presentation of the initiating works in this field (Umbarger, Brown, 1958; Yates, Pardee, 1956) led to the construction of different enzyme models which were able to describe some peculiarities of regulatory enzymes (Frieden, 1964; Monod et al., 1965; Atkinson et al., 1965; Sanwal et al., 1965; Koshland et al., 1966). These different models are not mutually exclusive and detailed physiological studies carried out on purified enzymes are necessary to confirm any of the different hypotheses.

Attempts to purify AAS from *Ps. aeruginosa* to a reasonable degree have failed but there are some results with gel filtration. These suggest that two equilibrium forms of acetohydroxy acid synthetase should be taken into account (Varga, Horváth, 1965, 1967b). One of them is a valine sensitive enzyme. It has a molecular weight high enough to be present in the void volume of Sephadex G200, it has a pH optimum of about 7.7, it is not inhibited by its substrate even in high concentrations, there is a break in the Arrhenius plot of temperature-

dependence. It is sensitive to repeated freezing and thawing resulting in a decrease of valine sensitivity, and a disappearance of the break in the Arrhenius plot, appearance of a pronounced substrate inhibition, a shift of the pH optimum beyond 8.0 and a disruption of the original valine sensitive enzyme molecule making able the enzyme to penetrate into the Sephadex G 200. Storage of the treated enzyme results in the partial restoration of the characteristics of valine sensitive enzyme. Gel filtration of the ammonium sulphate precipitate on Sephadex G 200 in the presence of valine led to the disappearance of the smoller valine insensitive enzyme form, while the presence of pyruvate caused a pronounced inactivation of the enzyme. The presence of the two enzyme forms is also supported by inactivation studies: in the course of heat inactivation valine sensitivity strongly increases.

We suppose that the existence and the equilibrium of the two enzyme forms may contribute to the kinetic characteristics of AAS and that different factors may effect the equilibrium:

$$\text{valine sensitive enzyme} \underset{\text{valine lower temperature}}{\overset{\text{dilution higher pH pyruvate}}{\rightleftharpoons}} \text{valine insensitive enzyme}$$

The phenomenon of the stimulatory effect of a feedback inhibitor has been found with other enzymes, e.g. threonine deaminase obtained from mutant strains of *E. coli* (Sanchez, Changeux, 1966), and it can be explained on the basis of an allosteric enzyme model proposed by Monod, Wyman, Changeux (1965) it extended to nonexclusive ligand-binding (Rubin, Changeux, 1966). In the case of AAS from *Ps. aeruginosa* there may be another explanation, namely: pyruvate shifts the enzyme-equilibrium towards the labile valine insensitive form resulting in an apparent substrate inhibition. Valine is supposed to counteract this effect by shifting the equilibrium in the opposite direction resulting in the stimulation of enzyme activity.

We suggest that these elaborate effects of substrate and feedback inhibitors on enzyme activity have a regulatory significance. This is supported by the results of cultivation studies carried out with *Ps. aeruginosa* (Varga, Horváth, 1967b).

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The role of acetohydroxy Acid Synthetase in the Regulation of Valine and isoleucine biosynthesis in Pseudomonas aeruginosa

III. Behaviour of the Enzyme on Gel-filtration and Freezing

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Acetohydroxy acid synthetase from *Pseudomonas aeruginosa* showed two activity maxima on gel-filtration on a P 300 polyacrylate column at pH 8.2. Significant differences were found between the two fractions as to their valine-sensitivity and activity measured at different pH values. If the gel-filtration was carried out at pH 7.4, the fractions did not separate. In the presence of L-valine only the higher molecular weight enzyme could be detected. In the presence of pyruvate the major part of the activity was lost.

On repeated freezing and thawing of the enzyme obtained by gel-filtration in the presence of valine, the susceptibility of the enzyme to inhibition by valine decreased, the pH-optimum increased, and substrate-inhibition appeared already at low pyruvate c n entration. On repeated gel-filtration the untreated enzyme emerged in the exclusion volume. After repeated freezing the enzyme emerged from the column with significant delay. The characteristics of the enzyme stored after freezing changed in the opposite direction.

The behaviour of the enzyme suggests the existence of an equilibrium between a valine-sensitive and a valine-insensitive form.

Introduction

It has been shown for several regulatory enzymes that the active enzyme molecule consists of identical or different subunits, and it has been assumed that the regulatory properties of the enzyme are determined by the association and dissociation (indirect interactions) of these subunits (Monod et al., 1965). With other regulatory enzymes the effect of the effectors and modifiers of the enzyme on the reaction rate could be interpreted solely in terms of (direct) interactions between binding sites (Atkinson et al., 1965). Since it has been shown that the complicated kinetics characteristic of regulatory enzymes can be explained by various enzyme models (Koshland et al., 1966), final conclusions as to a model or type of mechanism cannot be drawn from kinetic data. Therefore it seems to be important to carry out studies of non-kinetic nature, when a distinction between alternative reaction mechanisms is to be made.

Abbreviations: AAS = acetohydroxy acid synthetase; FAD = flavine adenine dinucleotide; TPP = thiamine pyrophosphate; V = reaction rate: μmoles o acetolactic acid formed per hour; specific activity = V/mg protein; ε_{520} = the extinction value at 520 mμ of the color-reaction according to Westerfeld (1945) of acetoin formed from the reaction product (α-acetolactic acid).

The inactivation and kinetic behaviour of acetohydroxy acid synthetase (AAS) from *Pseudomonas aeruginosa* led us to assume the existence of two enzyme forms (Varga and Horváth, 1967a,b). The present paper deals with experiments on gel-filtration and freezing of AAS from *Pseudomonas aeruginosa*, and with the properties of the enzyme obtained by gel-filtration. A preliminary report on this subject has already been published (Varga and Horváth, 1965).

Methods

The microorganism was grown and the activity of AAS was assayed as described in a previous paper (Varga and Horváth, 1967a). Gel-filtration was carried out as described earlier (Varga and Horváth, 1965) with the modification that the buffer-cofactor mixture used for gel-filtration contained also 2 μg FAD per ml. In some experiments, as indicated, Biogel P 300 polyacrylate gel was used instead of Sephadex G 200. All chemicals were commercial preparations of reagent grade. Protein concentration was determined according to the method of Lowry et al. (1951).

Results

Effect of pH on gel-filtration

The flow rate was found to vary considerably with the pH when gel-filtration was carried out on Sephadex G 200 columns. Therefore, the comparative experiments shown in Fig. 1 were performed on a Biogel P 300 column, the resistance of which changed only to a negligible extent with pH. At pH 8.2 upon gel-filtration on a Biogel P 300 column, AAS resolved into two peaks, similarly to the gel-filtration pattern obtained on Sephadex G 200 (Varga and Horváth, 1965). There were marked differences between the two fractions in their susceptibility to inhibition by valine, which disappeared during storage (Fig. 1, Table I). As to the enzymic activities of the fractions at pH 7.4 and 8.2, fraction A exhibited no significant differences in activity at the two pH values, while the activity of fraction B at pH 8.2 was about twice of that observed at pH 7.4. These results confirm our assumption made on the basis of kinetic experiments that the pH optimum of the valine-insensitive enzyme (E_B) is higher than that of the valine-sensitive enzyme (E_A). When the gel-filtration was carried out at pH 7.4, the separation of the two enzyme forms was not so clear-cut, but the enzyme fractions obtained in the first and final stages of gel-filtration showed obviously the characteristic of enzymes E_A and E_B, respectively, as shown by the pH-dependence of enzymic activity and susceptibility to inhibition by valine (Table I).

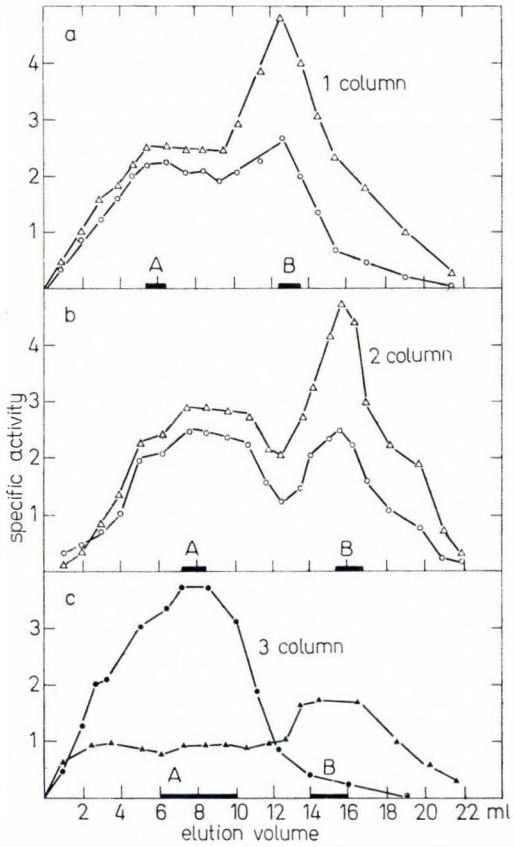

Fig. 1. Effect of pH, valine, and pyruvate on gel-filtration. Four columns (1.4×40 cm) were prepared from Biogel P 300. Column 1 was equilibrated with buffer-cofactor solution, pH 7.4, (0.2 M Tris. HCl; 0.1 M potassium phosphate; 100 μg/ml of TPP; 2 μg/ml of FAD). Columns 2, 3, and 4 were equilibrated with buffer-cofactor solution, pH 8.2. Column 3 contained 10 μM L-valine, in addition to buffer and cofactors, while column 4 contained 100 mM sodium pyruvate. The columns were put into a jacket containing melting ice. 10 l of a 2-hour-old culture of P. aeruginosa was centrifuged, the cells were washed and sonically disintegrated. The crude extract was precipitated by ammonium sulphate as described in a previous paper (Varga and Horváth, 1965). The ammonium sulphate precipitate was divided into four equal portions, and dissolved in the solutions used for the equilibration of columns 1, 2, 3, and 4, respectively, then after gel-filtration on Sephadex G 25 columns equilibrated with the same solutions, the protein solutions obtained were applied to the Biogel P 300 columns 1, 2, 3, and 4, respectively. The enzymic activity of fractions was measured without delay. Symbols: part A and B; ○—○ Enzymic activity measured at pH 7.4; △—△ Enzymic activity measured at pH 8.2; part C: Enzymic activity measured at pH 8.2; ●—● with fractions obtained by gel-filtration in the presence of L-valine; ▲—▲ with fractions obtained by gel-filtration in the presence of pyruvate. The 0 point of the abscissa means the 0 point of the exclusion volume (V_0) of columns, as determined by the aid of blue dextrane

Table I

Inhibition by valine of fractions A and B: effect of storage on inhibition

	Inhibition in the presence of 10 μM valine			
	fraction A		fraction B	
	measured			
	immediately	after 20 hrs	immediately	after 20 hrs
Gel-filtered at pH 7.4	74	31	18	30
Gel-filtered at pH 8.2	63	25	20	29
Gel-filtered at pH 8.2 in the presence of L-valine	75	78	—*	—*
Gel-filtered at pH 8.2 in the presence of pyruvate	67	30	19	39

The inhibition by valine of fractions A and B obtained by gel-filtration as described in Fig. 1 was determined before and after storage.

* Not evaluable.

Effect of pyruvate and valine on gel-filtration

When gel-filtration was carried out at pH 8.2 in the presence of L-valine, peak B disappeared. The valine-sensitivity of fraction A was similar to that observed normally, but the valine sensitivity failed to decrease in the way characteristic of enzyme A during storage. This apparent change in the property of the enzyme can be explained by the fact that during storage the valine, in concentrations used for elution, was present, and prevented desensitization, i.e. stabilized form A. The disappearance of fraction B can be explained by a shift of the $E_A \rightleftharpoons E_B$ equilibrium to the left. In case of gel-filtrations carried out in the presence of pyruvate fractions did not separate readily, and about 50 per cent of enzymic activity was lost as compared to the control. From the data shown in Fig. 1 we may assume that AAS from *Ps. aeruginosa* has at least two, but possibly more forms of different molecular weights, which differ from one another in valine-sensitivity as well. The equilibrium between the various enzyme forms may play a decisive role in valine sensitivity and other characteristics of the enzyme.

Effect of freezing

It was observed in our preliminary experiments that if the enzyme solution was frozen during storage, the thawed enzyme solution showed a higher activity, rather than being inactivated. This activation effect was especially conspicuous with enzyme A obtained after gel-filtration, at low temperature and low substrate concentration. Along with the increase in activity observed after repeated freezing and thawing, there was a considerable decrease in the valine sensitivity of the enzyme, but a few hours following treatment the valine sensitivity was restored to almost the original level (Table II). The inhibition by substrate, experienced in

Table II

Activity and susceptibility to inhibition by valine of frozen enzyme A

Enzyme	Inhibition in the presence of 2 μM valine, per cent	Specific activity*
Untreated enzyme A	56	0.79
Measured immediately after repeated freezing	21	1.05
Stored for 6 hrs after freezing	39	0.59

* Measured at pH 8.2.

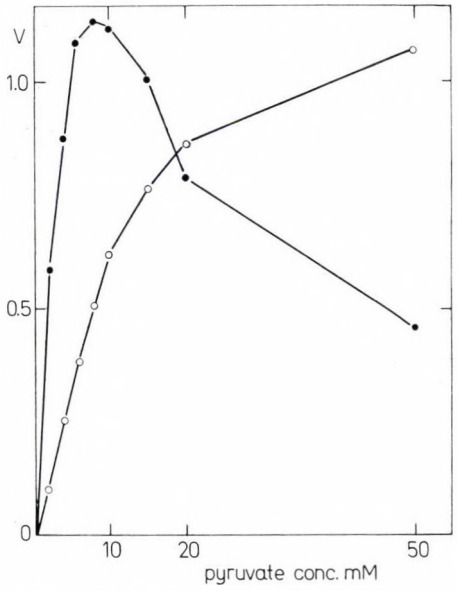

Fig. 2. Effect of freezing on substrate inhibition. The reaction rate was determined with enzyme A obtained by gel-filtration in the presence of L-valine as described in Fig. 1., at substrate concentrations indicated in the figure. Symbols: ○—○ untreated; ●—● enzyme solution was frozen and thawed successively five times; reaction rate was determined with solution obtained after the fifth thawing

our kinetic studies (Varga and Horváth, 1967b) could hardly, or not at all, be detected with enzyme A obtained by gel-filtration, while after freezing substrate inhibition occurred already at low substrate concentrations (Fig. 2). The pH optimum of enzyme A appeared in the less alcaline range, as compared to the crude enzyme, but after repeated freezing the pH-dependence curve exhibited marked changes and the pH optimum was displaced toward a more alcaline direction (Fig. 3).

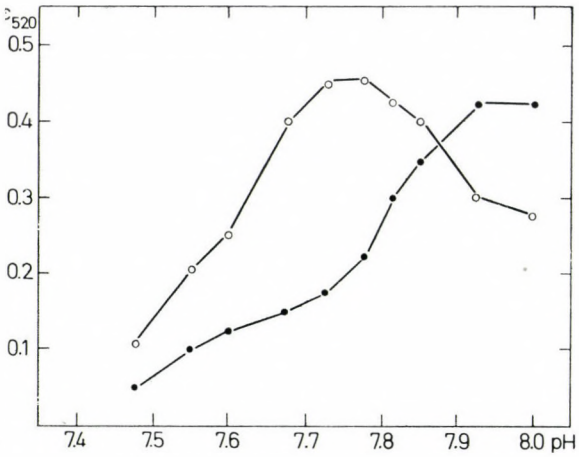

Fig. 3. Effect of freezing on the pH-optimum of the enzyme. Assays were carried out with the enzyme specified in Fig. 2, at pH values indicated in the figure. Symbols: ○—○ untreated enzyme; ●—● after repeated freezing

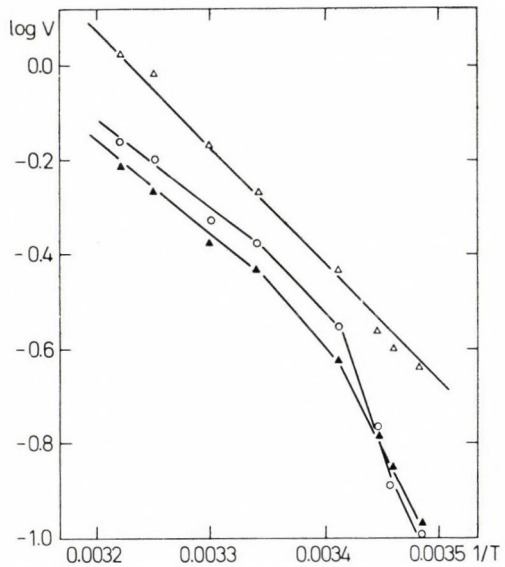

Fig. 4. Effect of freezing on the Arrhenius plot. Assays were carried out with the enzyme specified in Fig. 2, at temperatures indicated in the figure. Symbols: ○—○ untreated enzyme; △—△ immediately after repeated freezing; ▲—▲ 6 hours after repeated freezing

As it has already been described (Varga and Horváth, 1967b), the temperature dependence of the activity of the crude enzyme is irregular, there is a break in the Arrhenius plot. The break of the plot is more pronounced with enzyme A, but if examined immediately after freezing, the break cannot be found, while, in turn, it reappears after a few hours of storage (Fig. 4).

The results of the freezing experiments have suggested that on the destructive effect of freezing enzyme A was partly transformed into the valine-insensitive B form, which was still able of partial re-sensitization during storage, i.e. form A can be formed reversibly. Repeated filtration on a smaller column confirmed this assumption (Fig. 5). As it was already described (Varga and Horváth, 1965), and is seen also from the figure, enzyme A appeared in the exclusion volume of the

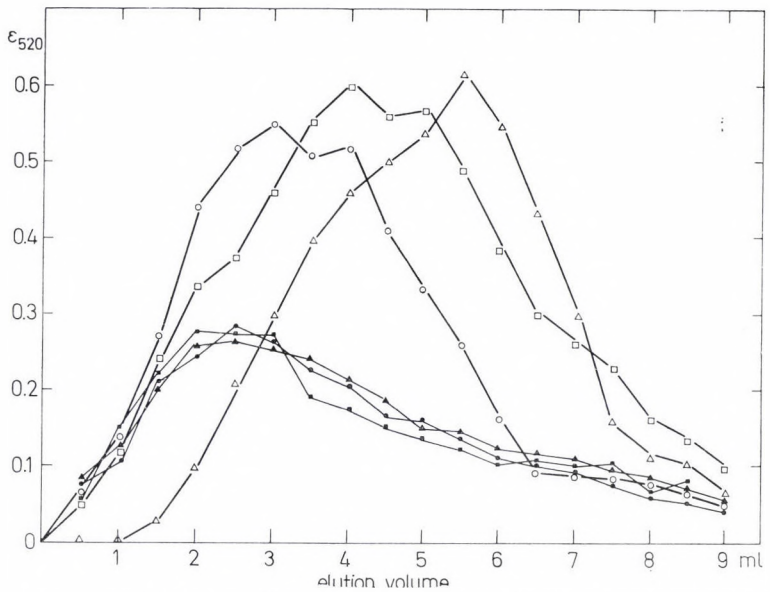

Fig. 5. Effect of freezing on gel-filtration. Enzyme A obtained in the presence of L-valine according to Fig. 1, C, was re-filtered on a 1×20 cm Sephadex G 200 column. The conditions of gel-filtration were previously described (Varga and Horváth, 1965). The 0 point of the diagram corresponds to the 0 point of the exclusion volume (V_0) determined by the aid of blue dextrane. The open symbols mean the optical density values of the color reaction according to Westerfeld (1945), which are proportional to enzymic activity, while the full symbols mean the optical density values of the color-reaction according to Lowry et al. (1951), which are proportional to protein concentration. Symbols: Circles: Values obtained with the refiltration of the untreated enzyme; Triangles: Values obtained with immediate gel-filtration after repeated freezing; Squares: Values obtained with gel-filtration after 6 hour storage of the freeze-thawed enzyme

gel when gel-filtered repeatedly on Sephadex G 200. After freezing, however, the enzymic activity emerged from the column with a marked delay. This result indicates that the less valine-sensitive enzyme obtained by the treatment is composed of fragments of lower molecular weight. If gel-filtration was repeated one hour after freezing, this delay could not be observed and the enzymic activity emerged again in the exclusion volume of the column.

These results explain our failure to detect by repeated filtration differences in the molecular weight of enzymes A and B obtained by gel-filtration. If the gel-filtered enzyme was re-filtered on the same column, 50 to 80 per cent of enzymic activity was lost in spite of the presence of stabilizing cofactors during gel-filtration.

If re-filtration was carried out on a smaller column, which did not provide complete separation of forms A and B, no clear-cut differences could be detected in the majority of experiments in the elution patterns when A or B was used as starting material, i.e. activity was observed in the exclusion volume of the gel on the re-filtration of the small molecular weight enzyme B as well. Taken into account, however, that the lower molecular weight, insensitive enzyme obtained by freezing recombined to the higher molecular weight form within one hour, no differences are to be expected on re-filtration by means of the rather slow Sephadex technique.

Discussion

The results obtained from gel-filtration and freezing experiments confirmed our earlier assumptions, made on the basis of stability and kinetic studies, according to which AAS from *Ps. aeruginosa* has two active forms in equilibrium with each other and these forms differ in molecular size and in their affinity towards the effectors and modifiers of the enzyme. The equilibrium between the two forms is determined in a complicated manner by the concentration of enzyme, temperature, and pH of the medium, the presence and absence of cofactors, substrate and inhibitor. Valine seems to stabilize the larger molecule, which can be inhibited allosterically. In the presence of valine the second (insensitive) peak does not appear. On the other hand, in the presence of pyruvate there is no qualitative change in the elution pattern but the majority of enzymic activity is lost. The inhibition by the substrate, which has been demonstrated earlier, is presumably due to this inactivation, and the results obtained with pyruvate are interpreted in terms of a shift of the $E_A \rightleftharpoons E_B$ equilibrium towards form B. An enhanced inactivation occurred as a result of high pyruvate concentration (cf. inactivation and kinetic studies, Varga and Horváth, 1967a,b).

Further evidence for an $E_A \rightleftharpoons E_B$ equilibrium was supplied by our results obtained in freezing experiments. After repeated freezing and thawing the valine-sensitivity of the enzyme decreased, the characteristic break of enzyme A in the temperature-dependence curve of enzymic activity disappeared, the pH-optimum was displaced towards higher pH values, and the enzyme emerged from the column on repeated re-filtration with a considerable delay as compared to enzyme A. During storage the original characteristics of the enzyme were restored to some extent, and the coincidence of emergence of the stored enzyme and enzyme A suggested that enzyme A, which had been split by freezing into small insensitive molecules, recombined to a larger, valine-sensitive form again. It may be assumed that the formation of the insensitive form of AAS from *Ps. aeruginosa* after freezing was brought about via the disruption of association between subunits, in accordance with similar phenomena observed with other enzymes composed of subunits (Chilson et al., 1965).

According to the theory of Monod, Wymon and Changeux (1965), allosteric effects are brought about by allosteric effectors through a shift of the equilibrium between the different protein forms possessing various affinities towards the substrate and inhibitors. Our results suggest that the $E_A \rightleftharpoons E_B$ equilibrium ob-

served is not identical with the equilibrium outlined in the above theory. On the contrary, we are dealing with an equilibrium between a desensitized and an allosterically sensitive enzyme form. Form A can at once be inhibited by valine, which appears to be a good example of allosteric inhibition. Thus if we accept the above assumption of Monod et al. (1965), the inhibition by valine may be brought about via indirect interactions between the subunits. We may assume that the substrate and inhibitor are bound through "non-exclusive" bonds to the different enzyme forms (Rubin and Changeux, 1966). Form B becomes susceptible to inhibition only after a relatively prolonged time. The alterations which take place in form B during this period render it possible for the system to undergo allosteric transition, and *vice versa*, during storage form A keeps on desensitizing, i.e. it gradually loses its "ability" to undergo the allosteric transition.

On the basis of our results it may be assumed that the equilibrium between the sensitive and desensitized enzyme forms is an important characteristic of AAS from *Ps. aeruginosa*. One has always to reckon with the presence of both sensitive and insensitive enzyme forms, and irrespectively whether one starts from the sensitive (E_A) or the insensitive (E_B) form, the same $E_A \rightleftharpoons E_B$ equilibrium is achieved. Furthermore, it may be assumed that the sensitive $\rightleftharpoons$ insensitive equilibrium of the enzyme studied represents an equilibrium between enzyme molecules of different subunit structure, and the proper organization or fit of the enzymes (or of some of them) of valine-isoleucine biosynthesis plays a decisive role in that that the allosteric transition can take place in the valine-sensitive A state. The low rate of the sensitive$\rightleftharpoons$insensitive transition may also indicate that recombination between various protein molecules might play a role in the transition between the two forms. This assumption is in accordance with recent results suggesting the existence of multi-enzyme systems among the regulatory enzymes ("polycephalic enzymes"). Data along these lines are accumulating with the refinement of methods applied (Ito and Yanofsky, 1966; Egan and Gibson, 1966). A somewhat similar organization of the enzymes of valine-isoleucine biosynthesis in Neurospora has recently been described (Wagner and Bergquist, 1963; Kiritani et al., 1965; Wagner et al., 1965). Our earlier results are in line with these observations (Varga and Horváth, 1965). Further investigations in this field are in progress in our laboratory.

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Periodate in Sequence Studies of Ribonucleic Acid*

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Experiments carried out by Ogur and Small only with AMP were extended to other mononucleotides. Both the liberation of inorganic phosphate and that of the base was followed. Cleavages around 95% were observed. With dinucleotides a temperature of 20° C proved to be insufficient. At 45° C, as suggested by Neu and Heppel, the cleavage of various di-, tri- and tetranucleotides was satisfactory. Instead of paper chromatography separation was carried out on DEAE cellulose column. This procedure was found to be more favourable both for the identification of the released base and for making the residue ready to the next cycle. The variation of time led to the unexpected observation that liberation of the base under certain conditions is much faster than that of the 3′-P group. This observation may serve as a new starting point in the investigation of the mechanism of the elimination reaction. Apart from the theoretical interest the observation has a practical significance as well: in order to attain fairly clear-cut cycles in the sequence determination we do not necessarily have to reach a cleavage value quite close to 100%.

Introduction

As soon as introduction of chromatographic methods gave rise to a rapid development in the investigation of nucleic acid structure, attempts have been made to determine nucleotide sequence. Progress, however, was very slow and the determination of overall base composition was the only way to characterize a preparation. The determination of the sequence in a trinucleotide seemed to be the limit for a long time. All the more remarkable was the brilliant achievement of Holley and his collaborators (1965, 1966) who established the complete nucleotide sequence of alanine specific t-RNA consisting of 77 nucleotides. Prerequisite of this success was, of course, the availability of homogenous polynucleotides the purification of which was based, among others, on their biological activity. Another important step was the application of T_1-RNase for partial digestion which yielded the necessary overlaps when combined with pancreatic

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** This is the VIII paper of the series Nucleic Acids from this institute. Paper I was published by Zsindely, Szabolcs and Tankó (1959), Paper VII by Zsindely and Berencsi (1966).

Abbreviations: RNA, ribonucleic acid; t-RNA, transfer RNA; R Nase, ribonuclease; DEAE, diethylaminoethyl; PME, prostate, phosphomonoesterase; CME, E. Coli phosphomonoesterase; AMP, adenosine-5-P; GMP, guanosine-5-P; Ap, Gp, Cp and Up the corresponding nucleoside-3-phosphates; A, G, C and U the corresponding nucleosides.

RNase. All these would not have been sufficient had not Holley, Madison and Zamir (1964) introduced a new method that involves the use of snake venom diesterase and an analysis of the successively shortening chains, instead of the analysis of the nucleoside released by this exonuclease from the 3′-OH end. This method, slightly modified, was used also by Zachau et al. (1966) in the determination of the complete sequence of two serine specific t-RNAs, another splendid success in this field.

The periodate method, originally suggested by Whitfeld (1954), and independently also by Brown, Fried and Todd (1955) appeared to be of little significance even after the contribution of Ogur and Small (1960). This method evidently could not compete with Holley's procedure at that time. More recent investigations published by Neu and Heppel (1964), however, appeared rather promising. Our aim was to study and improve the essential steps of this procedure, which seems to be more simple than Holley's method. Our investigations were made possible by the availability of oligonucleotides, to be used as models, being obtained in our laboratory as described in a previous paper (Zsindely and Berencsi, 1966).

Experimental

AMP was prepared from ATP-Ba by hydrolysis with baryta as described by Tankó (1951), the other 5′-nucleotides were obtained from the REANAL Fine Chemicals Factory. Oligonucleotides were prepared from pancreatic RNase (REANAL) digests of RNA as described by Zsindely and Berencsi (1966). The RNA was isolated from rabbit liver according to Zsindely, Szabolcs and Tankó (1959). Phosphomonoesterase was partly prepared from human prostate according to Davidson and Fishman (1959) but in the majority of cases a preparation from E. Coli according to Garen and Levinthal (1960) was used. We are indebted to our colleague M. Hauck in our institute for letting us use his preparation, free of diesterase activity. As our oligonucleotides had 3′-P terminals this had to be removed in each case before oxidation with periodate. Usually 0.2 – 1.0 μmoles of the oligonucleotide were dissolved in 0.1 – 0.5 ml 0.02 M acetate buffer pH 5 if dephosphorylation was performed with PME. In the case of CME 0.02 M NH_4HCO_3 pH 8.6 was used. 0.02 – 0.1 ml enzyme was sufficient to complete dephosphorylation at 37° C in 3 – 10 hours, as checked by measuring the inorganic P. The phosphatase was removed during chromatography on DEAE cellulose column from the oligonucleotide now ending in free 3′-OH and the amounts were given in the tables as μmoles.

Details of the experiments with periodate are given in the text of the tables and figures. A 0.1 M solution of Na-metaperiodate was used and its titer as well as its consumption in several experiments was determined arsenometrically according to Dyer (1956). Lysin was added only in experiments of Table 1 after a separate period of periodate oxidation; in all other cases it was applied together with the oxidant. Ethylene glycol was used as a 1 M solution and added in 20% excess to the introduced periodate for reduction of the latter. The elimination reaction was carried out at pH 6.7 – 7, at the temperature and for the time indicated, followed by the reduction with glycol. After dilution with water aliquots were

withdrawn for the determination of 3′-P with CME and identification of the base by paper or DEAE cellulose chromatography. Where two successive cycles of sequence determination were performed, the whole lot was treated with CME and chromatographed on a DEAE cellulose column to separate the residual oligonucleotide with free 3′-OH terminal from the base.

For paper chromatography either 70% isopropanol-water with ammonia in the vapour phase was used (Solvent A) as recommended by Markham and Smith (1952), or the mixture n-propanol–conc. NH_4OH – water (50 : 10 : 35, per volume, Solvent B) according to Alexander et al. (1961) with descendent technique. DEAE cellulose (Whatman DE 50) was prepared according to Staehelin (1961) in columns of 0.6×10 or 0.6×15 cm size. Elution was carried out with NH_4HCO_3 solutions of different molarities at pH 8.6 at a flow rate of 3 ml/5–10 minutes, collecting 3 ml fractions in a "Fractiomat" automatic fraction collector. The quantitative evaluation of the chromatography was made in the Beckman DU spectrophotometer (Tankó, 1959c).

Determination of total and inorganic phosphorus was principally carried out according to Martland and Robison (1926) but instead of 15 only 5 ml final volume was used and instead of sulfuric acid $HClO_4$ was employed. The reagents were 0.3 ml each of 70% $HClO_4$, 10% ammonium molybdate and 0.5% hydroquinon (dissolved in 20% Na_2SO_3). Using 1 cm cuvettes readings were taken in the Beckman spectrophotometer at 770 mμ exactly 30 minutes after mixing the reagents. Range: between 1–10 μg phosphorus.

Results

The usefulness of the periodate method was greatly improved by Neu and Heppel (1964) but the relatively drastic conditions applied by these authors (large excess of periodate and amine, temperatures as high as 45–60° C) might lead to breaks inside the chain, too, not only at the terminal. This danger seemed to exist with the large molecular weight TMV-RNA, as shown by Whitfeld (1965) and by Steinschneider and Fraenkel-Conrat (1966). Based on reports previous to Neu and Heppel we investigated whether satisfactory cleavage might be achieved under less drastic conditions.

First we studied Ogur and Small's method (1960) with AMP as substrate. Their short communication attracted attention at that time as it was shown that the reaction could be carried out almost to completion in much shorter time than described before. The breakdown of the dialdehyde formed by splitting between carbon atoms 2′ and 3′ was explained by the Todd school as a reaction of an alkali-labile β-aldehyde-ester, in analogy with the well-known conversion of the P-glyceraldehyde into lactic acid. This is the reason why the pH was adjusted with glycine to 10–10.5, a limit below which the internucleotide bond of RNA was regarded to be stable. Ogur and Small replaced glycine for lysine and found the optimum at least with AMP as substrate around pH 7.5–8. That the amine had a specific effect and not only that of a buffer, was later proved by Khym and Cohn (1961) and by Khym (1963). A Schiff-base like compound was formed as

an intermediate and so the reaction was not simply that of a β-aldehyde-ester. High pH even decreased the yield as it diverts the process to a Cannizaro-type reaction.

Mononucleotides as models simplify the experiments, as after elimination of the pentose residue inorganic P is left and there is no need of testing the release of 3′-terminal P as in the case e.g. of a dinucleotide. According to Ogur and Small we oxidized AMP in a final concentration of 0.003 M at 20° C for one hour at pH 5.5 with a 8 fold excess of Na-metaperiodate. Then the mixture was treated with a 80 fold excess of lysine for 90 minutes at various pH values. Based on the estimation of inorganic P the elimination at pH 7.2, 7.7 and respectively 8.7 amounted to 97, 96 and respectively 88%. Extending the work of Ogur and Small we titrated the periodate arsenometrically and at each pH value found the consumption of periodate to be equivalent to the amount of the nucleotide introduced. Thus it was not due to the primary reaction, to the formation of the dialdehyde, if the yield of elimination decreased.

We regarded it to be important to measure the release of the base too, since nothing was reported by Ogur and Small in this respect. Using solvent A for paper chromatography 80% of the adenine was recovered after elimination at the optimal pH, less than expected from the value obtained for phosphorus. Still the result cannot be regarded as unfavourable since Neu and Heppel did not obtain a higher yield of adenine even at 60° C using the same excess of the reagents. While Ogur and Small restricted themselves to the study of AMP we extended the experiments to GMP as well. Based on the liberation of inorganic P a cleavage of 97% was found at pH 7.4 with a periodate consumption close to the calculated figure.

It might have been possible to conclude from these findings that 20° C is sufficient and the high temperature (45° – 60° C as suggested by Neu and Heppel) can be avoided. However, with dinucleotides liberation of both the terminal base and the 3′-P group was far less than 100% as shown in Table 1. Here again, the periodate consumption corresponded well to the amounts of moles introduced. Thus the low yields were not due to a failure of oxidation leading to dialdehyde. Since the oligonucleotides used in our experiments were obtained from pancreatic RNase digest of RNA (Zsindely and Berencsi, 1966) we could not test dinucleotides with purines at the 3′-P terminal. It is unlikely that this would have improved the yield.

Based on the results presented in Table 1 the conditions suggested by Neu and Heppel were tested: the excess of periodate was raised to 10fold, that of the lysine to 100fold of the substrate. In this respect this method did not differ greatly from the previous one but the application of a higher temperature (45° instead of 20) is a significant change. During 90 minutes periodate and amine were present together in the reaction mixture, there being no separate oxidation phase. In Table 2. only experiments No. 1 and 2 refer to oligonucleotides tested also by Neu and Heppel; none of the others were used as substrates by these authors. The released base was determined by paper chromatography using solvent B except for experiments No. 6, 11 and 12 where separation on DEAE cellulose was introduced. This proved to be quicker and more accurate than paper

Table 1

Extension of the Ogur and Small method to dinucleotides

Substrate	μmole	Liberated base		Cleaved 3′-P	
		μmole	%	μmole	%
ApC	0.93	0.61	66	0.60	65
ApU	0.81	0.60	74	0.57	70
GpU	0.99	0.76	77	0.71	62
GpC	1.23	0.85	68	0.87	70
GpC*	0.63	0.52	82	0.49	78

Oxidation with an 8 fold excess of periodate at 20° C, two hours. Elimination with a 50 fold excess of lysine at 20° C, 20 hours. (E. g. in the case of ApC: in 0.2 ml final volume 0.08 ml 0.1 M $NaJO_4$ + 0.05 ml 1 M lysine pH 7).

* with glycine, pH 10 instead of lysine.

Table 2

Application of the Neu and Heppel method

No.	Substrate	μmole	Liberated base		Cleaved 3′-P	
			μmole	%	μmole	%
1	ApC	0.46	0.42	91	0.43	93
2	ApU	0.19	0.20	104	0.18	95
3	GpC	0.47	0.44	94	0.45	96
4	GpU	0.59	0.53	92	0.55	95
5	GpC	0.56	0.47	84	0.52	93
6	GpC	0.94	0.97	103	0.88	94
7	ApGpU	0.25	not determined		0.24	96
8	ApGpC	0.70			0.68	97
9	GpApC	0.35	0.31	89	0.35	100
10	GpApU	0.21	0.18	86	0.20	95
11	GpGpU	0.51	0.49	96	0.51	100
12	GpG	0.31	0.29	94	0.30	97
13	ApGpGpC	0.15	0.13	87	0.14	93
14	Gp(ApGp)U	0.20	0.19	95	not determined	

Oxidation and elimination with simultaneous addition of a 10fold excess of periodate and 100fold excess of lysine at 45° and pH 6.7–7, 2 hours (e. g. in case of No. 1: in 0.15 ml final volume 0.05 ml 0.1 M $NaJO_4$ + 0.05 ml 1 M lysine). In experiments No. 6, 11 and 12 DEAE cellulose, otherwise paper chromatography was applied for separation.

chromatography. Neu and Heppel stated that DEAE cellulose was applicable only to oligonucleotides of chain length 6 or greater (although the longest chain we find in their tables was a tetranucleotide). In our hands the columns worked well even with mono- and dinucleotides. We have found that from a suitably pretreated column iodate and the base can easily be eluted with 0.01 M NH_4HCO_3. Actually they are not bound on the column and by this washing of the column the base is quantitatively recovered. The bases were identified by measuring the

optical density at two different wavelengths. Control cuvettes contained the same amount of periodate reduced with ethylene glycol in the same way with no substrate present. Reduction was necessary since periodate absorbs strongly in this region. Any phosphomonoesterase present was washed off the column along with the base and iodate. As to the residue an eluent of 0.1 M proved necessary, as e.g. in experiment No. 6, to remove Gp. In the case of longer chains, more concentrated NH_4HCO_3 were used according to the chain length, or more correctly according to the charge due to the phosphate groups.

Except for the related experiments No. 11 and 12 elimination was restricted to one cycle only. Release of both the base and the 3′-P group was quite satisfactory in most cases. Lower yields of the bases obtained in some cases may be due to the limitations of the paper chromatographic technique (losses during elution). The remarkable difference we have found between mono- and dinucleotides regarding their sensitivness to elimination (see above) was not observed from the dinucleotides onwards, up to the tetranucleotides at least.

In experiments No. 11 and 12, two cycles were performed with DEAE cellulose for separation. Starting with GpGpU, after the combined oxidation-elimination reaction, the excess of periodate was reduced with ethylene glycol, the whole lot (not only an aliquot) was treated with CME and applied to the column. 0.01 M NH_4HCO_3 washed off the iodate, base and CME, a stronger solution (0.1 – 0.25 M) eluted the GpG which was ready for the next cycle after the removal of the volatile salt in the usual way. In the second cycle we were satisfied with the determination of the base and no CME was used at this stage (No. 12).

The described modification of the Neu – Heppel method seems to be suitable for sequence determination of oligonucleotides. With increasing length – up to tetranucleotides at least – one has only to apply stronger solutions of NH_4HCO_3 to elute the residual oligonucleotides. By fitting in CME in the right phase, only one separation on column is needed in each cycle. It appears that a similar modification of the Neu – Heppel method was performed by Smith and Herbert (1966).

In further experiments we tested whether or not the chromatographic separations could be omitted or at least carried out only after several cycles. A number of tri- and tetranucleotides were degraded in the first step as described (cleavages 95 – 100%), then after treatment with ethylene glycol and CME the terminal 3′-OH was set free. The phosphatase was inactivated and with more periodate added, the second cycle was performed (no amine was added since sufficient amounts remained in the reaction mixture from the previous stage). The determination of the 3′-P group so released indicated that a yield of only 70% was achieved. A plausible explanation can be that the great excess of the first portion of periodate, after having been reduced to iodate, inhibited the effect of the second portion. To perform several cycles in this way would be of course out of question. Besides, the identification of the newly liberated base would be exceedingly difficult. Such an illusory "simplification" would be meaningless indeed.

The introduction of the separation step on DEAE cellulose enabled us to obtain an insight into the kinetics and mechanism of the elimination reaction. It was observed that after treating the dinucleotide GpC with periodate, lysine

and ethylene glycol in the usual way, and applying the reaction mixture to a column, the easily eluted fraction containing iodate and base was followed by a small peak, before the Gp itself appeared. A contamination of GpC was out of question since the parent substance, GpCp was obtained on chromatography as

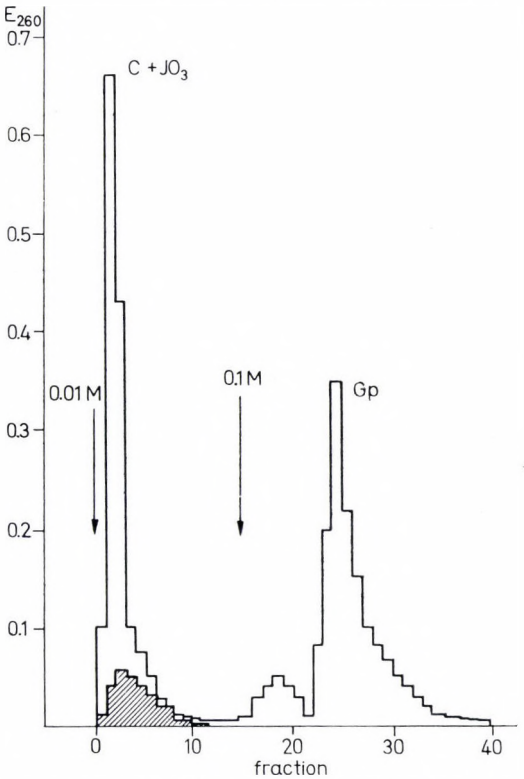

Fig. 1. Separation of the oxidation-elimination mixture of GpC after incubation for 2 hours. In all three experiments (Fig. 1—3) 0.52 μmole GpC was treated with a 10 fold excess of periodate at 20° C and pH 5.6 (this lasted 15 minutes in each experiment). Then incubation with a 100 fold excess of lysine followed at 45° and pH 6.6—6.8 (this time varied). After reduction with 0.1 ml ethylene glycol at 20° for 15 minutes the mixture was applied to a DEAE cellulose column of 10 cm × 0.6 cm size, using 0.01 M and 0.1 M NH_4HCO_3 solutions, respectively, as eluents, at pH 8.6. Change of eluent concentration, as indicated by the arrow. Shadowed peak refers to the absorption by iodate in the control which was taken into account in the quantitative evaluation

a single peak. The possibility of the occurrence of intermediate(s) of incomplete elimination had to be considered. Therefore in a further experiment less time was allowed, deliberately, for the combined action of periodate + lysine at 45°C. After 2 hours, 30 or 6 minutes, respectively the mixture was cooled, the reduction with glycol was carried out, the sample was diluted with water and at once applied to the column. In neither case was the 3′-P split off with phosphatase, as in the meantime the elimination reaction might have gone further. The main interest was focussed on the intermediates themselves. Figs 1 to 3 show how the first peak

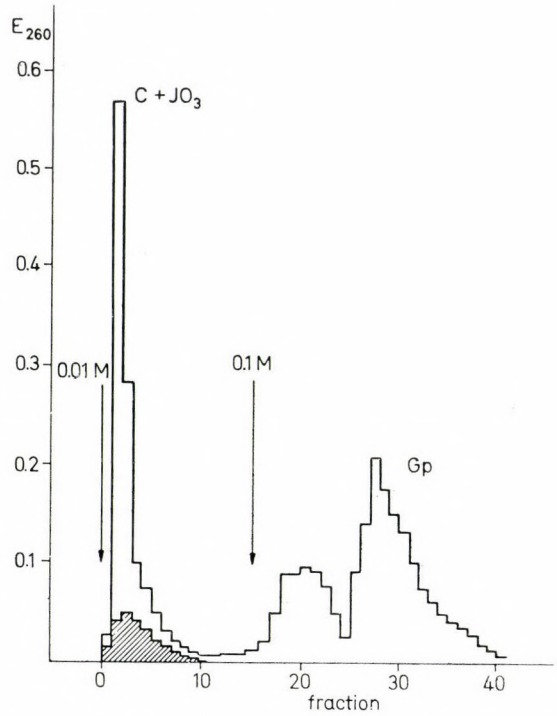

Fig. 2. Elution profile after 30 minutes elimination of GpC

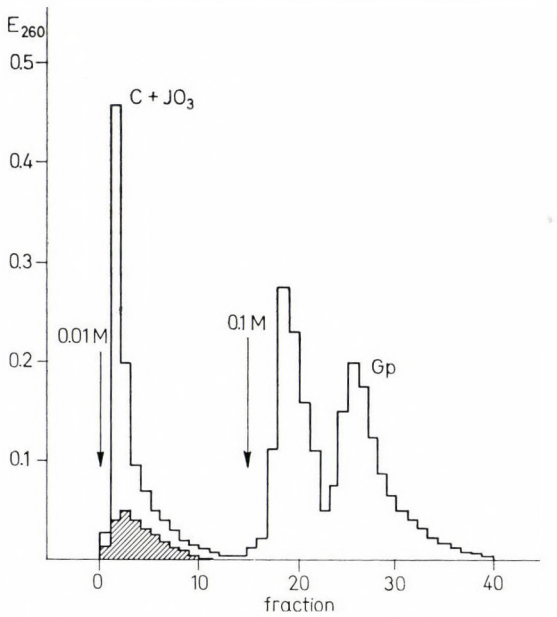

Fig. 3. Elution profile after 6 minutes elimination of GpC

Table 3

Variation of time during degradation of GpC

Minutes	Cytosine		Intermediates in OD_{260} units		Gp	
	μmole	%	found	calculated	μmole	%
6	0.44	85	3.36	3.11	0.30	57
30	0.47	91	2.14	2.39	0.34	65
120	0.53	102	0.68	0.47	0.50	96

These are quantitative data belonging to Figs 1—3. For the experimental details see text of figures. Calculation was based on the UV absorption at pH 1. Molar extinctions: cytosine $\varepsilon_{274} = 10{,}200$; Gp $\varepsilon_{257} = 12{,}200$; GpC $\varepsilon_{257} = 18{,}300$ (Venkstern and Bayev, 1965). All these values were transformed to the value at λ_{260} according to the graphs published in the literaturte since the actual determination of absorption of the peaks (after combining the appropriae, fractions) was performed at λ_{260}.

increased with the decrease of incubation time. In Table 3 the quantitative data presented in the three figures are summarized. In all three cases 0.52 μmole dinucleotide was introduced. In 6 minutes 85% of the cytosine, but only 57% of the Gp was set free: in 30 minutes the difference between 91% and 65% was still remarkable. The first peak should therefore consist of a mixture of intermediates that do not contain cytosine and Gp in equivalent amounts. The compounds involved should contain phosphate as the charge of this group prevents the compound(s) from being eluted along with the base, but apparently not both dissociating loci of the phosphate are free, as is the case of Gp.

There might be several types of intermediates of such a character. 1. GpC oxidized to dialdehyde and combined with lysine to form a Schiff-base, or possibly a further oxidized derivative of this compound; 2. the same compound(s) but without cytosine, eventually still further oxidized; 3. a derivative of Gp still containing a fragment of the pentose residue. It is possible that just owing to this fragment the phosphate group carries only one negative charge. Without attempting to separate these possible intermediates we could assume — in order to characterize them analytically — for simplicity that type 1 absorbs like GpC itself, type 2 and 3 on the other hand absorb like Gp. It can be worked out that in the first peak after 6 minutes 0.08 μmoles of cytosine are present as type 1, since 0.44 μmoles of cytosine are found in free state out of 0.52 μmoles GpC introduced. On the other hand 0.30 μmoles are found as Gp and so 0.22 μmoles are in the first peak, of which 0.08 μmoles belong to the 1 type and thus $0.22 - 0.08 = 0.14$ μmoles are present in the first peak as type 2 and 3. So from the data representing pure cytosine (first column) and pure Gp (last column) the composition of the intermediates in the first peak can be calculated. The molar extinction values are known. Taking into account the pH and the particular wavelengths we can calculate how many OD Units should be in the total peak of the intermediates. These figure can be compared with the OD Units actually found in this peak and as Table 3 illustrates the calculated and observed values agree fairly well.

From these findings it can be concluded that the release of the base is faster than the liberation of the 3′-P group. It is true that the mixture in the intermediate peak was not tested to see whether or not the phosphate group was blocked indeed. Still there was a similar experiment of a duration of 6 minutes where – after treatment with glycol – without any separation on column the amount of 3′-P was determined with CME and, as expected, it was less than the calculated value.

As to the reaction mechanism no definite conclusions can be drawn from the results described. It is possible that the first step in the degradation is the splitting of the oxygen bridge in the dialdehyde-amine complex. Then the terminal base would still carry a fragment from the ribose residue and similarly a fragment would still be attached to the newly formed 3′-P group. The rate of removal of these fragments would be different. Our findings indicate a higher rate for the liberation of the base. It has to be investigated whether the base obtained from the column is free indeed if tested by other chromatographic methods, or it still contains a fragment of the pentose. Should an intermediate of this kind be found to accompany the free base, this would corroborate the assumption that the primary reaction is the splitting of the oxygen bridge in the dialdehyde-amine complex with the base still attached to it.

The difference in the rate of liberation of the base and the P was observed also with AMP as a model with no CME being necessary to check the formation of phosphate free of any fragments. The amount of inorganic P directly indicates if this is the case. As illustrated in Table 4, 51% of the adenine was set free in 2

Table 4

Variation of time during degradation of AMP

Minutes	AMP μmole	Adenine		Inorganic P	
		μmole	%	μmole	%
2	0.52	0.55	51	0.21	20
5	0.33	0.74	69	0.42	39
30	0.10	0.98	91	0.89	84
120	0.06	1.02	94	1.00	94

Reaction was performed with a 10fold excess of periodate and a 100fold excess of lysine at 45°C, pH 6. 0.45 ml aliquots were withdrawn at interwals indicated and reduced with 0.05 ml M ethylene glycol. In one of the parallel aliquots the amount of total and inorganic P was measured, the other aliquot was diluted with water to 2 ml and chromatographed on a DEAE column (10 cm × 0.6 cm) using 0.01 M (for adenine) and 0.1 M (for AMP) NH_4HCO_3 pH 8.6, respectively. UV absorption was determined at pH 1 and λ_{260} in the appropriately combined fractions.

minutes whereas only 20% of the phosphate appeared in inorganic form. The difference decreased with the time of course. Thus mononucleotides are also suitable models in the investigation of the problem, whether or not the rupture of the oxygen bridge takes place prior to the splitting off of the base. This experiment with AMP proved to be an important one since in the case of Gp it might be argued that in the intermediate compound one of the negative charges

of the phosphate group was missing not because a fragment was attached to it, but owing to the formation of a cyclic 2′ : 3′-diester. Such a diester could not possibly be formed in the experiment with AMP.

Discussion

Reports previous to Neu and Heppel did not preclude the possibility that satisfactory cleavage of oligonucleotides might be achieved at temperatures lower than 45°C. Brown, Fried and Todd (1955) have found in one experiment 53% splitting of P from AMP in 5 minutes and 85% in 3 hours at 20°, pH 10.5 at 1.5 fold molar concentration of periodate and 35fold glycine. As to the release of base, observations with paper chromatography were reported which hardly permitted an evaluation even qualitatively. Whitfeld (1954) studied the degradation of dinucleotides at 37°C, pH 10. No data regarding the amount of periodate and glycine applied or the release of base and 3′-P were given. In paper chromatographic experiments, starting with ApC, finally a spot corresponding to cytosine was obtained and hence it was concluded that elimination was successful. It might have happened, however, that intermediates of an incomplete cleavage moved together with cytosine.

Ogur and Small, as stressed earlier in this paper, following Brown et al., also worked at 20°C. Our investigations gave a more complete information of the possibilities of this method as we extended the investigations besides AMP to other mononucleotides and measured quantitatively the release of the base, too. This was found to be as much as 80% at 20°C, a finding not expected on the basis of the results of Neu and Heppel who observed a yield of only 32–66% at 24°C with approximately the same excess of periodate and lysine. We have shown that such a cleavage cannot be achieved with dinucleotides and even the formation of 3′-P is decreased to 65–78%. It was proved by simultanous titrations that the consumption of periodate, i.e. the oxidation to dialdehyde is not the limiting step. The limiting step is the elimination itself.

The temperature had to be raised to 45°C. In that case we observed satisfactory cleavages as determined both by the formation of 3′-P and liberation of the base. As compared to paper chromatography, as employed by Neu and Heppel we found the separation on DEAE cellulose column to be superior. The base can be identified by its spectral properties directly in the solution obtained from the column and at the same time the chain left for the next cycle without P at the terminal 3′ is obtained readily by elution with an NH_4HCO_3 solution of suitable molarity. Increase of the chain length up to tetranucleotide revealed no limitation.

Reports in the literature regarding the optimum pH are contradictory and poorly documented. The pH optima may be different for the individual intermediary steps. Thus the values found to be suitable are probably results of a compromise. Consequently the limits are rather broad, e.g. pH 5–9 according to Khym and Cohn and 6–9 according to Neu and Heppel, who nevertheless used pH 8.5–9. We performed our experiments generally on the slightly acid side,

at pH 6.6–7. As we observed, the change in pH may have an opposite effect depending on the amine used.

Our results regarding the different rate of liberation of the base and the 3′-P seem to be in striking contrast to the observations reported by Neu and Heppel. According to their Table VII the 3′-P was equally released to the extent of 93–97% although the amount of periodate was varied between 1–16fold excess. Still, their yield of adenine was only 26, 56 and 82%, respectively as measured by paper chromatography, while the excess of periodate was 2, 3.3 and 6.7 fold, respectively. All this pointed to a lower rate of liberation of the base. Our figures 1–3 and Table 3 indicate just the opposite: as judged from the amount of Gp the liberation of 3′-P lags behind in comparison with the release of base. Neu and Heppel's findings support their idea that the base can be released only at the cost of a certain "overoxidation". This is the reason why a suitable excess of periodate has to be present during the elimination. For the liberation of 3′-P an elevated temperature is sufficient. Actually the contradiction between our findings and those of Neu and Heppel is only an apparent one as in our case not the excess of periodate was varied but the reaction time, at a given optimal amount of periodate.

Based on these observations a closer insight into the mechanism of elimination may be obtained, a problem which has not been elucidated yet. Khym and Cohn (1961) and Khym (1963) proved that the amine was not merely a buffer but it was condensed to the aldehyde. Their conclusions referring to AMP and methylamine may not be valid with other substrates and reagents: e.g. it might depend on the chain length, the type of the RNA and the amine itself whether 1 or 2 molecules of amine are bound by the oxidized pentose residue, as shown by Dulbecco and Smith (1960), by Hunt (1965) and by Steinschneider and Fraenkel-Conrat (1966). The breakdown of the intermediate Schiff-base like compound, leading to the free base and the new 3′-P terminal is not explained. We only know that the theory put forward by the Todd school – alkali-lability of a β-aldehyde ester – no longer holds.

We feel that shortening of the reaction time might lead to the separation and identification of the intermediates. It is practically significant that even if the base eluted from the column still retained a fragment of the oxidized pentose residue, its optical characteristics corresponded to those of a "free" base. This can be concluded from the agreement between the calculated and actually found values in Table 3. So this fraction can be used directly for the identification of the base. In paper chromatography, a base still retaining a fragment of the oxidized ribose behaved like a nucleoside and moved like the base itself only after acid treatment (Khym and Cohn, 1961). The case would be more complicated of course if we had to identify a rare or entirely new base as it happened during recent investigations of t-RNAs (Holley, Zachau).

Another significant conclusion that may be drawn from our findings is that the liberation of the base precedes that of the 3′-P. There is no need to force by all means an elimination quite close to 100%. This is not necessary for the identification of the base. In addition, the products of an incomplete elimination can well be separated on the column and so for the next cycle a perfectly split,

homogeneous product is available. The accumulation of incompletely split products that make the results unreliable even after a few cycles was regarded to be the main drawback of the periodate method for a long time. This drawback can now be overcome even if we do not strive for arriving at new 3′-P terminals to an extent quite close to 100%.

It is possible, that based on the present results the Neu and Heppel method could be rendered less drastic. The use of smaller excess of reagents, the use of amines not or inadequately tested so far, the lowering of temperature, taken together with the fact that there is no need to adhere to a breakdown close to 100%, might reduce the danger of splits inside long chains. Naturally in case of RNAs with several hundred or thousand nucleotides the same principle will have to be followed that enabled brilliant investigators to establish the complete sequence of t-RNAs, i.e. to get shorter, overlapping chains by partial, limited enzymic degradation. In the analysis of such shorter chains the periodate method will probably successfully compete with the Holley method. What chain length will be the limit, cannot yet be decided either from our experiments or from those reported by Smith and Herbert (1966). It should be stressed, in how original and ingenious way such partial digests were obtained by Smith, Smith and Herbert (1966) with t-RNAs and by Lee and Gilham (1966) with large molecular weight phage RNA.

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On the Mechanism of Calcium Transport in the Sarcoplasmatic Reticular Fraction

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The sarcoplasmatic reticular fraction (SRF) prepared from fish muscle binds a significant quantity of Ca^{++} even in the absence of oxalate. This loosely bound Ca^{++} can be easily removed from SRF. No binding of Ca^{++} occurs upon readdition of Ca^{++} to pre-treated SRF, even though the activation of adenosine triphosphatase by Ca^{++} is not diminished in the pre-treated preparations.

Caffeine, nicotine and d-tubocurare inhibit the binding of Ca^{++} to the natural binding sites (acceptors) of Ca^{++}, however, they do not affect the accumulation of Ca^{++} in the form of Ca-oxalate. These inhibitors also promote the mobilization of Ca^{++} already bound to the acceptors. Caffeine only inhibits the uptake of Ca^{++} when a certain amount of Ca^{++} has already been accumulated in SRF; this inhibition is presumably not the only result of an effect on the transport mechanism.

SH-inhibitors inhibit the activity of adenosine triphosphatase which plays an important role in the uptake of Ca^{++}. They also bring about the mobilization of Ca^{++} by abolishing the interaction between Ca^{++} and Ca^{++}-acceptor.

Introduction

It is known that the contraction and relaxation cycles of muscle are strictly related to the increase and decrease, respectively, of the level of Ca^{++} in the intracellular space. According to recent investigations, a concentration of intracellular Ca^{++} higher than 10^{-7} M is necessary for muscle contraction, while muscle relaxation is possible only at a lower level of Ca^{++} (Weber, Winicur, 1961; Seidel, Gergely, 1964).

The sarcoplasmatic reticulum around the myofibrills can reduce the concentration of free intracellular Ca^{++} by accumulating Ca^{++} (Ebashi, 1961; Hasselbach, Makinose, 1961; Ebashi, Lipmann, 1962; Fanburg, Gergely, 1965) and this, in turn, can bring about the relaxation of muscle. It is still an unsettled question, however, how Ca^{++} once accumulated in the sarcoplasmatic reticulum is mobilized again.

In our present model experiments, we have investigated the influence of caffeine, nicotine, d-tubocurare and certain SH-inhibitors on the uptake of Ca^{++} by the sarcoplasmatic reticular fraction (SRF), and also on the release of Ca^{++} already bound to SRF. A part of these results was presented at the 2nd Meeting of the Hungarian Biochemical Society (Szabolcs et al., 1965).

Methods

Sarcoplasmatic reticular fraction (SRF) was prepared from fish muscle (*Amiurus nebulous*) as described in our previous publication (Szabolcs et al., 1966). A perfusion technique was employed for studying the kinetics of uptake and mobilization of Ca^{++}. This was done as follows: columns of 0.8 cm in diameter and 5.0 cm in height were prepared from 0.70 g of cellulose powder (Whatman) in Razotherm glass tubes (Szabolcs, Kövér, 1966). Following the application of native or heat denatured (Control experiments) SRF, the glass tube was plugged with a rubber cork with a central hole. A well-fitted plastic tube, 0.2 cm in diameter, was driven through the hole of the rubber cork, and fixed in a position above the surface of the column. The other end of the plastic tube was submerged into one of the adjoining containers, which were filled to the same level with washing, incubation and ^{45}Ca-containing incubation solutions, respectively.

The rate of flow through the cellulose column was adjusted to a standard value of one drop per nine seconds during an equilibration of the column with 3 ml of the washing solution (0.055 M KCl, 0.002 M $MgCl_2$, 0.01 M TRIS-maleate buffer, pH 7.0), or of incubation solution (0.05 M KCl, 0.002 M $MgCl_2$, 0.002 M ATP, 0.01 M TRIS-maleate buffer pH 7.0). Following this equilibration, the upper end of the plastic tube was temporarily closed and transferred into the incubation solution containing ^{45}Ca (0.06 – 0.24 mM ^{45}Ca). Starting with the perfusion of the incubation solution containing ^{45}Ca, the effluent from the cellulose column was collected into glass tubes calibrated to 1.3 ml. Of the incubation solution containing ^{45}Ca, 5.2 ml ($4 \times 1 \cdot 3$ ml) was passed through the column in each experiment. Thereafter, the incoming plastic tube was connected to the container holding each incubation solution without ^{45}Ca, or washing solution. A total volume of 10.4 ml ($8 \times 1 \cdot 3$ ml) of the latter solutions was passed through the column. Each 1.3 ml fraction was collected in three minutes, which means that the constant velocity of perfusion was 0.433 ml/min. 0.5 ml aliquots of the 1.3 ml fractions were used for the determination of ^{45}Ca, and inorganic phosphate, respectively. The technique of measuring ^{45}Ca was previously published (Szabolcs, Kövér, 1966). Inorganic phosphate (P_i) was measured according to Taussky and Shorr (1953).

All the three solutions (washing, incubation with and without ^{45}Ca, respectively) contained the substance studied in a given concentration in experiments on the inhibition of uptake of ^{45}Ca. In experiments on the release (mobilization) of already bound ^{45}Ca, the studied compound was only present in the incubation solution without ^{45}Ca.

The following experimental details are common to all experiments shown in Figs 2 – 7: from the beginning of the perfusion of the incubation solution containing ^{45}Ca, twelve fractions (No. 1 – 12) were collected, each of a volume of 1.3 ml. After collection fractions 1 – 4, during which time the incubation solution containing ^{45}Ca was passed through the column, perfusion of the incubation solution without ^{45}Ca was started. Because of a dead volume of 3.5 ml of the cellulose column, the first fraction shown on the abscissa of Figs 2 – 7 is fraction 3. On the ordinates, ^{45}Ca and inorganic phosphate contents of fractions are

indicated. The distribution of ^{45}Ca in the Figs 2–7 is similar to a Gauss' curve. By contrast, inorganic phosphorus does not show a similar distribution, because ATP was present in the incubation solutions both with and without ^{45}Ca.

Analytical grade reagents were used. Ca^{++} was removed from ATP according to Seidel and Gergely (1963) before use. $^{45}CaCl_2$ with a specific activity of 423 μC/0.12 mM was purchased from the Isotopic Institute of the National Atomic Energy Committee.

Protein content of SRF was determined with the micro-Kjeldahl method.

Results

It was shown in a previous publication (Szabolcs et al., 1966) that SRF prepared from fish muscle accumulates a significant amount of ^{45}Ca even in an oxalate-free medium. We also made the observation that the accumulation of ^{45}Ca is increased but slightly by increasing the concentration of Ca^{++} in the incubation solution. These observations have led us to the tentative conclusion that the amount of some kind of acceptor in SRF is the limiting factor in the binding or accumulation of Ca^{++}. In order to study the relation between the "natural" Ca-acceptor and the structure of SRF, we investigated the changes in the activity of ATPase and in the Ca-binding capacity of SRF when the same preparation of SRF was repeatedly treated with Ca^{++} and washed free of Ca^{++}.

Changes in the characteristics of SRF in experiments repeated on Ca^{++} uptake

5.2 ml of an oxalate-free incubation mixture containing ^{45}Ca and an oxalate-free washing solution was used in these experiments (Table 1, series A). The difference between the amount of ^{45}Ca applied to SRF and that found in the

Table I

Uptake of Ca^{++} by SRF when treatment with Ca^{++} and washing is repeated in successive cycles

Uptake of Ca^{++} by SRF without any pre-treatment: 0.26 μmole ^{45}Ca per 1.4 mg protein from an oxalate-free medium, and 1.02 μmole ^{45}Ca per 1.4 mg protein from a medium containing oxalate. — Experimental Conditions: Washing solution = 0.055 M KCl, 0.055 M KCl, 0.005 M $MgCl_2$, 0.01 M TRIS/maleate buffer, pH 7.0; with or without 0.005 M potassium oxalate. Incubation solution containing ^{45}Ca = 0.05 M KC 1, 0.005 M $MgCl_2$, 0.005 M ATP, 0.01 M TRIS/maleate buffer, pH 7.0, 0.24 mM $^{45}CaCl_2$, with or without 0.005 M potassium oxalate. The washing solution was applied following the incubation solution which contained ^{45}Ca. Total protein of SRF was 1.4 mg. Further details in next.

Series	Oxalate (A)	+ Oxalate (B)
	μmol ^{45}Ca taken up by SRF	
I	0.251	0.951
II	0.04	0.501
III	0.05	0.545

collected fractions reveals the quantity of Ca^{++} bound to the acceptor (Table 1, I. A). After washing on the column, another 5.2 ml aliquot of the incubation solution containing ^{45}Ca was applied to the same SRF preparation and the washing was repeated (Table 1, II. A). Finally, the above procedure was repeated once more (Table 1, III. A). Parallel with these experiments, we also checked whether the amount of Ca^{++} taken up from an oxalate-containing medium will change as a consequence of repeated experiments on the uptake of Ca^{++} from an oxalate-free medium (Table 1, I. B–III. B). Thus, in series B one, two and three experiments, respectively, were performed with the SRF on the uptake of Ca^{++} from an oxalate-free solution before the amount of Ca^{++} taken up from the oxalate-containing solution was determined.

Based on Table 1, our results can be summarized as follows: 0.251 μmole Ca^{++} was bound to the "natural" acceptor in SRF in the course of experiment I. A. When carrying out a second experiment on the uptake of Ca^{++} with the same SRF, the amount of bound Ca^{++} did not increase, moreover, the Ca^{++} which had been accumulated in the first experiment was practically removed (experiment II. A). Of the applied 2×1.25 μmoles of ^{45}Ca, only 0.04 μmole remained bound to SRF. No further change in the amount of bound ^{45}Ca occurred in experiment III. A. It would follow from these observations that the natural Ca-acceptor is easily washed out from SRF.

Following experiments I. A which was performed in the absence of oxalate, 0.95 μmole of Ca^{++} accumulated in the presence of oxalate (expt. I. B) in a second experiment with the same SRF. This indicates that the active transport of Ca^{++} was not impaired in the course of experiments I. A and I. B In experiment II. B, which was carried out in the presence of oxalate following two experiments (I. A, II. A) in the absence of oxalate, the quantity of accumulated Ca^{++} was reduced about to half the original value, i.e. to 0.501 μmole. A similar value was obtained in experiment III. B (0.54 μmole bound ^{45}Ca), which was carried out following experiment III. A. The latter results indicate that even the mechanism of active transport of Ca^{++} was damaged.

Some further data pertinent to the mechanism of Ca-accumulation were obtained in the above experiments by measuring the inorganic phosphate content of the fractions, since the ATPase activity of SRF was responsible for the production of P_i. The results of these measurements are shown in Fig. 1. It is evident from a comparison of the first columns (Fig. 1a,b) that the basal ATPase activity determined in the absence of Ca^{++} is higher in the oxalate-free medium, than in the presence of oxalate. This can be explained by the presence of a low concentration of Ca^{++} in our incubation solutions in spite of using an assumedly Ca-free ATP, and the concentration of ionized Ca is obviously reduced in the presence of oxalate, respectively, it is also seen that if Ca^{++} is added to the medium, both in the absence (1a) and in the presence (1b) of oxalate, this results in a roughly 50 per cent increase in the activity of ATPase, even though the corresponding basal activities are different. There is no change in ATPase activity in the course of experiments I. A and I. B (Fig. 1, I. A and I. B), and this is in accordance with the observation that no marked change in the uptake of Ca^{++} occurs either. ATPase activity does not change even in the course of experiment

II. A (Fig. 1, II. A) though as seen from Table 1 II. A SRF can no longer take up or even retain Ca^{++}. A marked decrease in ATPase activity is apparent in the course of experiment II. B (Fig. 1, II. B) and this indicates a damage to the transport mechanism. The dominating role of this phenomenon can be seen from the results we have obtained in the succeeding experiments (Fig. 1, III. A and III. B). Parallel with the decrease in ATPase activity the uptake of Ca^{++} is also reduced (Table 1, III. A and III. B).

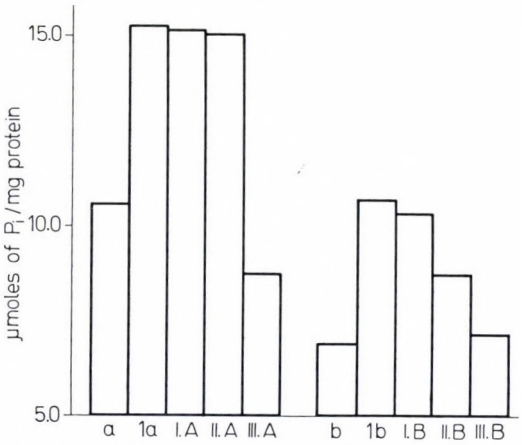

Fig. 1. ATPase activity of SRF when treatment with Ca^{++} and washing is repeated in cycles. — *a)* ATPase activity in oxalate-free medium. *b)* ATPase activity in a medium containing oxalate. 1*a)* ATPase activity in oxalate-free medium with 0.24 mM $CaCl_2$. 1*b)* ATPase activity in a medium containing oxalate plus 0.24 mM $CaCl_2$. I. A) ATPase activity in the first cycle of uptake and washing in oxalate-free medium. I. B) ATPase activity in a cycle of uptake and washing with oxalate, which followed expt. I. A. II. A) ATPase activity in the second cycle of uptake and washing in oxalate-free medium. II. B) ATPase activity in a cycle of uptake and washing with oxalate, which followed expt. II.A. III.A) ATPase activity in the third cycle of uptake and washing in oxalate free medium. III. B) ATPase activity in a cycle of uptake and washing with oxalate, which followed expt. III. A. — Experimental conditions as for Table 1

Effect of caffeine, nicotine, d-tubocurare, sodium azide and SH-inhibitors on the release of bound ^{45}Ca and on the ATPase activity of SRF

Following the perfusion of 5·2 ml incubation solution which contained ^{45}Ca, a Ca-free incubation solution was passed through the column which also contained one of the above compounds in the concentration shown in Table 2 and Figs 2–4. The effects of caffeine, nicotine and d-tubocurare were studied both in the presence and absence of oxalate in the incubation solution. Other inhibitors were only applied in the absence of oxalate. It is seen from Table 2 that mobilization of already bound Ca^{++} is observed with caffeine, nicotine and d-tubocurare, and there is no marked effect of oxalate on the induced release of Ca^{++}. The amount of Ca^{++} which is released depends on the concentration of the studied compounds. SH-inhibitors are apparently the most effective, since

these compounds induce 60–100 per cent mobilization in concentrations as low as 0.1 mM. Sodium azide does not induce release of already bound Ca^{++} in concentrations up to 2–5 mM.

Table 2

Effect of caffeine, nicotine d-tubocurare, sodium azide and SH-inhibitors on the release of bound ^{45}Ca by SRF

Experimental conditions: Incubation solution = 0.05 M KCl, 0.002 M $MgCl_2$, 0.002 M ATP, 0.01 M TRIS /Maleate buffer, pH. 7.0, 0.06 mM $^{45}CaCl_2$ (Series A): and the same with 0.12 mM $^{45}CaCl_2$ and 0.002 M potassium oxalate (Series B). Following the application of the above incubation solution, such a one was used which was prepared without $^{45}CaCl_2$ but with the given inhibitor

Series	Treatment	Concentration mM	Ca^{++} taken up	Ca^{++} released
			umole ^{45}Ca per mg protein	
A	control	—	0.13	—
	caffeine	5.0		0.0437
	caffeine	50.0		0.101
B*	control	—	0.144	—
	caffeine	5.0		0.041
	caffeine	50.0		0.091
A	control	—	0.146	—
	nicotine	50.0		0.128
B	control	—	0.26	—
	nicotine	50.0		0.132
A	control	—	0.145	—
	d-tubocurare	10.0		0.110
B	control	—	0.26	—
	d-tubocurare	10.0		0.105
A	control	—	0.12	—
	p-CMB	0.01		0.052
	p-CMB	0.10		0.12
A	control	—	0.14	—
	Salyrgane	0.01		0.07
	Salyrgane	0.10		0.14
A	control	—	0.14	—
	sodium azide	5.0	—	0.00

*With 0.06 mM $CaCl_2$; p-CMB = para-chloro-mercuribenzoate

We have obtained some indications as to the possible mechanism of induced release by studying the relationship between mobilization of Ca^{++} and ATPase activity of SRF. Maximal release of bound Ca^{++} and maximal inhibition of ATPase are observed in the same fractions (fractions 9–10, Fig. 2). This might indicate a strict relationship between mobilization of Ca^{++} from, and activity of ATPase in SRF. However, a re-evaluation is necessary in the light of observations made with caffeine. As seen from Fig. 3 maximal mobilization of Ca^{++} coincides with maximal activity of ATPase when caffeine is perfused through the column. Further, ATPase activity decreases in the presence of sodium azide (Fig. 4) with

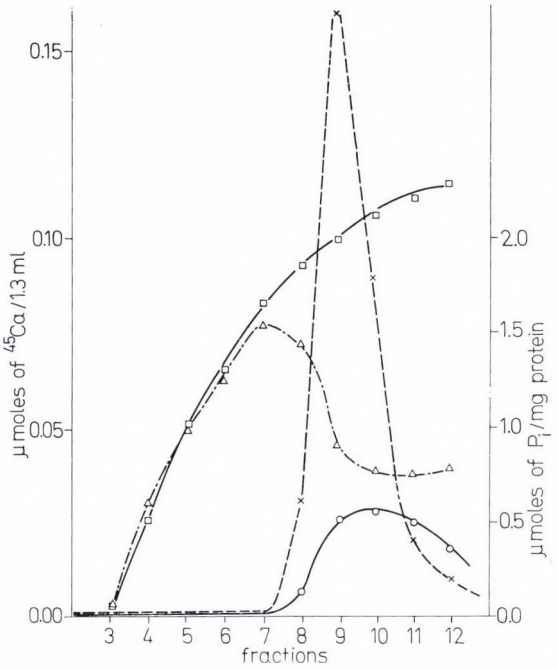

Fig. 2. Effect of p-CMB on the release of already accumulated ^{45}Ca. Total amount of $^{45}CaCl_2$ applied = 0.312 μmole. Composition of the incubation solution = 0.05 M KCl, 0.002 M $MgCl_2$, 0.002 M ATP, 0.01 M TRIS/maleate buffer, pH 7.0 and 0.06 mM $^{45}CaCl_2$. This was followed by the application of a similar solution without $^{45}CaCl_2$, ○—○ μmoles $^{45}CaCl_2$ recovered; ×—× μmoles $^{45}CaCl_2$ recovered with 0.1 mM p-CMB; □—□ ATPase activity of SRF; △—△ ATPase activity of SRF with 0.1 mM p-CMB present

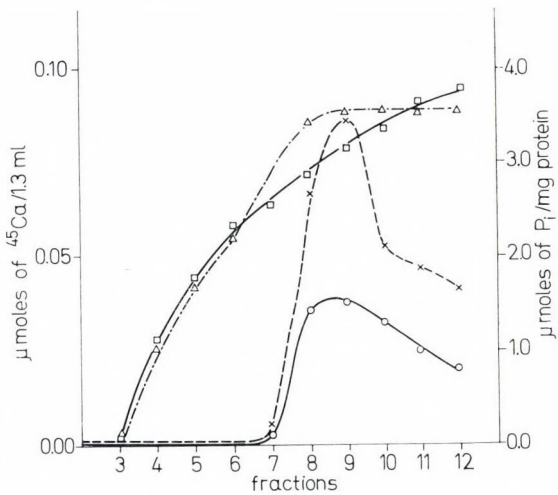

Fig. 3 Effect of caffeine on the release of already accumulated ^{45}Ca. — As in Fig. 2 with 50 mM caffeine instead of 0.1 mM p-CMB

no accompanying effect on bound Ca^{++}. Therefore, it is tentatively concluded that there is no close correlation between the activity of SRF-ATPase and the mobilization of Ca^{++} bound by SRF.

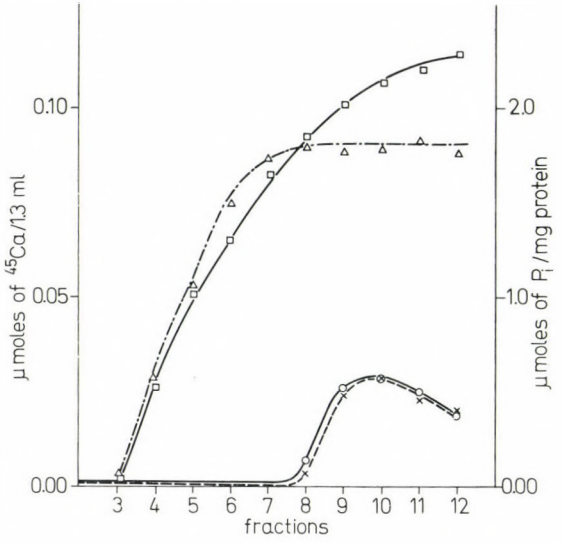

Fig. 4. Effect of sodium azide on the release of already accumulated ^{45}Ca. — As in Fig. 2 with 2 mM sodium azide instead of 0.1 mM p-CMB

Effect of caffeine, nicotine, d-tubocurare, sodium azide and SH-inhibitors on the uptake of ^{45}Ca and on the ATPase activity of SRF

It is seen from Table 3 that there is a concentration-dependent inhibitory effect of these compounds on the uptake of Ca^{++} by SRF. It can be concluded from the comparison of the results obtained in the presence and absence of oxalate that first of all an inhibition of the interaction between Ca^{++} and the "natural" acceptor is involved. This is shown by the following calculations: When the concentration of ^{45}Ca in the incubation solution is 0.06 mM, 0.144 μmole ^{45}Ca per mg protein is bound in the presence of oxalate and 0.13 μmole is bound in the absence of oxalate. It follows from the result of the successive uptake experiments described above that only the difference, that is 0.014 μmole, can be present in an oxalate-bound state. The same figure is obtained in the presence of 5 mM caffeine; since 0.1 μmole ^{45}Ca per mg protein is taken up with oxalate present and 0.086 μmole ^{45}Ca per mg protein is taken up in the absence of oxalate ($0.1-0.086 = 0.014$). When the external concentration of ^{45}Ca is raised to 0.12 mM, there is a slight increase in the amount of ^{45}Ca bound to the acceptor (0.145 μmole ^{45}Ca per mg protein), while the amount bound as Ca-oxalate is greatly increased ($0.26-0.145 = 0.115$ μmole ^{45}Ca per mg protein). These values show a good agreement with those published earlier (Szabolcs et al., 1966). It is seen from Table 3 that the same difference of 0.115 μmole ^{45}Ca per mg protein is obtained in the presence of the uptake-inhibitors nicotine and d-tubocurare.

Table 3

Effect of caffeine, nicotine, d-tubocurare, sodium azide and SH-inhibitors on the uptake of ^{45}Ca by SRF

Experimental conditions: Incubation solution = 0.05 M KCl, 0.002 M $MgCl_2$, 0.002 M ATP, 0.01 M TRIS/Maleate buffer, pH 7.0 and 0.06 $^{45}CaCl_2$ (series A), and the same with 0.12 mM $^{45}CaCl_2$ plus 0.002 M potassium oxalate (series B). Following the application of the above incubation solution prepared with ^{45}Ca, another incubation solution was applied which did not contain ^{45}Ca. All incubation solutions also contained the given inhibitor.

Series	Treatment	Concentration	Ca^{++} taken up	Reduction of Ca^{++} taken up.
			μmole ^{45}Ca per mg protein	
A	control	—	0.13	—
	caffeine	5.0	0.086	0.044
	caffeine	50.0	0.024	0.106
B*	control	—	0.144	—
	caffein	5.0	0.100	0.044
	caffein	50.0	0.043	0.101
A	control	—	0.145	—
	nicotine	50.0	0.013	0.133
B	control	—	0.26	—
	nicotine	50.0	0.124	0.136
A	control	—	0.145	—
	d-tubocurare	10.0	0.005	0.140
B	control	—	0.26	—
	d-tubocurare	10.0	0.11	0.15
A	control	—	0.12	—
	p-CMB	0.01	0.00	0.12
	p-CMB	0.10	0.00	0.12
A	control	—	0,14	—
	Salyrgane	0.01	0.03	0.11
	Salyrgane	0.10	0.00	0.14
A	control	—	0.145	—
	sodium azid	5.0	0.146	0.00

* 0.06 mM $^{45}CaCl_2$; p-CMB = para-chloro-mercuribenzoate

In some of the experiments on the inhibition of Ca-uptake we have also determined ATPase activity from aliquots of the collected fractions. Fig. 5 shows that under the given conditions ATPase activity as well as the uptake of ^{45}Ca is inhibited by p-CMB. As expected, there is no binding of Ca^{++} to the Ca-acceptor. On the other hand, it would follow from the observed increase in ATPase (Fig. 6) in the presence of caffeine that the active transport mechanism should not be damaged in this case. Indeed, Ca^{++} is apparently bound to Ca-acceptor when caffeine is present (Fig. 6, cf. Discussion).

Even the highest concentration of sodium azide does not inhibit the uptake of ^{45}Ca. However, ATPase activity is significantly inhibited (Fig. 7). Therefore, even, though active transport of Ca^{++} is slightly inhibited by sodium azide, the accumulation of acceptor-bound Ca^{++} is not affected.

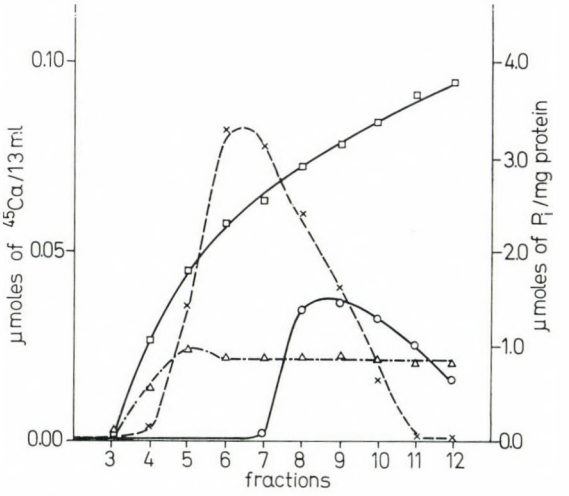

Fig. 5. Effect of p-CMB on the uptake of ^{45}Ca by SRF. — Total amount of $^{45}CaCl_2$ applied = = 0.312 μmole. Composition of the incubation solution = 0.05 M KCl, 0.002 M $MgCl_2$, 0.002 M ATP, 0.01 M TRIS/maleate buffer, pH 7.0, 0.06 mM $^{45}CaCl_2$ and 0.1 mM p-CMB. This was followed by a second solution from which $^{45}CaCl_2$ has been omitted. Further details in text. — ○—○ μmoles $^{45}CaCl_2$ recovered, ×—× μmoles ^{45}Ca Cl_2 recovered with 0.1 mM p-CMB, □—□ ATPase activity of SRF, △—△ ATPase activity of SRF with 0.1 mM p-CMB present

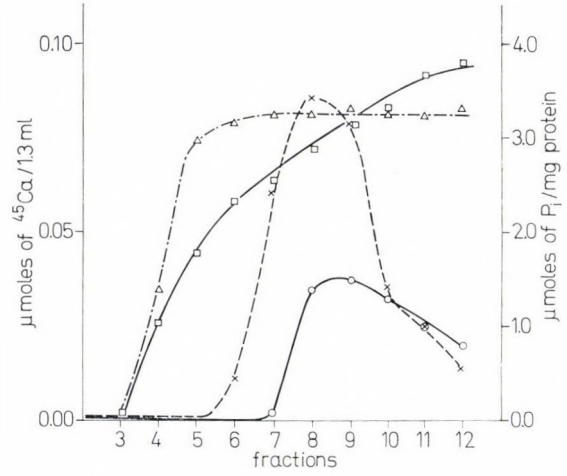

Fig. 6. Effect of caffeine on the uptake of ^{45}Ca by SRF As in Fig. 5 with 50 mM caffeine instead of 0.1 mM p-CMB

Discussion

The exact mechanism whereby Ca^{++} is taken up by SRF is not known. It has been shown by Hasselbach and Makinose (1961, 1962) that it is a process coupled to the consumption of ATP and to a ^{32}P exchange reaction between ADP and ATP, and that anions which form a precipitate with Ca^{++}

increase the accumulation of Ca^{++} in SRF. However, Ca^{++} is taken up by SRF even in the absence of ATP if the incubation solution contains a complete phosphate transfer system, such as creatine phosphate and creatine phosphotransferase (Molnár, Loránd, 1962; Weber et al., 1963). Molnár and Loránd concluded that active Ca-uptake by SR fragments is due to the operation of structurally bound phosphate acceptor groups. Ebashi and Endo (1964) showed that the ability of SRF to bind Ca^{++} in the presence of an ATP-regenerating

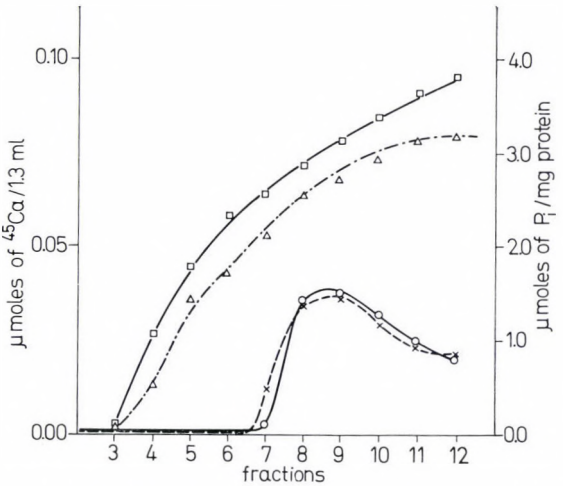

Fig. 7. Effect of sodium azide on the uptake of ^{45}Ca by SRF. — As in Fig. 5. with 2 mM sodium azide instead of 0.1 mM p-CMB

system (pyruvate kinase and phosphoenol pyruvate) is similar and even more than the chelating power of EGTA.

Martonosi (1964) studied the role of phospholipids in the uptake of Ca^{++} by SRF, and also studied the phospholipids as structural components of SRF. He found that SRF could no longer accumulate Ca^{++} after a treatment with phospholipase C. The capacity of SRF to accumulate Ca^{++} was restored by the addition of lecithine or lysolecithine.

Accordingly, the following mechanisms were suggested to explain the uptake of Ca^{++} by SRF: 1. Chemical bonds are involved (Ebashi and Lipmann, 1962; Molnár, Loránd, 1962), 2. it is an active transport through the membrane of SRF (Hasselbach, Makinose, 1961), and 3. it is a combination of active transport and bond formation (Martonosi, Feretos, 1964).

It certainly depends on the choice of experimental conditions whether one or the other of the above mechanisms will be reflected. The fact that Ca^{++} accumulates in SRF even in the absence of oxalate necessarily involves the postulation of the existence of "natural" Ca-acceptors. Were there no acceptors inside the SRF particles, the Ca^{++} taken up by active transport would be lost through the opened end of the particles. It is suggested that oxalate and other anions which precipitate Ca^{++} are, to some extent, analogues of the "natural Ca-

acceptor". The repetition of uptake experiments in our studies yielded further support to this view. Table 1 shows, namely, that Ca^{++} bound to the natural acceptor is retained in SRF even if it is subsequently treated with an ATP-free solution (Table 1, I. A). This means that no energy is required for the storage of Ca^{++} in the form of a Ca-acceptor complex. Experiments I. A and II. A in Table 1 show that the interaction between the natural acceptor and the membrane structure of SRF can only be a week one. Removal of the acceptor does not lead to an immediate damage of membrane structure or Ca-transport mechanism. Activation of the transport mechanism by Ca^{++} can be observed for a while after removal of the acceptor (Fig. 1, II. A).

By studying the effect of some inhibitors on the intensity of active Ca-uptake and the release of already accumulated Ca^{++} we have obtained some additional clues as to the nature of the natural acceptor and to further details of the mechanism of Ca-transport. Theoretically, it is possible to prevent the accumulation of Ca^{++} from an oxalate-free medium by inhibiting ATP ase activity of SRF, by reducing the permeability of the membrane of SRF, and by inhibiting the formation of the complex of natural acceptor and Ca^{++}. However, a mobilizing effect of the inhibitors on already accumulated Ca^{++} can only be due to an abolishment of the interaction between Ca^{++} and the natural Ca-acceptor. This is so, because, as already discussed, no energy is required for the storage of acceptor-bound Ca^{++}. Moreover, the release of already accumulated Ca^{++} once it is liberated from the acceptor-complex, can not be limited by membrane permeability under the given experimental conditions, since ionized Ca^{++} can freely leave the particles through their opened ends. Table 2 shows that the difference in the amount of Ca^{++} taken up from solutions which did, and did not contain oxalate, respectively, is not changed if various inhibitors are present. This means, in turn, that caffeine, nicotine, and d-tubocurare only inhibit the binding of Ca^{++} to the natural acceptor, but not the accumulation of Ca-oxalate. Based on these considerations it is suggested that the active transport mechanism is not adversely affected by the inhibitors. This view is supported by the observation that ATPase activity of SRF is not decreased but even increased by Ca^{++} if caffeine is present (Fig. 3, 6). It is interesting to note that inhibition of Ca-uptake by caffeine is delayed with respect to inhibition by p-CMB (c.f. fraction 8, Fig. 6 and fraction 6, Fig. 5), while release of Ca^{++} is brought about simultaneously with caffeine and p-CMB (fraction 9, Fig. 3 and fraction 9, Fig. 2). This indicates that the action of caffeine is delayed until a certain amount of Ca^{++} has already been accumulated. Thus even in the uptake experiments, the mobilizing effect of caffeine is manifested. We suggest that this argument and a variation in the experimental conditions would explain the contradictory results in the literature as to the action of caffeine (Hasselbach, Makinose, 1964; Weber et al., 1963; Seidel, Gergely, 1964; Carvallhio, 1966; and Herz, Weber, 1965).

As to the point of action of caffeine, Bianchi (1961) has already concluded from experiments with frog sartorius muscle that the association constant of membrane-bound Ca^{++} or Ca^{++} bound to the sarcoplasmatic reticulum is decreased in the presence of caffeine. A similar interpretation will hold for the finding of Ahmad and Lewis (1962) according to which uptake of Ca^{++} by frog

muscle is inhibited by 0.05 mg per ml d-tubocurare, and release of already accumulated Ca^{++} is brought about by 1 mg per ml, d-tubocurare.

Apparently, treatment with an SH-inhibitor results in an inhibition of Ca-uptake (Table 3) because of a primary inhibitor of the transport mechanism (SRF ATPase, Fig. 5), and a release of already accumulated Ca^{++} (Table 2) is due to a destruction of the acceptor-Ca^{++} complex. Therefore, it is possible that SH groups play some role in the complex involving the natural acceptor.

It is an outstanding feature of these results that the affinity of the natural Ca-acceptor to Ca^{++} can be decreased by reagents the point of attack of which is presumably different. Indeed, this is the very characteristic of the natural Ca-acceptor which may enable it to regulate the intercellular level of Ca^{++} (Fujino et al., 1960; Lee et al., 1966; Kalamkarova, 1966).

Acknowledgement

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Studies on the Postnatal Changes in the Sarcoplasmatic Reticular Fraction of Rabbit Muscle

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We studied adenosinetriphosphatase activity, cholinesterase activity and Ca-uptake of the 1 000 × g, 8 000 × g and 25 000 × g fractions of the homogenate of rabbit muscle. Rabbits of different ages were used. The fractions were obtained by differential centrifugation. It was found that Ca-uptake and the above enzyme activities are characteristic of the fractions 1 000 × g—8 000 × g (RF. II) and 8 000 × g—25 000 × g (RF. IV or SRF). During the first five days of postnatal life adenosinetriphosphatase and cholinesterase activities were high, and Ca-uptake was low. Beginning with the sixth day of postnatal life there was a significant uptake of Ca^{++}, and a progressive decline of the above enzyme activities. Also from the sixth day on, adenosinetriphosphatase of SRF was increasingly activated by Ca^{++}. The activation reached the extent characteristic of fully grown animals with 27—39 day-old rabbits. In conclusion we suggest the existence of a correlation between the observed changes in the functional characteristics of sarcoplasmatic reticulum, and the alteration of the character of muscle function with postnatal age.

Introduction

In the course of ontogenesis in mammals there is a specific differentiation in the sarcoplasmatic reticulum of striated muscle (Verratti, 1902) and an alteration of the character of muscle function (Rückert, 1930; Kostoyants, Rabinovskaya, 1936; Leibson, 1939; Varga et al., 1957). It is also known that the contraction-relaxation cycle of muscle is first of all determined by the sarcoplasmatic reticulum which regulates the intracellular level of Ca^{++} (Hasselbach, Makinose, 1961; Ebashi, Lipmann, 1962). Therefore, the question arises whether or not the histological alteration of sarcoplasmatic reticulum (SR) with postnatal development is reflected in a functional alteration of SR. In the present paper results obtained in studies on the uptake of Ca^{++} and some enzymatic properties of the sarcoplasmatic reticular fraction (SRF) of rabbit muscle, prepared from animals of different ages will be described. Part of these observations were presented at the 1965 Meeting of the Hungarian Physiological Society (Szabolcs et al., 1966a).

Methods

Preparation of subcellular fractions. — Details of the procedure have been published (Szabolcs et al., 1966b). RF. I is the supernatant of centrifugation at 1000 × g, RF. II is the precipitate obtained by centrifugation at 1 000 − 8 000 × g,

RF. III is the supernatant and RF. IV (SRF) is the precipitate, respectively, which are obtained by centrifugation at $8\,000-25\,000\times g$.

Uptake of Ca^{++}. – Details of the measurement have been published (Szabolcs, Kövér, 1966). The procedure is based on the use of a cellulose column and ^{45}Ca. Results are expressed in μmoles $CaCl_2$ per mg protein.

ATPase activity. – ATPase activity was determined in the same incubation mixture which we used in uptake experiments with ^{45}Ca (0.05 M KCl, 0.002 M $MgCl_2$, 0.002 M potassium oxalate, 0.002 M ATP and 0.01 M TRIS/maleate buffer, pH 7.0). The assay was carried out at 23°C. Protein concentration was different with different centrifugal fractions. 1-ml aliquots were withdrawn from the incubation mixture at different time intervals, the reaction was stopped by the addition of trichloroacetic acid, and the concentration of inorganic phosphorus (P_i) was determined according to Taussky and Shorr (1953).

Cholinesterase activity – Cholinesterase activity was determined at 37°C. Composition of the incubation mixture was as follows: 0.05 M KCl, 0.002 M $MgCl_2$, 0.002 M potassium oxalate, 0.001 M acetylcholin chloride and 0.01 M TRIS/maleate buffer, pH 7.0. Acetylcholin content of aliquots of the incubation mixture was determined at 0 and 30 min according to Hestrin (1949).

Commercial ATP (Reanal, Budapest) was purified on Dowex 50 ion exchanger by the method of Seidel and Gergely (1963). $^{45}CaCl_2$ was purchased from the Isotope Institute of the National Atomic Energy Committee. The specific activity of this preparation was 423 μC per 0.12 mM.

Protein content of SRF was determined with the micro-Kjeldahl method.

Table 1

Distribution of protein, cholinesterase (ChE) activity, ATPase activity and the capacity to take up Ca^{++} in the subcellular fractions of rabbit muscle obtained from animals of different ages

Experimental details are given in *Methods*

Age of animals, days	Fraction	g protein / 100 g muscle	ChE activity μmoles ACh/ mg protein/h	ATPase act. μmoles P_i/mg protein/5 min	Ca-uptake μmoles Ca/mg protein	
					+oxalate	−oxalate
5	RF. I	2.01	0.70	0.85	0.01	—
	RF. II	0.155	2.70	1.80	0.06	—
	RF. III	1.723	0.45	0.25	0.00	—
	RF. IV	0.12	3.25	2.16	0.06	—
10	RF. I	2.16	0.80	0.80	0.06	—
	RF. II	0.21	1,80	1.75	0.27	—
	RF. III	1.82	0.50	0.33	0.007	—
	RF. IV	0.105	2.93	2.20	1.45	0.032
27	RF. I	1.96	0.44	1.10	0.20	—
	RF. II	0.128	0.61	1.40	0.58	—
	RF. III	1.75	0.14	0.39	0.00	—
	RF. IV	0.11	0.53	1.20	1.74	0.042
Fully grown	RF. IV	0.15	0.35	1.65	2.40	0.05

Results

Table 1 shows the protein content, ATPase activity, cholinesterase (ChE) activity and Ca-uptake of some centrifugal fractions of rabbit muscle. The animals used in these studies were offsprings of the same mother, and they were 5, 10 and 27 days old, respectively. Protein content of the different fractions does not show a marked variation with the age of the animals. The activity of the enzymes ChE and ATPase is significantly lower in preparations obtained from 27-day-old

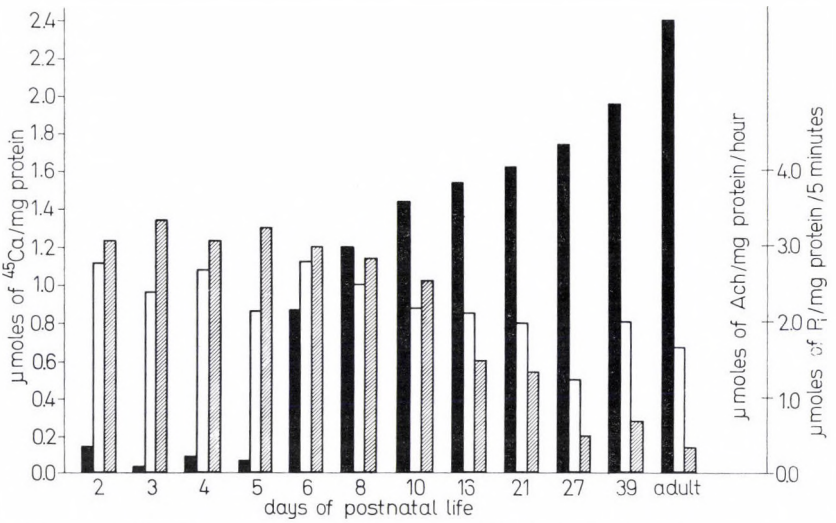

Fig. 1. Alteration of ATPase activity, cholinesterase (ChE) activity and the capacity to take up Ca^{++} of rabbit muscle SRF in the course of postnatal development. — Experimental details are given in *Methods*. ■ Ca-uptake; □ ATPase activity; ▨ ChE activity

animals, than in those isolated from 5—10-day-old ones. Ca-uptake of fractions RF. II and RF. IV shows a progressive increase from the 5th to the 27th day of postnatal life.

These results indicated some connection between the simultaneous alteration of the two enzyme activities and Ca-uptake. Therefore these changes were studied in more detail.

Ca-uptake, ATPase activity and ChE activity of SRF (RF. IV). — The rabbits used in these studies all derived from our own brood, but from different mothers. Groups of younger animals of the same age were used in each experiment. Fig. 1 shows the time course of changes in ATPase activity, ChE activity and Ca-uptake of SRF. It is seen that a low level of Ca-uptake is characteristic for the first days of postnatal life. It is interesting to note another characteristic of this stage of postnatal development, namely that the ratio of ATPase and ChE activity is higher than with older animals. For instance, SRF obtained from two-day old animals takes up 0.15 µmole ^{45}Ca per mg protein, has an ATPase activity of 2.8 µmoles P_i per mg protein per 5 min, and a ChE activity of 3.1 µmoles ACh per

mg protein per h; the corresponding values with SRF obtained from three-day-old animals are 0.04 μmoles ^{45}Ca, 1.95 μmoles P_i and 3.35 μmoles ACh, respectively. Beginning with the sixth day of postnatal life there is a sharp increase in Ca-uptake, while ATPase and ChE activities are decreasing.

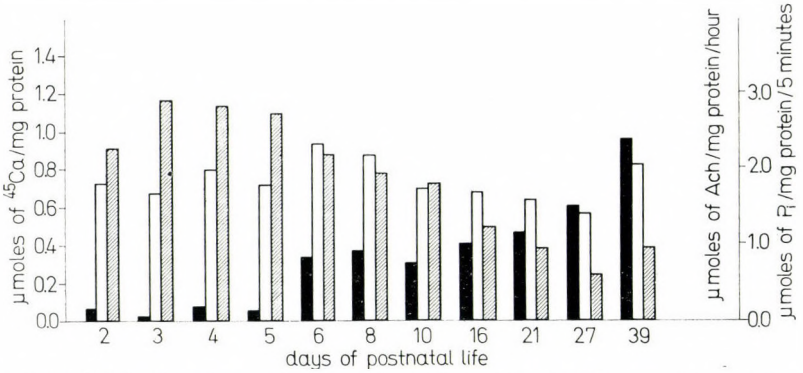

Fig. 2. Alteration in the course of postnatal development of ATPase activity, cholinesterase activity and the capacity to take up Ca^{++} of rabbit muscle fraction RF. II. (8000 × g). — Experimental details are given in *Methods*. ■ Ca-uptake; □ ATPase activity; ▨ ChE activity

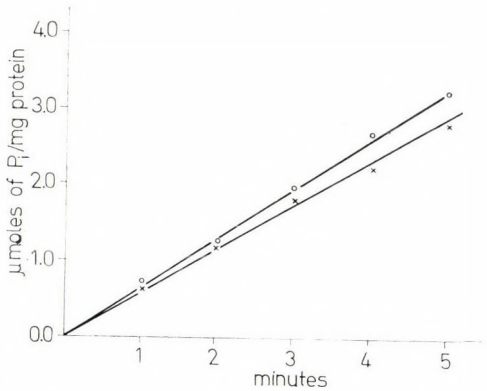

Fig. 3. ATPase activity of SRF obtained from six-day-old rabbits. — ×—× ATPase activity without Ca^{++}; ○—○ ATPase activity in the presence of 0.12 mM $CaCl_2$. Composition of the incubation mixture is given in *Methods*

Ca-uptake, ATPase activity and ChE activity of RF. II. — Fig. 2 shows the observed changes in RF. II, which is the fraction sedimenting at 1 000 – 8 000 × g. It is seen that the time courses show the same tendency as that observed in SRF (Fig. 1), however, the initial specific activity of ATPase and ChE is lower than in SRF. Also, the increase in Ca-uptake during the 6th to 8th day of postnatal development is less dramatic than in SRF. From the above comparison of SRF and RF. II we tentatively conclude that Ca-uptake of RF. II is due to a contamination with SRF of this fraction, the main components of which are mitochondria.

Activation by Ca^{++} of the ATPase of SRF. — Fig. 3 shows the ATPase activity of 6-day-old rabbits, Fig. 4 that of 27-day-old animals, and Fig. 5 that of fully grown animals. There is a slight activation of ATPase by Ca^{++} even with SRF prepared from 6-day-old animals (Fig. 3). It should be remembered that this

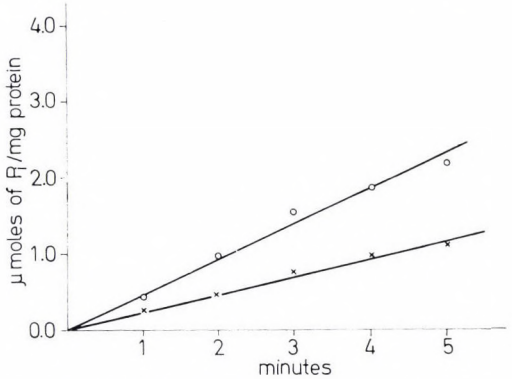

Fig. 4. ATPase activity of SRF obtained from 27-day-old rabbits. — ×—× ATPase activity without Ca^{++}; ○—○ ATPase activity in the presence of 0.12 mM $CaCl_2$. Composition of the incubation mixture is given in *Methods*

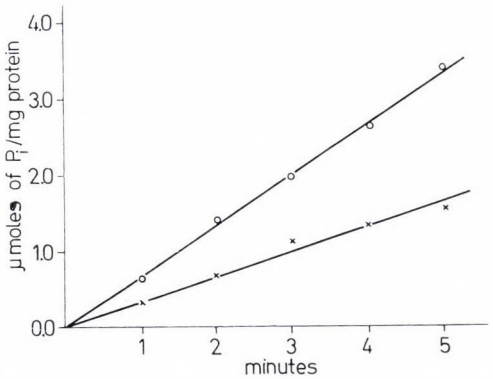

Fig. 5. ATPase activity of SRF obtained from fully grown rabbits. — ×—× ATPase activity without Ca^{++}; ○—○ ATPase activity in the presence of 0.12 mM $CaCl_2$. Composition of the incubation mixture is given in *Methods*

is the age at which there is a sharp increase in Ca-uptake (Fig. 1). It is also seen from a comparison of Figs 3, 4 and 5 that the activating effect of Ca^{++} increases until an age of about 27 days is reached.

Discussion

We have indicated in the Introduction that in the course of ontogenesis there is a progressive change in the character of the function of striated muscles. During the first days of postnatal life muscle function is predominantly tonic,

and this changes to a tetanic character with age. This functional character of muscle is reflected in acetylcholin sensitivity, acetylcholin content, ChE activity, contractile structure, differentiation of the neuro-muscular junction, and so on (Ginetsinki, Michelson, 1937a,b; Martini, Torda, 1937; Lissák, Endrőczi, 1950; Hermann, Nicholas, 1948; Robinson, 1952; Varga et al., 1957; Kovács et al., 1961; Kövér, Kovács, 1961). Sréter and Gergely (1964) found characteristic differences in the uptake of Ca^{++}, ATPase activity and even in the electron microscopic structure of SRF prepared from the red (tonic) and white (tetanic) muscles of fully grown rabbits. They have shown that white muscle is characterized by lower ATPase activity, and higher Ca-uptake of SRF. This indicates a strict correlation between the functional character of the muscle and the properties of SRF. Obviously, these observations have a bearing on the postnatal changes described in this paper. Figs 1 and 2 show that the ATPase activity of SRF declines from the 6th day of postnatal development, while it is apparent from Fig. 3 that between the 6th and 8th days there appears an activation of ATPase by Ca^{++} as well as a significant uptake of Ca^{++} by SRF. There is a 10 per cent activation of ATPase by Ca^{++} on the 6th day and a 80–90 per cent activation on the 27th day. In our opinion, this indicates that during this time the active center of ATPase is transformed into another steric structure which is favourable with respect to an effective interaction with Ca^{++}, and ATP, respectively. Further, there is a progressive decrease in the basal ATPase activity (determined in the absence of Ca^{++}) of SRF between the 6th and 27th days, and this decrease in the basal activity is in fact roughly compensated by the enhanced activation of the enzyme by Ca^{++}. According, an energetically favourable situation is created by a structural rearrangement at the molecular level which takes place in an early stage of postnatal life. This conclusion is in agreement with data by Sréter and Gergely (1964) according to which ATPase activity of SRF from tetanic (white) muscle is comparatively low, more over even this low activity is greatly reduced in the presence of EGTA, while the ATPase activity of SRF prepared from tonic muscle has a higher basal level and is not affected by the addition of EGTA.

In our studies on fish muscle SRF we found that during the so-called "ageing" process the increase in ATPase activity is accompanied by a parallel increase in cholinesterase activity (Szabolcs et al. 1966b). This and some further observations from other laboratories (Karnovsky, 1964; Miledi, 1964; Ulbrecht, Kruckenberg, 1965) indicate that the molecule of cholinesterase is a structural component of SR. In this case, "ageing" would represent another instance in which during the observed rise in cholinesterase activity SRF undergoes a structural rearrangement. This rearrangement is in fact a partial disorganization, which is also reflected in reduced uptake of Ca^{++}. Bennett (1960) suggested that a primitive organizational form of SRF is present in embryonal muscle and in the muscle of new-born animals. Our data show that this primitive form of SRF can be characterized by a high activity of both ATPase and cholinesterase and a hardly detectable Ca-uptake. Summarizing our observations, there is a correlation between the reduction of ATPase and cholinesterase activities and the enchancement of Ca-uptake in ontogenesis, but also a reciprocal correlation between an increase in these enzyme activities and a decrease of Ca-uptake in "ageing". We

suggest that all these properties of SRF indicate the grade of structural organization of SR. The data presented justify the conclusion that there is a correlation between the alteration of some functional characteristics of SR, and the alteration of the character of muscle function which occurs in the course of postnatal life.

Acknowledgement

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A Myofibrillar Protein Probably Localized in the Z-lines

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The dense substance of the Z-lines can be removed from the myofibrils by treatment with lipase at pH 6. The disappearence of the Z-substance from the striation pattern is accompanied with a simultaneous release of protein. The quantity of the released protein is not more than the amount of the Z-substance. The results of sedimentation, gel electrophoretic and spectrophotometric experiments are described.

Introduction

According to a number of recent reports (Ernst et al., 1958; Guba et al. 1960, Garamvölgyi et al., 1962) the Z-lines could be isolated from vertebrate and insect myofibrils as "Z-discs" (cf. Ernst, 1963). The method adopted for isolation (i.e. actomyosin extraction performed with Weber–Edsall's solution followed by mounting on electron microscopic supporting film) excluded the possibility that myosin, or actomyosin would be involved, but it has not been excluded that they may contain actin. Huxley (1963) suggested that tropomyosin may be the Z-substance, on the basis of the similar arrangement visible in the electron micrographs of Z-lines and tropomyosin crystals.

A method of isolation of myofibrils by tryptic digestion has been introduced by Schick and Hass (1949). The isolation of myofibrils by this method resulted in the disapprearence of the Z-lines (Ashley et al., 1951). Guba (1954) treated myofibrils with lipase and observed the lack of I-bands together with the Z-lines. It was concluded from these observations that they contain lipids. Garamvölgyi (1961) carried out enzymatic treatments with both trypsin and lipase on insect flight muscle and has come to the conclusion that both kinds of enzymes remove the dense material of the Z- and M-lines without breaking the myofibrils (cf. Garamvölgyi, 1965). From these data we concluded that the Z-lines might be built up from protein and lipid constituents and that the decomposition of one of the constituents may result in the collapse of the whole structure. Thus, we expected the release of the protein constituent of the Z-lines after treating the purified myofibrillar suspensions with lipase. Results of preliminary experiments performed on bee muscle, at pH 7 have already been reported (Garamvölgyi, 1967). As will be shown we succeeded in extracting protein by the procedure outlined above, with a concomitant removal of the Z-substance, while in the control samples (in the last supernatant before the treatment) no protein was found. These observations are supported by ultracentrifugal analysis.

Materials and Methods

The experiments were performed with rabbit psoas muscle. Myofibrillar suspensions were prepared either in a Turmix, or in an ice-cooled MSE blendor, in a physiological solution, or in a standard KCl solution buffered with 0.0067 M phosphate buffer, pH 7 (Huxley and Hanson, 1957). The suspensions were checked by phase contrast microscopy. In order to remove the water-soluble sarcoplasmic

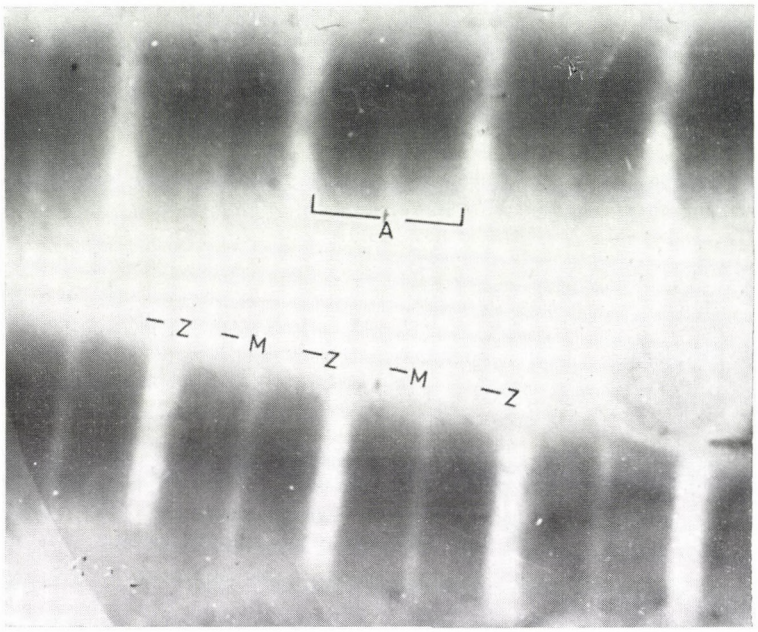


Fig. 1. Low magnification electronmicrograph of two rabbit myofibrils treated with lipase. Dried, unfixed, unstained. Note the lack of the Z- and M-lines

proteins the medium was changed 4–5 times by centrifugation in a refrigerated centrifuge. By centrifugation the coarse connective tissue elements were also removed. After stirring in ice for half an hour the medium was exchanged with 300 ml of 0.067 M phosphate buffer pH 6. The washing was repeated 5–6 times. 9–10 centrifugations were performed. The last sediment was resuspended in 100 ml of the same buffer and stirred in ice for 15 to 20 minutes. 20 ml of the suspension was kept for control. To the residual suspension 20 ml of 0.1 per cent lipase solution in pH 6 phosphate buffer was added thus bringing the final enzyme concentration to about 0.02 per cent.

The control as well as the test suspension were stored at 0°C overnight. The myofibrils were removed by a last centrifugation and those of the digested sediment were checked in the phase constrast microscope and in some cases in the electron microscope, too (Fig. 1). The protein content of the supernatant was determined spectrophotometrically by the biuret reaction. The yield was about

100 ml solution containing 1–1.5 mg protein per ml. If higher concentrations were needed, the solutions were dialyzed against a 50 per cent dextran solution in pH 6 phosphate buffer of 0.0067 M.

The enzyme preparation used was liophilized pancreatic lipase (Reanal Fine Chemicals Ltd. Budapest).

For sedimentation analyses a Beckman–Spinco Model E ultracentrifuge equipped with a synthetic boundary cell–capillary type was used. The usual speed was 50 740 r. p. m. and photographs were taken in 4, or 2 minute-intervals starting from 2 or 3 minutes after full speed had been reached. The protein was stored in a refrigerator at least for one day before the sedimentation experiments were carried out. In other experiments we used a PhyWe-Göttingen ultracentrifuge, also equipped with a synthetic boundary cell. The photographs were taken in 5-minute intervals starting 15 minutes after 45 000 r. p. m. was reached.

Polyacrylamide gel electrophoresis was carried out according to a slightly modified method introduced to the Biochemistry Group, Department of Phylogenetics and Genetics, Eötvös Loránd University, Budapest (Bálint and Bíró, in preparation). The gel columns were photographed and evaluted in a Schnellfotometer III of Carl-Zeiss, Jena, or in a Kipp Delft-Holland automatic recording photometer.

The ultraviolet absorption spectra were taken with a Unicam SP 700 type recording spectrophotometer using resolution 3, scan speed 1 and chart speed 90 in per minute.

The solutions containing 0.2 mg protein/ml were assayed in 1 cm quartz cells.

Results

The released protein seems to have a fairly low molecular weight. The sedimentation records indicate low sedimentation constants of about $S = 3 \times 10^{-13}$. The sedimentation patterns indicate the presence of a single peak (Fig. 2) which could not be separated into two, or more distinct ones, although the assymetry of the peak indicates a certain degree of heterodispersity. We do not attribute absolute significance to the obtained sedimentation constants because of the possibility of protein-lipid interactions. It is noteworthy that after storage of the protein at room temperature, for 2–3 hours, a marked flotation was observed instead of sedimentation.

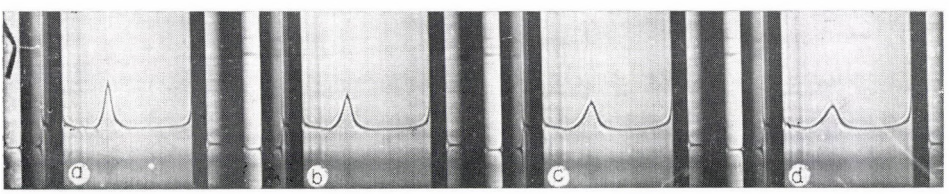


Fig. 2. Sedimentation patterns of the released protein. Concentration: 2.5 mg per ml, t = 17.5 C°, Philpot angle: 50°. Photographs taken in intervals of 4 minutes from 3 minutes after reaching 50,470 r. p. m. $S = 2.72 \cdot 10^{-13}$

By polyacrylamide gel electrophoresis we obtained one main component and 2, or 3 smaller ones (Fig. 3). In the control preparations, stored without previous digestion sometimes a single minor component was detected which does not correspond to the major component of the test solution. The comparison of the electrophoretic patterns of the control and the test solutions eliminates the objection that the release of the protein may be simply the result of extraction and is not due to the enzymatic treatment. The protein content of the test solutions due

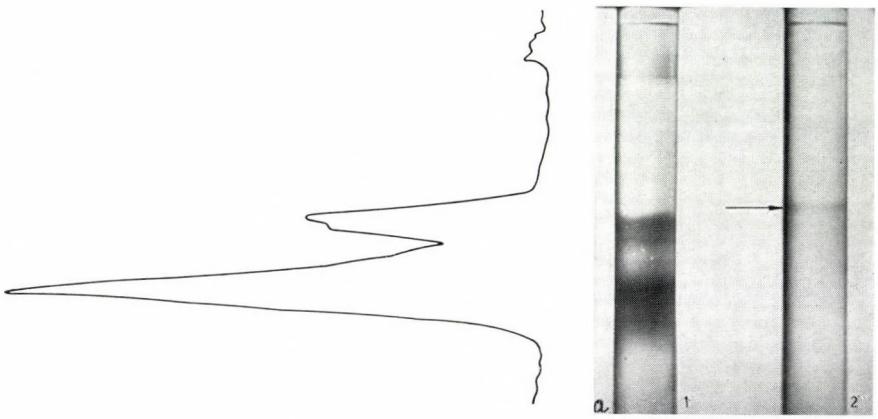
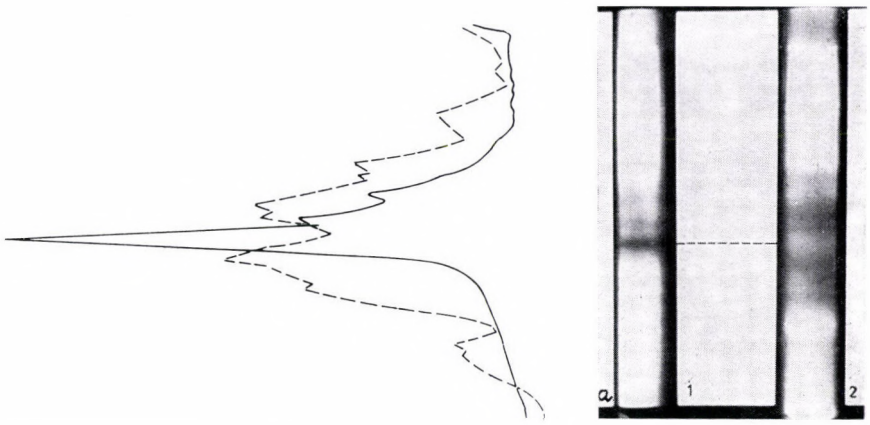


Fig. 3. *a)* Polyacrylamid gel electrophoresis patterns of the released protein (1) and of the undigested control (2) (arrow). *b)* Densitometric tracing of gel column (1) of Fig. 3a

to the enzyme preparation added is also negligible from this point of view. In some controls containing only the corresponding amount of lipase an extremely pale component appeared near the bottom of the column, which could not be reproduced photographically.

In the gel electrophoretic experiment presented in Fig. 4 we compared the pattern of the protein released by lipase treatment with that of unpurified F-actin prepared from acetone dried muscle powder, polymerized and digested with lipase under the same conditions as myofibrils were treated in the usual experiments. It should be pointed out that the peak probably representing the Z-line protein corresponds to a minimum on the densitometric tracing of digested F-actin (Fig. 4).

The ultraviolet absorption spectra (Fig. 5) taken at pH 6 exhibited a maximum at 267–268 mμ, sometimes extending from 267 to 273 mμ. The spectra taken at pH 13 did not exhibit any marked maximum. The difference spectra had two maxima, at 240–244 mμ and at 294–296 mμ. The position of the minimum corresponded to 276–277 mμ. The value of the specific extinction was $E_{1cm}^{1\%} =$



b a

Fig. 4. *a)* Gel electrophoresis patterns of the released protein (1) and of the product of lipase-digestion of unpurified F-actin (2). *b)* Densitometric tracing of gel columns 1 (continuous) and 2 (interrupted) of Fig. 4.a. Note the peak of curve 1 to correspond to a minimum of curve 2

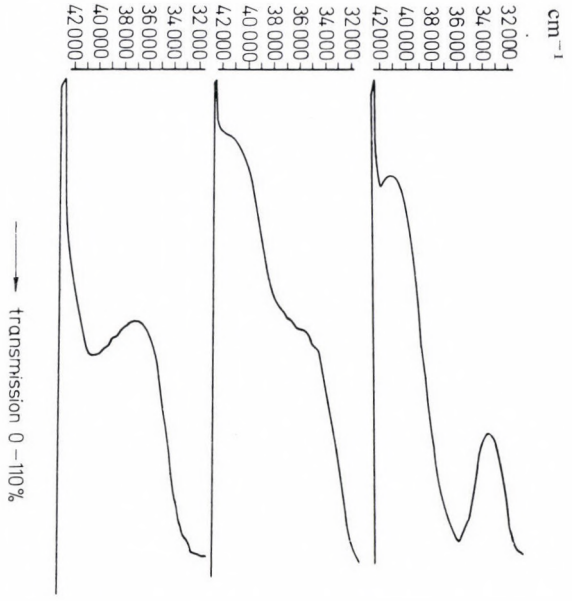


Fig. 5. Ultraviolet absorbtion spectra of the released protein. Right: difference spectrum, middle: pH 13, left: pH 6. Concentrations: 0.2 mg per ml

14.7 (at 267 mμ). The amount of tyrosine and tryptophane was calculated according to Beaven and Holiday (1952) from the extinction at 280 and 294 mμ. 34–35 moles tyrosine and 16 moles tryptophane were found per 10^5 g protein. These values are very high as compared to those published for fibrous muscle proteins (Kominz et al., 1957). It is also clear that the maximum at pH 6 is shifted towards lower wavelengths in relation to the minimum of the difference spectrum. This fact, as well as the lack of a maximum in the spectrum at pH 13, might be explained by the presence of bound nucleotides.

Discussion

The amount of protein released by lipase is not more than that found for the Z-substance in interference microscopic measurements (i.e. 6.2 per cent of total myofibrillar substance, Huxley and Hanson, 1957). This can be calculated from the weight of a pair of carefully prepared mm psoas (25 g, average of 27 muscles) and from the amount of the released protein (150 mg, maximum yield). If we accept that the soluble fraction (i.e. the sarcoplasmic proteins) represents about 30 per cent of total muscle protein (Hasselbach and Schneider 1951, Huxley and Hanson, 1957) the protein released by lipase represents 4.6 per cent of total myofibrillar proteins. This is commensurable with the protein content of Z-lines.

In order to be able to assess the effect of the eventual proteolytic contaminations we used the lipase itself in a very low concentration and performed the digestion at pH 6 i.e. very far from the pH optimum of trypsin. At this pH trypsin has been found practically ineffective on myosin at low ionic strength (Bíró, 1966). (To eliminate any chance that the protein released by lipase treatment arises from the myosin moiety of the myofibrillar structure, experiments are in course with myosin-extracted myofibrils. The results of these experiments will be discussed in detail in another publication.)

The question arises whether or not the released protein is identical with actin. This could be expected on the basis of our previous work (Garamvölgyi, 1965) which has shown the lack of secondary filaments after a treatment with lipase. However, these latter experiments were done on insect muscle, at pH 7, with higher lipase concentration (0.05 per cent) and much more dilute suspensions, corresponding to much higher enzyme : substrate ratios. In the present experiments this supposal was not verified. The amount of released protein was found to be very low as compared to that of actin (Huxley and Hanson, 1957). Moreover, the gel electrophoresis patterns (Fig. 5) do not support the idea that the bulk of liberated protein should be identical with actin. The smaller components may, however, represent one, or another fragment of actin. One of the smaller components might be identical with the substance of the M-line.

A number of papers [Ebashi, 1967; Masaki (cited by Ebashi, 1967) and that of the team of Mommaerts (Briskey et al., 1967)] suggested that α-actinin might be identical with the Z-line protein. However, it is necessary to check whether or not the method used for the preparation of actinin removes the Z-lines. If this

question remains unsolved, no final conclusion as to the localization can be reached. At any rate, the substance of the Z-line represents a problem of increasing importance in the biochemistry of myofibrillar proteins.

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Effect of Secondary Fluorescence on the Emission Spectrum and Quantum Yield of Fluorescence in Chlorophyll-a Solutions and Algal Suspensions

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The spectral effect of secondary fluorescence of chlorophyll-a in benzene is negligible up to $c \times d = 10^{-5}$ mole. cm/l (c and d are concentration and layer thickness of the solution). Under the same conditions the effect of secondary fluorescence on the absolute value of the intensity of fluorescence is, however, about 10 per cent. This effect is negligible only up to $c \times d = 10^{-6}$ mole. cm/l allowing for a difference of 2 per cent between the measured and true intensities. Practically the same results are obtained with chlorophyll-*a* in other solvents. In suspensions of Chlorella, Anacystis and Porphyridium there is no spectral effect of the secondary fluorescence and the effect on the quantum yield of fluorescence is negligible (less than 1 per cent even in suspensions of optical density of 0.5). From the saturation of the secondary fluorescence, an absolute maximum spectral effect of 5—6 per cent is estimated for the secondary fluorescence and 18 per cent for the quantum yield in chloroplast.

1. In complicated organic compounds of biological interest, the absorption and fluorescence spectra of their solutions strongly overlap. As a result, the fluorescence originating in the solution is reabsorbed and the measured intensity of fluorescence is smaller in this (overlap) spectrum range than the true intensity. Förster (1951) and Duysens (1952) have pointed out that the reabsorption can be taken into consideration by simple calculations.

The reabsorption of fluorescence photons give rise to new excited molecules. The secondary fluorescence of these photons has been studied by Budó and Ketskeméty (1956, 1957), Budó et al. (1957), Lavorel (1957), Agranovitch and Konobejev (1959), Melhuish (1961), Kravtsov (1963) and Rohatgi and Singhal (1966). In this paper we report investigations on the secondary fluorescence in chlorophyll *a* after the method of Budó and co-workers (Budó, Horvai, 1956; Dombi, Horvai, 1956; Budó, Ketskeméty, 1962). We have used this method bacause all the parameters and functions (needed for calculations by Budó's method) are available in a tabulated form in our laboratory (Szeged) and because some of the methods listed above are either tedious or inexact. Budó and Ketskeméty (1956) have shown that in case of longitudinal observation from the side of excitation (i.e. from the front face) the true fluorescence spectrum $f(\lambda')$ is correlated with the

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measured spectrum $dB(\lambda')$ in the following way:

$$dB(\lambda') = \frac{\rho}{4\pi n^2} E(\lambda)\, \Phi(\lambda) f(\lambda') \frac{\alpha}{\alpha+\beta} \frac{1-e^{-(\alpha+\beta)}}{1-\kappa} d\lambda'. \tag{1}$$

In equation (1), $E(\lambda)$, $\Phi(\lambda)$, n, λ and λ' denote the intensity of the exciting light, the true quantum yield of fluorescence, the refractive index of the solution, the wavelength of absorption (λ) and emission (λ'), respectively. $\rho < 1$ is a factor accounting for the reflection from the front surface of the cuvette. α and β are related to concentration (c), layer thickness (d) and molar decadic extinction coefficient $\varepsilon(\lambda)$ as shown below:

$$\alpha = 2.3 \times \varepsilon(\lambda)\, cd; \qquad \beta = 2.3 \times \varepsilon(\lambda')\, cd; \tag{2}$$

κ in equation (1) is the ratio of the intensity of secondary fluorescence (S) to the intensity of primary fluorescence (P). This equation becomes identical with the Förster-equation (Förster, 1951; equations 8 and 14 on page 41) for reabsorption when the secondary fluorescence is negligible compared to the primary fluorescence ($S \ll P$, $\kappa \to 0$).

When the directions of excitation and observation form an angle of ϑ with the normal of the front face of the cuvette, the path length of the exciting light and/or the thickness of the observed layer is increased. This can be considered by taking $d/\cos\left(\text{arc sin}\,\frac{\sin\vartheta}{n}\right)$ instead of d in Equ. (2). (For small ϑ, $d/\cos\vartheta/n$ should be introduced for d in Equ. (2) as a sufficient approximation; this holds if $\vartheta < 15°$ and an error of 1–2 per cent is allowed.)

According to the calculations in (9) and (11):

$$\kappa = \int_0^\infty \Phi(\lambda'') f(\lambda'') M(\alpha, \beta, m)\, d\lambda'', \tag{3}$$

where M is a function of the depth (d) and the radius (R) of the cylindrical cuvette and

$$\gamma = 2.3 \times \varepsilon(\lambda'')\, cd; \qquad m = \frac{R}{d}. \tag{4}$$

Actual measurements [i.e. in (12)] showed that κ increases with concentration and layer thickness from $\kappa = 0$ up to an upper limit from which it becomes constant.

On neglecting κ in Equ. (1) we obtain the quantum yield $\Phi^\circ(\lambda)$ and fluorescence spectrum $f^\circ(\lambda')$ corrected for reabsorption. Consequently, the true fluorescence spectrum and the true quantum yield are given by the following equations:

$$f(\lambda') = N(1-\kappa) f^\circ(\lambda') \tag{5}$$

(where the constant N is given by the condition $\int_0^\infty f(\lambda')\, d\lambda' = 1$)

$$\Phi(\lambda) = \frac{\Phi^\circ(\lambda)}{1-\kappa}. \tag{6}$$

Instead of this a somewhat more complicated but more exact relationship is given by Budó, Dombi and Szöllősy (1956).

2. Our measurements and calculations were carried out for a solution of chlorophyll *a* in benzene and for suspensions of *Chlorella pyrenoidosa*, *Anacystis nidulans*, and *Porphyridium cruentum*. The corrections for secondary fluorescence were given at different concentrations or optical densities [$\alpha(\lambda)$]. The details of the experimental work are given by Szalay et al. (1967) and those of the spectrofluorometer by Govindjee and Yang (1966) and Govindjee (1966). The wavelengths of excitation are shown in Table 1. In the concentration range where the

Table 1

	λ_{exc} A	λ_{max} A	Φ_{max}	$\kappa(\alpha, 0)$	$\kappa(\alpha, \beta_{max})$
Chlorophyll-*a*	4 320	6 665	0.300	0.190	0.121*
Anacystis	4 400	6 750	0.002	0.000_6	0.005
Chlorella	4 400	6 750	0.030	0.017	0.014
Porphyridium	4 300	6 793	0.006	0.002	0.008

λ_{max} — location of the absorption maximum in the overlap region.
* This figure refers to a value of $\beta_{max} = \alpha$

effect of secondary fluorescence is not negligible (e.g. for higher concentration) κ does not depend on the radius of cuvette (more precisely on the ratio $m = R/d$) to a great extent. According to the Equs (2) and (4) κ depends on the excitation coefficients at the wavelength of excitation and observation and on the product $c \times d$, or (in algal suspensions where the extinction coefficient is unknown) on the optical density.

Chlorophyll-*a* in solution

3. κ as a function of $\log c \times d$ for chlorophyll-*a* dissolved in benzene is shown in Fig. 1. The exciting wavelength $\lambda = 4320$ Å and therefore $\alpha(\lambda) = 2.3 \times \varepsilon(4320\ \text{Å}) \times c \times d$. Since $\varepsilon(4320) = 1.02 \times 10^5$ (see Seely and Jensen, 1965), $\alpha(\lambda) \approx 2.3 \times 10^5\ cd$. The α-values are given in terms of multiples of β-values, and we assumed that the quantum yield, $\Phi(\lambda) = 0.3$ [Weber and Teale, (1957) and Latimer et al. (1957)] was independent of the concentration. On account of the concentration quenching $\Phi(\lambda)$ begins to decrease with increased concentration from about $c = 2.10^{-3}$ mole/l (Rabinowitch, 1951). This means that the κ-functions are exact only for concentrations of $c < 2.10^{-3}$ mole/l. At higher concentrations the effect of secondary fluorescence with κ-functions calculated with $\Phi(\lambda) = 0.3$ is overestimated. This overestimation, however, should not be too much because self-quenching reduces the intensity of the fluorescence of chlorophyll-*a* in butyl ether only to about 70 per cent of the maximum (Rabinowitch, 1951), i.e. to about $\Phi = 0.20$ instead of $\Phi = 0.30$ even at a high concentration (10^{-2} mole/l).

In order to correct a given experimentally obtained result (e.g. a fluorescence spectrum or a quantum yield) for secondary fluorescence, first the correction for reabsorption should be made. Then the $\kappa(\alpha,\beta)$-values should be taken for the given $c \times d$ product from Fig. 1 and plotted versus $\beta(\lambda')$. From this new plot the

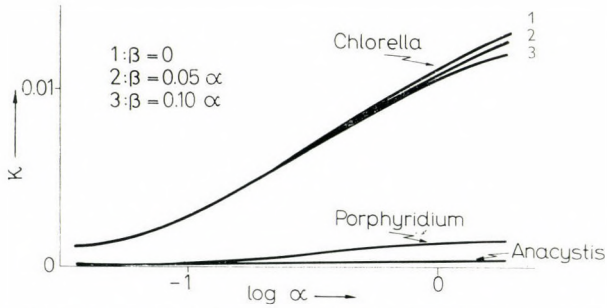

Fig. 1. The correction term κ (α, β) for calculating the true fluorescence characteristics as a function of log $c \times d$. (c — concentration of solution, d — layer thickness). α and β denote the optical densities of the solution at the excitation and observation wavelengths

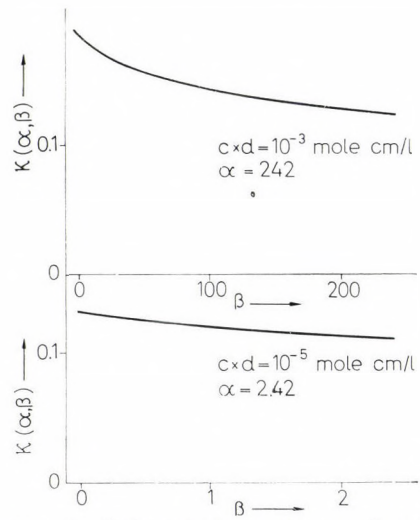

Fig. 2. The correction term κ (α, β) for calculating the true fluorescence characteristics as a function of $\beta = 2.3\ \varepsilon\ (\lambda') \times c \times d$ for two values of $c \times d$ (c — concentration of solution, d — layer thickness). and denote the optical densities of the solution at the excitation and observation wavelengths

values of $\kappa(\alpha,\beta)$ at the proper λ'-values can be read and the corrected fluorescence spectrum $f(\lambda')$ can be calculated with Equ. (5). (See Fig. 2 for $d = 0.1$ cm and $c = 10^{-4}$ or 10^{-2} mole/l). Similarly, from Equ. (6) the true absolute quantum yield can be calculated. These calculations were programmed and carried out by means of a Minsk-3 computer in the Laboratory for Cybernetics of the University of Szeged.

The spectral effect of secondary fluorescence in the case of chlorophyll *a* in solution is shown in Fig. 3. The corrected spectra are labeled f_1^0, f_2^0, f_3^0, whereas the uncorrected ones are labeled as f_1^*, f_2^*, f_3^*. All the spectra were "normalized" at 700 mμ since no spectral effect of the secondary fluorescence is expected in this wavelength range. The emission spectra corrected for reabsorption are given for $d = 0.1$ cm and for $c = 10^{-5}(f_1^0)$, $10^{-4}(f_2^0)$ and $10^{-2}(f_3^0)$ mole/l. [These were calculated from Equ. (5) assuming that the shape of the true spectrum was independent of the concentration.] In the concentration range considered this assump-

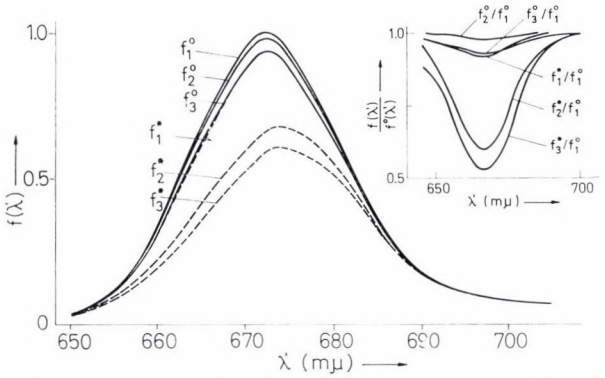

Fig. 3. The effect of reabsorption on the fluorescence spectrum of chlorophyll *a*. f_1^0, f_2^0, f_3^0: fluorescence spectra corrected for reabsorption at concentration of 10^{-5}, 10^{-4} 10^{-3} mole/l. f_1^*, f_2^*, f_3^*: uncorrected fluorescence spectra for concentrations of 10^{-5}, 10^{-4}, 10^{-3} mole/l, layer thickness 0.1 cm

tion may be valid. No significant difference between the absorption spectrum of chlorophyll *a* in dilute butyl ether solution and in a solution of 2.10^{-2} mole/l is observed (Rabinowitch, 1951; p. 774). However, Brody and Brody (1961) have reported increased absorption at 6820 Å in 3×10^{-2} M chlorophyll *a* in ethanol when compared with 10^{-5} M chlorophyll *a* in ethanol.

All the spectra denoted by f_1^0, f_2^0 and f_3^0 (Fig. 3) ought to coincide provided only the reabsorption exists. Since f_1^0, f_2^0 and f_3^0 do not coincide, there is the remaining spectral effect of secondary fluorescence. The shape of the true emission spectrum is not independent of the concentration as was assumed above; the measured spectrum does change with the concentration on account of reabsorption and at higher concentrations also because of secondary fluorescence. This spectral effect of the secondary fluorescence is, however, not great (see below). The insert in Fig. 3 shows the plot of f_2^0/f_1^0, etc. It shows that the spectral effect of secondary fluorescence does not exceed 2 per cent at 10^{-4} mole/l and $7-8$ per cent at 10^{-2} mole/l. Fig. 1, however, shows that the secondary fluorescence in chlorophyll-*a* solution (in benzene) does not exert a spectral effect up to a log $c \times d$ value of about -5.00. This means that in a layer thickness of 0.1 cm up to a concentration of 10^{-4} mole/l there is no spectral effect of secondary fluorescence.

The absolute value of the fluorescence intensity can be very much influenced by the secondary fluorescence. For $\kappa > 0.1$ for $d = 0.1$ cm and $c = 10^{-4}$ mole/l,

the true intensity of fluorescence is about 10 per cent less than the intensity of fluorescence obtained by correcting only for the reabsorption. Fig. 1 shows that $\kappa > 0.02$ in solution of $\log c \times d = -6.00$! This means that in a layer thickness of 0.1 cm, even at a concentration of 10^{-5} mole/l, a secondary fluorescence of about 2 per cent (in the absolute value of the intensity of emission) is found.

Chlorophyll-*a* in Algae

4. Calculations – similar to those with solutions – were made in the case of suspensions of *Chlorella pyrenoidosa*, *Anacystis nidulans* and *Prophyridium cruentum*. Since the true concentration of the pigment and the optical density value for cells are unknown, it is *not* possible to apply corrections for reabsorption and secondary fluorescence within single cells. However, it is possible to apply "outer" corrections for reabsorption and secondary fluorescence. The correction for secondary fluorescence given in the above manner yields the "true" emission spectrum of a suspension which is considered to be a true solution in the sense of the two consecutive applications of Beer's law by Rabinowitch (1951).

On assuming a concentration of 10^{-2} mole/l in the chloroplast (or the whole cell of Anacystis, since blue-green algae do not contain chloroplast) a further correction would be necessary. For 10^{-2} mole/l chlorophyll *a* and 10 μ maximum layer thickness in the chloroplast, $\log cd = -4.00$. According to Fig. 1, this is about the saturation of κ. This means that the maximum value of $\kappa = 0.18$ to 0.19 is attained for chlorophyll *a* for $\beta = 0$ (at a wavelength in emission where there is no absorption) and $\kappa = 0.12$ for $\beta = \alpha$ (at a wavelength in emission where the optical density is equal to that at the wavelength of excitation). Even if we assume a concentration of 10^{-1} mole/l, the situation does not change because the saturation of κ has already been attained. Consequently in any case (different concentration of chlorophyll and different size of chloroplast), the effect of secondary fluorescence on the spectral distribution of fluorescence should not exceed 5–6 per cent and that on the quantum yield should not exceed 18 per cent. This is a fairly conservative estimate.

On account of the small quantum yield of fluorescence by algae (Latimer, Bannister and Rabinowitch (1957)) the effect of secondary fluorescence was found to be comparatively small though the overlap of the two spectra is considerable. Fig. 4 shows the κ-factor as a function of α at different β-values. In Chlorella for $\log \alpha = -1.0$ ($\alpha = 0.10$), $\kappa = 0.003$, in Porphyridium, $\kappa = 0.000_4$ and in Anacystis much less. In all cases the effect of secondary fluorescence is negligible. If the optical density of suspension is as high as 0.50 ($\log \alpha = 0.70 - 1$), $\kappa = 0.010$ in Chlorella, $\kappa = 0.002$ in Porphyridium, and $\kappa = 0.000_3$ in Anacystis. There is no spectral effect of secondary fluorescence. This means that even in these dense suspensions the effect of secondary fluorescence on the quantum yield is less than 1 per cent. Of course, we have considered only the "outer" correction. The comments made above are valid for the "inner" correction.

Table 1 summarizes some of the data. $\kappa(\alpha, 0)$ denotes the κ-function at a wavelength of observation where there is no reabsorption, $\kappa(\alpha, \beta_{max})$ means a

κ-function for a wavelength of observation where the reabsorption is maximum; α represents the optical density for the exciting wavelength.

The effect of secondary fluorescence is practically independent of the observation wavelength. Thus the shape of the emission spectrum is not influenced by

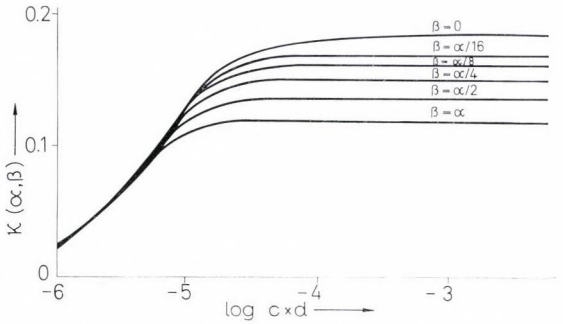

Fig. 4. The correction term κ (α, β) for calculating the true fluorescence characteristics as a function of α (optical density of the suspension) at different β-values (β-optical density at the observation wavelength) 1 : $\beta = 0$; 2 : $\beta = 0.05$; 3 : $\beta = 0.10$ α

secondary fluorescence even at longer waves but the absolute quantum yield is influenced, especially at higher optical densities.

Acknowledgement

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Strength-Duration Relation in the Nerve of the Freshwater Mussel

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The cerebrovisceral connective of the freshwater mussel *(Anodonta cygnea L.)* was stimulated with square wave impulses and the action potential recorded. An empiric approximation to the strength-duration relation, corresponding to the experimental values with an error less than 5 per cent, was obtained. The formula has a structure which is only partly exponential:

$$V = R\,[1 - d^{\beta d} \times e^{-2{,}3\alpha d}]^{-1}$$

The relation between some of the phenomenological theories of excitation and the relation obtained is discussed. It is assumed that the non-exponential structure is a consequence of the non-linear cable structure of the nerve.

Introduction

Numerous empiric and theoretical approximations to the strength-duration relation (SDR) characteristic of the excitation and excitability of different excitable tissues are known (Hoorweg, 1892; Weiss, 1901; Nernst, 1908; Blair, 1932a, 1932b, 1936; Rashevsky, 1936; Hill, 1935; Young, 1937; Nasonov, 1958). Recently, on the basis of Young's-model (1937), Fitz-Hugh (1966) has given an explicit formula for SDR. Its experimental control is difficult because of the highly indirect parameters.

In the present study we start from Blair's simple expression (1932). The insufficient agreement between the experiments and Blair's model will be pointed out. Furthermore, as an outcome of the experiments, we shall present an empiric modification of the model which is more precisely smoothed to the experimental average. A possible interpretation of the modification will be discussed to show how it can be related to other theories.

Method

The experiments were carried out on the cerebrovisceral connective (CVc) of *Anodonta cygnea L.*, stimulated with DISA Multistim equipment with constant voltage output. The arrangement of the silver electrodes used was constant (1 – 10 – 1 – 10 mm, see Fig. 1). For the leading off of the action potentials we used

the following system: DISA 14C0021 preamplifier ($2 \times 200\ M\Omega$; 12 pF; 2–500 cps) and DISA 51B01 amplifier with DISA Universal Indicator. Temperature: 24–28°C. During the recording the nerve was kept in liquid paraffin. When establishing the strength-duration relation (SDR) we did not measure the threshold in the most excitable fibers, but worked on the linear stretch of the activation strength curve between 40 and 80 per cent activations (Fig. 2). Under these con-

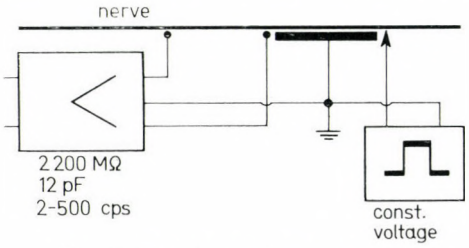

Fig. 1. Experimental arrangement

ditions the error of amplitude measurement was less than 1–2 per cent. For this reason the accuracy of voltage threshold measurement was better, too. The proximal grounded stimulating electrode was the cathode. This method of threshold determination did not influence the value of K measured by us.

Table 1

Experimental and theoretical threshold-voltage values and

For explanations

1	2	3	4	5	6
0.15	33.13	660	36.46	34.23	+10 per cent
0.20	26.85	565	27.53	26.61	+ 2.5
0.30	18.47	617	18.77	18.78	+ 1,62
0.50	12.05	558	11.22	12.13	— 6.9
0.70	9.19	527	8.14	9.28	—11.5
1	7.07	483	5.81	6.89	—18.9
3	3.02	413	2.21	2.96	—26.9
10	1.29	378	1.02	1.40	—19
30	0.85	310	0.76	0.90	—11.8
100	0.82	107	0.75	0.76	— 8.6
300	0.75	52	0.75	0.75	0
1000	0.75		0.75	0.75	0
d (msec)	V (volt)	10^4k	V_{Blair} (volt) $k = 660$	V_{theor} (volt)	δ Blair (volt) $k = 660$
average of deviations from the experiment					— 7.5 per cent
standard error of theory					±13.3 per cent
$\log \frac{\Sigma + \delta}{\Sigma - \delta}$					+ 0.84

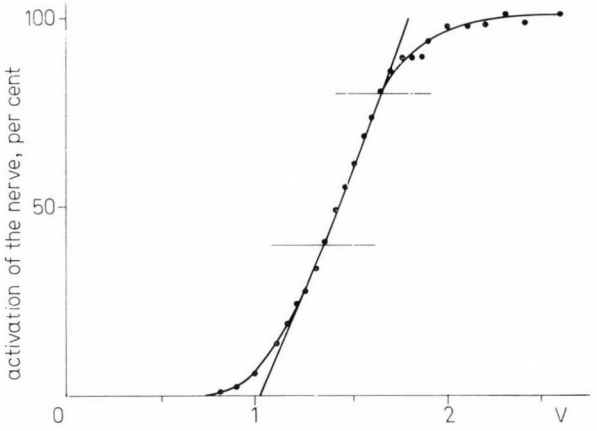

Fig. 2. Experimental work-points on the activation curve of stimulus intensity. Abscissa in volt, ordinate in per cent of total activation

Experimental Results

1. In Table 1 column 2 shows the mean values of threshold voltages obtained in 13 series of measurements. In column 1 the numbers indicate the duration of

their errors at different durations of impulses

see the text

7	8	9	10	11	12
+3.32 per cent	55.89V	+68.7	32.05	— 3.36 per cent	±35.3 per cent
—0.90	41.95	+55.16	25.61	— 4.62	±43.5
+1.68	28.10	+52.1	19.17	+ 3.79	±43.3
+0.66	17.02	+41.2	13.22	+ 9.71	±45.4
+0.98	12.27	+33.5	10.32	+12.29	±46.1
—2.55	8.71	+23.2	7.86	+11.21	±43.6
—2	3.16	+ 4.6	3.89	+28.8	±39.4
+8.53	1.26	— 2.4	1.98	+53.5	±37.9
+5.88	0.80	—15.9	1.29	+51.76	±35.5
—7.32	0.75	—18.3	0.96	+17.07	±31.4
+1.35	0.75	0	0.84	+13.51	±36.6
0	0.75	0	0.78	+ 4	±26.6
δ theor.	V_{Blair} (volt) $k = 391$	δ Blair $k = 391$	V_{HW} (volt)	δ HW	standard error of experimental results
+0.8 per cent		+20.2 per cent		+16.5 per cent	
±4.2 per cent		±36.5 per cent		±25.7 per cent	
+0.27		+ 0.88		+ 0.97	

pulses in msec. In column 3 the following values are given:

$$k = \frac{1}{d} \lg \frac{V}{V - R} \tag{1}$$

where stimulus voltage V (in volt) and duration d (in msec) belong together. R is the rheobasis-voltage measured at $d = 1$ sec.

According to Blair's model this quantity k is constant. But actually there is a strong negative linear correlation between k and the logarithm of d. Approximating to this relation the following regression equation ($k = \alpha - \beta \lg d$) is obtained

$$10^4 k = 501 - 168 \lg d \tag{2}$$

with a correlation coefficient of -0.989.

In column 4 of Table 1 we used the value k as a constant obtained with short duration and calculated V in this way. In column 5 the V values were calculated with k according to equation (2). In columns 6 and 7 the deviation of the obtained theoretical V-values from the experimental average is shown in per cent.

In columns 8 and 9 the Blair-approximation and its error are demonstrated, calculating with the mean of experimental k ($k = 391$). For comparison, columns 10 and 11 (values V and their errors) show the Hoorweg–Weiss hyperbolic formula ($V = ad^{-n} + R$) generalized by Nasonov. The parameters: $R = 0.75V$, $a = 7210$ (msec). ${}_mV = 7.21$ (msec), $n = 0.768$; $T_{100V} = 33$ μsec (calculated). The chronaxie is 8.7 ± 3.5 msec (*s.d.* = 40.3 per cent).

2. In column 12 the standard deviations (*s.d.*) of the experimental results are indicated. The circled theoretical values are out of the *s.d.* (in columns 9 and 11). The remaining values are within the *s.d.* It is remarkable that the *s.d.* of R is not greater than those of the other threshold values.

At the lower part of Table 1 (Columns 6, 7, 9 and 11) the average of errors and *s.d.* in the case of the four hypotheses used are given. The majority of deviations from the experiment lie within the experimental dispersion. The approximation carried out with $k = \alpha - \beta \lg d$ is the nearest to the average. In order to characterize the deviations we introduced the quantity $\log [\Sigma + \delta / \Sigma - \delta]$ where the + and − errors are compared. In the case of a suitable theory this value comes near 0. The best result was obtained when working with the equation (2).

3. The present method of SDR-determination includes the possibility of a hidden mistake, because the measurement set was directed from the values of shorter to those of higher duration. The decrease in k might be due to the effect remaining after a previous stimulus. In this case measuring from 1000 to 0.1 msec also a decrease of k should be expected. But we observed an increase in k with the decrease of d even in the latter case. In Fig. 3 we demonstrate cases with ascending and descending measurements. In the form of correlation there is no significant difference depending on the above-mentioned condition. Therefore it is proved that the demonstrated deviation k does not originate in the residual effect of a previous stimulus.

4. In order to lend further support to the deviation k we measured k at the two close-lying short time-values of 1 and 3 msec. On 37 nerves the threshold

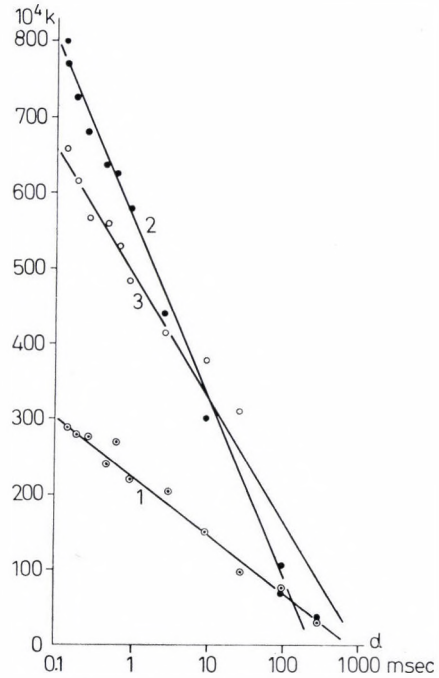

Fig. 3. Dependence of value of value k on impulse-duration (d) 1. descending measurement: $10^4k = 229—79 \lg d$ 2. descending measurement: $10^4k = 577—243 \lg d$ 3. ascending measurement: $10^4k = 501—168 \lg d$

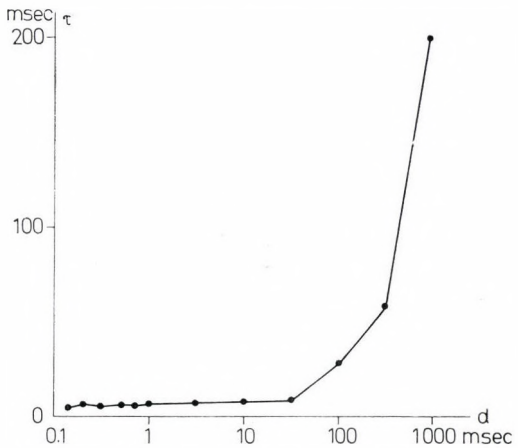

Fig. 4. Dependence of Blair-chronaxie (τ) on impulse duration (d)

voltages at these durations and the rheobasis were determined 1 – 10 times:

$$10^4\, k_{1\text{msec}} = 568 \pm 215 = 100 \pm 37.9 \text{ (per cent)}$$
$$10^4\, k_{3\text{msec}} = 464 \pm 173 = 81.7 \pm 30.5 \text{ (per cent)} \quad (3)$$
$$R = 0.84 \pm 0.35\, V \ (s.d. = 41 \text{ per cent})$$

The difference between k_1 and k_3 is statistically significant: $0.02 < P < 0.05$. As in 34 cases of the $37 k_3 < k_1$, and as the experiment is of self-control type, by calculating with relative numbers we obtained $0.001 < P < 0.01$. In both cases the Student test was applied.

Discussion

The SDR for square pulses in Blair's model (1932a, 1932b, 1936) is

$$I = R\,[1 - \exp(-d/T)] \tag{4}$$

where I = current-threshold; d = pulse-duration; $T = 0.435 k^{-1}$ (ms). It is 7–8 ms in our experiments.

Applying the k-decrease expressed by equation (2) we obtain a new SDR of non-totally exponential structure

$$V = R\,[1 - d^{0.0168d} \exp(-0.1152d)]^{-1}. \tag{5}$$

One of the possible causes of deviation is the difference between voltage-SDR and current-SDR due to the duration-dependence of resistance in the stimulating circuit. Though Rashevsky (1961) when discussing Blair's model does not believe that this distinction is important we could not exclude it to our satisfaction.

If we regard our SDR as equivalent to current-SDR, as Rashevsky did, we obtain a model similar to that of Young (1937). The decrease of K is the result of an accomodation-like process. Starting with Blair (1932a) from his equation for square wave impulses but modifying it by the introduction of (2)

$$\dot{c}_t = AV - kc = AV - \alpha c + \beta c \lg d \tag{6}$$

where $\dot{c}_t$ is the velocity of increase of the exciting agent concentration at the place of excitation; A, k, α, β, are parameters; V and d are voltage and duration.

A consequence of our experiments with increasing d is the appearance of a secondary process $+\beta c \lg d$. This accelerates start and slows down elimination. Compared with Blair's model the inertia of the nerve is elevated. For example the chronaxie of Blair is not a constant (Fig. 4), however, chronaxie as an empiric factor remains constant.

The criterion of excitation according to Blair is: $c > h$, where $R = kh/A$. As R is constant according to its definition ($R = \lim V$, if $d \to \infty$), equation (2) has further consequences; $1 - h$ increases with d and/or $2 - A$ decreases with increasing d.

Ad 1. The threshold flees before excitation. This forms the basis of accomodation, but our measurements are not determinations of the accomodation-constant, because the stimulus steepness is high and constant. The two-factor theories are based on this interpretation. They are equivalents of each other (Rashevsky, 1936; Young 1937; Hill, 1935; Offner 1937; Fitz-Hugh, 1966).

Ad 2. Production of the excitation substance is slowed down.

The obtained SDR-formula (5) is not exponential. Comparing it with the most general two factor-model, i.e. with that of Young (Young, 1937; Fitz-Hugh, 1966) we obtain

$$1 - d^{\beta d} \times e^{-2.3\,\alpha d} = C_1 - C_2 + exp(p_1 d) + exp(p_2 d). \tag{7}$$

Its evaluation for the parameters is a computer-task. Hodgkin and Rushton (1946) pointed out (cited by Noble, 1966) that a Blair-type relation could not be right if h was constant. The cause of this would be the non-exponential rising of voltage on point polarization. Taylor (1963) criticizes Lorente de No (1947), because he assumes a longitudinal polarization in the nerve to explain the non-exponential spreading. According to Taylor the connective tissues present in the nerve supplies a sufficient explanation.

In our opinion the non-exponential phenomena, thus our non-exponential SDR, are somewhat related to the non-linear cable properties. The part $d^{\beta d}/e^{2\cdot 3\alpha d}$ is similar to the gamma-function and this coincidence is suggestive of nonelementary mechanisms.

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Thermoosmotic Model Experiments on Anorganic Membranes and their Thermodynamic Interpretation

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In model experiments performed on semipermeable membranes of anorganic $Cu_2Fe(CN)_6$ it has been demonstrated that in the case of solutions of initially equal concentrations but different temperatures water will pass from the warmer to the colder solution while the warm solution becomes more concentrated and the cold more dilutes. The phenomenological coefficients of the membrane (L) were determined, and on the basis of these, the measured and calculated values of the two steady states were compared with the help of irreversible thermodynamics. In the case of binary, non-electrolyte (sucrose) solution the transport processes of the model may be suitably described and interpreted by the help of 3 forces, 3 flows and 9 phenomenological coefficients.

Introduction

The model experiments on organic membranes performed with the Ernst type of apparatus (1952) verified the assumption that in the case of solutions of initially equal concentrations but different temperatures water passes through the semipermeable membrane from the warmer to the colder solution, while the warmer solution becomes more concentrated, and the cold one more diluted. These experiments qualitatively supported the working hypothesis. The next step was to perform experiments by which a quantitative evaluation became possible on anorganic membranes, and thus the direct relation between hypothesis and practice could be investigated. We tried to solve this problem by the help of adequate, irreversible thermodynymics. Below an account is given of the first results of experiments serving this purpose. It is a particularly important and urgent task to see clearly at least in the field of model experiments, especially if we take into consideration that the aim, on the basis of these model experiments, is exactly to understand more complicated biological transport processes. As indicated by other experiments (Vető, 1963, 1966, 1967) in these biological mechanisms of fluid mobilization, thermoosmosis may also play a role.

Experimental Method

In the present experiments the model apparatus described several times by Ernst (e.g. 1952) was used. Of the more than one hundred thermoosmotic model experiments performed by us the course and results of one will be reported here.

The porosity of the clay cylinder used was determined by measuring the quantity of water dripping through it at 1 meter water head pressure in 1 hour. This value was 103 ml. Then a membrane of $Cu_2Fe(CN)_6$, to be placed in the wall of the cylinder, was prepared and was dried in a thermostat at 60°C for two days. Only then was the first measurement performed.

1. The apparatus was fitted together, filled up with water inside and outside, and the inside was placed under pressure, that is to say, a hydrostatic pressure difference ΔP was produced, which was $\Delta P = -1.00$ water m. The quantity of water passing through as a result of this pressure difference in a certain time was measured. The surface of the whole cylinder was the surface unit. The mean value of several measurements generally gives a more reliable result, but now the performance of several measurements was intentionally avoided. The reason for this was the earlier experience that the membrane deteriorated as a result of repeated measurements, which we wanted to avoid in order to perform the following other measurements, if possible, on the same membrane. This measurement was repeated only after the performance of the other measurements to ascertain the deterioration and change of the membrane.

2. After the accomplishment of the previous measurement, 0.5 Mol (16 per cent) sucrose solution was filled inside the apparatus, while the distilled water remained outside. After a few hours the liquid was poured out of both the inside and outside to prevent dilution of the solution by the water left over from a previous measurement. Following this, the apparatus was re-filled with fresh sucrose solution of 0.5 Mol inside, and with distilled water outside. In this way, volume flow was produced by the concentration difference ΔC. At the end of the experiment the dilution of the sucrose solution inside and the concentration of the distilled water outside were determined by refractometer.

3. For the evaluation following later it was necessary to measure, or rather, to calculate how much sucrose had passed out from inside, and how much water flowed in from outside in the previous experiment. This could be done on the basis of the volumes and concentrations.

4. The apparatus was soaked inside and outside for many hours with 0.1 Mol (3.4 per cent) sucrose solution changed several times. After that both compartments were filled with such 0.1 Mol sucrose solution. Thus, there was no difference in concentration and hydrostatic pressure. Following this, the apparatus was heated outside with hot water (80°C) and cooled inside with cold water (15°C). Since this temperature difference acted at about 1 cm distance, the temperature gradient was 6.5°C/mm. After the solutions had taken up the given temperature, and further heat dilatation did not occur, the volume flow caused by the effect of ΔT was measured. Concentration measurement was also performed by refractometer at the end of the experiment.

5. The sucrose and water quantities which passed through will be calculable here also from the previous data.

6. It would have been necessary to measure the heat flow from outside to inside in response to the former ΔT when both ΔP and ΔC were zero. As this was not done, the value will be calculated approximately, since the thermal conduction coefficient of water, the temperature gradient, the surface of the cylinder, that is,

the surface of the membrane (159 cm^2) too, are known. Of course the result obtained is only an approximate estimate of the true value.

7. The purpose of the next measurement was to determine how large a hydrostatic pressure difference ΔP counterbalanced a temperature difference ΔT, in the steady state when there was no concentration difference ΔC. To perform this the inside and outside of the apparatus were filled up with the same 0.1 Mol sucrose solution, and a long thin manometer was fixed inside of it. The outside was heated with hot water (82°C) and the inside was cooled with cold water (15°C). Thus, $\Delta T = 6.7$°C/mm. Thermal equilibrium was attained in about half an hour.

8. Following this, we measured how large a hydrostatic pressure ΔP was needed to keep equilibrium against a definite concentration difference ΔC when $\Delta T = 0$. Therefore 0.5 Mol sucrose solution was poured into the inside of the cylinder (after rinsing it several times) and a manometer tube was fitted on to it. The outside was filled up with distilled water, after washing it out several times. Thus, the result was $\Delta C = 0.5$ Mol.

From the data measured according to the first six items, the further values can be calculated, and the results of special experiments can be approximately predicted. The measurements described in items 7 and 8 were performed to enable us to compare their results with the "predicted" values calculated on the ground of the results of the previous six items.

Results and Discussion

(a) 1. The measurements showed that as a result of $\Delta P = -1.00$ water m the volume flow directly proportional to time was:

$$J_v = -2.0 \text{ ml}/4 \text{ hours} = -0.5 \text{ ml/hour}$$

At the end of the whole measuring period the same was:

$$J_v = -2.3 \text{ ml}/3 \text{ hours} = -0.8 \text{ ml/hour}$$

Thus, J_v in mean value is:

$$J_v = -0.65 \pm 0.15 \text{ ml/hour}$$

2. $\Delta C = 0.5$ Mol concentration difference resulted in a volume flow of

$$J_v = 3.8 \text{ ml}/44 \text{ hours} = 0.086 \text{ ml/hour}$$

According to the measurements the concentration of the solution had become 15.1 per cent (0.47 Mol) inside, and 0.2 per cent (0.006 Mol) outside. Thus, the initial 16.0 per cent $\approx$ 0.5 Mol concentration difference had decreased to 14.9 per cent $\approx$ 0.464 Mol by the end of the measurement. Thus, during the whole experiment there was a concentration difference of approximately $\Delta C = 0.482$ Mol $\approx$ 15.5 per cent. This difference had caused the above flow.

3. The volumes and the concentrations were as follows:

At the beginning of the experiment		At the end of the experiment	
Inside	79 ml 16.0 per cent	83 ml	15.1 per cent
Outside	90 ml 0 per cent	86 ml	0.2 per cent

4. In response to the ΔT applied the volume flow from warm to cold was:

$$J_v = 12 \text{ ml}/21 \text{ hours} = 0.57 \text{ ml/hour}$$

The concentration of the cold solution inside became 2.4 per cent, that of the cold one outside 4.5 per cent. Thus, while ΔP was 0 during the whole experiment, the concentration difference ΔC increased to 2.1 per cent from the initial 0 value. According to this the mean value was $\Delta C \approx 0.03$ Mol ≈ 1 per cent.

5. The volumes and concentrations were as follows:

At the beginning of the experiment		At the end of the experiment	
Inside	75 ml 3.4 per cent	87 ml	2.4 per cent
Outside	90 ml 3.4 per cent	78 ml	4.5 per cent

6. –

7. After the attainment of thermal equilibrium, $\Delta P = -1.00$ water m. In one hr this value decreased to -0.78 water m, and then remained practically unchanged for five hours, according to measurements performed every half hour. Thus equilibrium was maintained by $\Delta P = -0.77 \pm 0.03$ water m.

8. According to the measurements $\Delta C = 0.5$ Mol ≈ 16 per cent concentration difference kept equilibrium with -0.18 ± 0.03 water m hydrostatic pressure difference for 6 days.

(b) The application of thermodynamics of irreversible processes to physical and biological transport processes has been flourishing in the last years, because it renders possible a quantitative description founded also on theory. This is done, among others, by Kedem and Katchalsky (1958), Ogilvie, McIntosh and Curran (1963), Dainty and Ginzburg (1964). Their works, however, generally deal only with isotherm systems, although transport of substance usually means also transport of heat. Although the theory which takes into consideration thermal conditions as well is fairly well clarified, e.g. De Groot (1952) and Katchalsky and Curran (1965), yet its practical and concrete experimental application is rare. We are trying to apply the above theory in connection with our experiments.

Let us take a non-equilibrium system of solutions which has two components, is anelectrolytic and heterothermal. This means that on the two sides of a membrane there is a solution of the same solvent and solute, however, at two different concentrations, hydrostatic pressures, and temperatures. The membrane is more permeable to water and less so to solute. Let us assume a diluted, ideal solution. The system is closed, only thermal energy exchange may take place between the system and its surroundings. According to De Groot (1952) and Gyarmati (1960) the entropy production of the process taking place in it is:

$$\frac{d_i S}{dt} = \frac{dN_w}{dt}\left(\frac{\Delta\mu_w}{T} - \mu_w \frac{\Delta T}{T^2}\right) + \frac{dN_s}{dt}\left(\frac{\Delta\mu_s}{T} - \mu_s \frac{\Delta T}{T^2}\right) + \frac{dE}{dt}\frac{\Delta T}{T^2} \qquad (1)$$

where μ_w stands for the chemical potential of water and μ_s for that of the solute, N for the number of moles passing through, and E for internal energy. According to Kedem and Katchalsky (1958) the dissipation function more often used in practice is:

$$\Phi = \frac{d_i ST}{dt\, A},$$

.e. the dissipation of energy on a unit of surface (A) in a unit of time. From this

$$\Phi = \left(\Delta\mu_w - \mu_w \frac{\Delta T}{T}\right) \dot{n}_w + \left(\Delta\mu_s - \mu_s \frac{\Delta T}{T}\right) \dot{n}_s + \frac{\Delta T}{T} \dot{e} \tag{2}$$

where

$$\dot{n}_w = \frac{1}{A}\frac{dN_w}{dt}\,;\quad \dot{n}_s = \frac{1}{A}\frac{dN_s}{dt}\,;\quad \dot{e} = \frac{1}{A}\frac{dE}{dt}.$$

Thus equation (2) is the product of the flows per units of surface ($\dot{n}_w$, $\dot{n}_s$, $\dot{e}$) and the appropriate forces (chemical potential difference and the conjugated force of thermal flow). In the general way customary in irreversible thermodynamics $\Phi = \Sigma_i J_i X_i$ where J_i is the i^{th} flow and X_i is the i^{th} thermodynamic force. In the case of ideal diluted solutions the chemical potential difference for water, according to Kedem and Katchalsky's (1958) and Katchalsky and Curran's (1965) equations is approximately:

$$\Delta\mu_w = \bar{v}_w \Delta P - \frac{Q_w}{T} \Delta T - RT \frac{\Delta C_s}{C_w} \tag{3}$$

phere $\bar{v}_w$ is the partial molar volume of water (18 ml/Mol), ΔP the hydrostatic eressure difference, ΔT the temperature difference, ΔC_s the concentration difference of the solute on the two sides of the membrane, C_w is the concentration of water ($\approx 1/\bar{v}_w$), Q_w a quantity of molar energy dimension in relation to water. The chemical potential difference may also be written for the solute:

$$\Delta\mu_s = \bar{v}_s \Delta P - \frac{Q_s}{T} \Delta T + RT \frac{\Delta C_s}{C_s} \tag{4}$$

where $\bar{v}_s$ is the partial molar volume of the solute, Q_s the molar energy relating to the solute, and C_s the mean value of concentration of the solute in the two compartments of the apparatus. If equations (3) and (4) are introduced into equation (2) we obtain, partly on the ground of Kedem and Katchalsky's equations (1958)

$$\Phi = (\bar{v}_w \dot{n}_w + \bar{v}_s \dot{n}_s) \Delta P + \left(\frac{\dot{n}_s}{C_s} - \frac{\dot{n}_w}{C_w}\right) RT \Delta C_s +$$

$$+ \left[\dot{e} - (Q_w + \mu_w) \dot{n}_w - (Q_s + \mu_s) \dot{n}_s\right] \frac{\Delta T}{T}. \tag{5}$$

Here new forces and flows make their appearance.

The forces are: $\Delta P = X_v$, hydrostatic pressure difference,
$RT\Delta C_s = X_\pi \approx \Delta\pi$, osmotic pressure difference,
$\Delta T/T = X_E$, relative temperature difference.
The flows are: $J_v = \bar{v}_w \dot{n}_w + \bar{v}_s \dot{n}_s$, flow of volume,

$$J_\pi = \frac{n_s}{c_s} - \frac{\dot{n}_w}{c_w}, \text{ diffusional exchange flow,}$$

$$J_E = \dot{e} - (Q_w + \mu_w)\dot{n}_w - (Q_s + \mu_s)\dot{n}_s, \text{ energy flow.}$$

By applying the usual assumptions:

$$\begin{aligned} J_v &= L_{vv}\,\Delta P + L_{v\pi}\,\Delta\pi + L_{vE}\frac{\Delta T}{T} \\ J_\pi &= L_{\pi v}\,\Delta P + L_{\pi\pi}\,\Delta\pi + L_{\pi E}\frac{\Delta T}{T} \\ J_E &= L_{Ev}\,\Delta P + L_{E\pi}\,\Delta\pi + L_{EE}\frac{\Delta T}{T}. \end{aligned} \tag{6}$$

According to Onsager $L_{v\pi} = L_{\pi v}$; $L_{vE} = L_{Ev}$; $L_{\pi E} = L_{E\pi}$

Further conditions are: $L_{vv}L_{\pi\pi} - L_{v\pi}^2 > 0$

$$L_{\pi\pi}L_{EE} - L_{\pi E}^2 > 0$$

$$L_{vv}L_{EE} - L_{vE}^2 > 0$$

and $L_{vv} > 0$; $L_{\pi\pi} > 0$; $L_{EE} > 0$.

The value of the phenomenological coefficients depends on the quality of the membrane and that of the solution. If their values for a given membrane and solution have been determined by measurement, then various special circumstances – among them the conditions of steady states, and rates of flow as result of given forces, etc. – may be calculated from these values. From function system (6) several important relation may be deduced in the usual way.

Instead of further analysis let us determine the value of the phenomenological coefficients L by using the former relations and the data of the first 6 items of our results of measurements.

1. When $\Delta\pi = \Delta T = 0$ and $\Delta P = -1.00$ water m, the mean value for flow of volume is

$$J_v = -0.65 \text{ ml/hour} = L_{vv}\,\Delta P.$$

From this

$$L_{vv} = J_v/\Delta P = 0.65\ ml/hour\ m$$

2. $\Delta P = \Delta T = 0$ and $\Delta C = -0.482$ Mol. From this, on the ground of $RT\Delta C_s = \Delta\pi$, $\Delta\pi = -119.5$ water m is obtained. $J_v = 0.086$ ml/hour $= L_{v\pi}\Delta\pi$. From this $L_{v\pi} = J_v/\Delta\pi = -0.086/119.5 = -7.2 \times 10^{-4}\ ml/hour\ m$.

3. $\Delta P = \Delta T = 0$; $\Delta\pi = -119.5$ m. From J_π we may calculate $L_{\pi\pi}$.

$$J_\pi = \frac{\dot{n}_s}{C_s} - \frac{\dot{n}_w}{C_w}.$$

For this $C_w \approx 0.055$ mole/ml; $C_s = 2.3 \times 10^{-4}$ mole/ml; and $\dot{n}_s = -1.14 \times 10^{-5}$ mole/hour calculated from the change of concentration of the distilled water. $\dot{n}_w$ can be calculated from the relation $J_v = \bar{v}_w \dot{n}_w + \bar{v}_s \dot{n}_s$. For this $\bar{v}_w = 18$ ml/mole and $\bar{v}_s = 202$ ml/mole according to Kedem and Katchalsky (1958). Thus $\dot{n}_w = 4.9 \times \times 10^{-3}$ mole/hour, therefore $J_\pi = -0.139$ ml/hour and

$$L_{\pi\pi} = J_\pi / \Delta\pi = 1.16 \times 10^{-3}\ ml/hour\ m.$$

4. Initially $\Delta P = 0$ and $\Delta C_s = 0$. Thus, $\Delta\pi = RT\Delta C_s = 0$ but during the process only $\Delta P = 0$, $\Delta\pi$ increases gradually from zero owing to the effect of constant ΔT. Therefore the actual flow of volume is

$$J_v = L_{v\pi}\,\Delta\pi + L_{vE}\frac{\Delta T}{T},$$

in which everything is known, so L_{vE} can be calculated. $\Delta\pi \approx 8.1$ water m, $J_v = 0.57$ ml/hour $= -7.2 \times 10^{-4} \times 8.1 + L_{vE}\,6.5/320$

$$L_{vE} = 28\ ml/hour = 6.6 \times 10^{-5}\ kcal/hour\ m$$

(on the ground of volume work).

5. The diffusional exchange flow from the former state is now:

$$J_\pi = L_{\pi\pi}\,\Delta\pi + L_{\pi E}\frac{\Delta T}{T}.$$

From this $L_{\pi E}$ will be obtained. J_π will be calculated for it on the ground of

$$J_\pi = \frac{\dot{n}_s}{C_s} - \frac{\dot{n}_w}{C_w}.$$

For this $C_w = 0.055$ mole/ml; $C_s = 10^{-4}$ mole/ml; $\dot{n}_s = -6 \times 10^{-5}$ mole/hour from the change of concentration. Further, from $J_v = \bar{v}_w \dot{n}_w + \bar{v}_s \dot{n}_s$ $\dot{n}_w$ is $\dot{n}_w = 0.032$ mole/hour. Thus, $J_\pi = -1.18$ ml/hour $= 1.16 \times 10^{-3} \times 8.1 + L_{\pi E} 0.0203$. And from this

$$L_{\pi E} = -58.6\ ml/hour = -1.38 \times 10^{-4}\ kcal/hour\ m.$$

6. $\Delta P = \Delta\pi = 0$ and $\Delta T = 6.5$ °C/mm. How much would be the flow of heat through the membrane in this case?

$$J_E = \frac{\lambda F \Delta T}{d} = \frac{0.5 \times 0.0159 \times 6.5}{10^{-3}} = 51.6 \text{ kcal/hour.}$$

Since $J_E = L_{EE}\frac{\Delta T}{T}$; $L_{EE} = J_E T/\Delta T = 2540\ kcal/hour$.

Thus we have all the 9 coefficients and the simultaneous equations will be:

$$\begin{aligned} J_v \text{ ml/hour} &= 0.65 \text{ ml/hour m} \times \Delta P \text{ m} - 7.2 \times \\ &\times 10^{-4} \text{ ml/hour m} \times \Delta\pi \text{ m} + 28 \text{ ml/hour} \times \Delta T/T \\ J_\pi \text{ ml/hour} &= -7.2 \times 10^{-4} \text{ ml/hour m} \times \Delta P \text{ m} + 1.16 \times \\ &\times 10^{-3} \text{ ml/hour m} \times \Delta\pi \text{ m} - 58.6 \text{ ml/hour} \times \Delta T/T \\ J_E \text{ kcal/hour} &= 6.6 \times 10^{-5} \text{ kcal/hour m} \times \Delta P \text{ m} - 1.38 \times \\ &\times 10^{-4} \text{ kcal/hour m } \Delta\pi \text{ m} + 2.54 \times 10^3 \text{ kcal/hour} \times \Delta T/T. \end{aligned}$$

I. Now let us calculate from this how large a hydrostatic pressure difference ΔP maintains the steady state ($J_v = 0$), if there is no concentration difference, i.e. $RT\Delta C_s = \Delta\pi = 0$, and $\Delta T = 6.7°\text{C/mm}$. Is this calculated value in agreement with the measured value reported in the 7th item?

$$0 = 0.65 \times \Delta P + 28 \times 6.7/322$$

$$\Delta P = -28 \times 6.7 : 322 \times 0.65 = -0.90 \text{ water m (calculated)}$$

$$\text{Measured: } \Delta P = -0.77 \text{ water m} .$$

The difference is 17 per cent, which is not too bad agreement, because the accuracy of the measurements is not better either (about 23 per cent).

II. Let us calculate how large a hydrostatic pressure difference ΔP would keep equilibrium with a given concentration difference in the case of $\Delta T = 0$, which would correspond to $\Delta\pi = -124$ water m of osmotic pressure difference, when $J_v = 0$. This is indicative of the quality and degree of semipermeability of the membrane. Thus

$$0 = 0.65 \times \Delta P + 7.2 \times 10^{-4} \times 124$$

$$\Delta P = -7.2 \times 10^{-4} \times 124/0.65 = -0.14 \text{ water m (calculated)}$$

$$\text{Measured: } \Delta P = -0.18 \text{ water m} .$$

The difference is 22 per cent, which is within the accuracy of measurement. The membrane is very strongly permeable and is very far from the degree of ideal semipermeability.

These last two comparisons show that hypothesis and practice overlap within the range of the measuring error, and it is worth while to progress along this road with more measurements of greater accuracy.

III. As a following step, let us calculate the formal concentration difference, or rather the $\Delta\pi$ which is large enough to bring about Dufour's osmotic thermo-effect (1873) so that it is measurable; in short how large a ΔC is necessary to cause, e.g. ΔT of a 0.02°C? During the process $\Delta P = 0$ and also $J_E = 0$, i.e. the solution transported by the concentration difference carries the same amount of heat with it as the reverse conduction of heat, caused by ΔT, does

$$J_E = 0 = L_{E\pi} \Delta\pi + L_{EE} \Delta T/T .$$

From this

$$\Delta\pi = -\frac{L_{EE}\,\Delta T}{L_{E\pi}\,T} = \frac{2.54 \times 10^3 \times 0.02}{300 \times 1.38 \times 10^{-4}} = 1230 \text{ water } m \approx 4.8 \text{ Mol}.$$

Thus, according to this, formally a concentration difference of 4.8 Mol, constantly maintained can bring about the temperature difference of 0.02°C/mm. This is, of course, not a case of diluted solution any more, so the calculation cannot be quite right either, but the result still indicates, that it is worth while to deal with the question.

IV. As a last calculation let us examine, the quantity of energy transported by the solution, which is extremely important and characteristic. Let $\Delta T = \Delta\pi = 0$. Then a first approximation would be

$$E^* = J_E/J_v = L_{Ev}/L_{vv} = 0.1 \text{ cal/ml},$$

which would correspond to the value of 1.8 *cal/mole* water. However, here also solute diffuses, hence the calculation is not so simple. The determination of energies of transport and heats of transport is a following task.

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Short Communications

Specificity of Acetylornithinase in Escherichia Coli K 12 and B

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It is known that different organisms are synthesizing ornithine from glutamate through a number of acetylated intermediates and arginine is formed from ornithine by the well-known mechanism (Umbarger, Davis, 1962). The immediate precursor of ornithine is α-N-acetylornithine (Vogel, 1953). In E. coli α-N-acetylornithine is hydrolyzed to ornithine and acetate by α-N-acetyl-L-ornithine amidohydrolase (EC 3.5.1.16), referred to hereafter as acetylornithinase (Vogel, Bonner, 1956). We have previously observed that the arginine-less mutant BC 28^{-1} of E. coli is growing with the same doubling time, 55 min., in the presence of α-N-acetyl- or α-N-propionyl- or α-N-butyrylornithine (Dénes, Gorini, 1962). It is a characteristic property of this mutant that the enzymes of the arginine pathway are derepressed except the first one which is lost by a single mutation (Sercarz, Gorini, 1964).

The present paper reports kinetic experiments on the specificity of acetylornithinase purified from E. coli K 12 and B to explain the biochemical reasons of the non-specific α-N-acylornithine utilization observed.

The α-N-acylornithines were prepared chemically (Staub, Dénes, 1966). Wild-type cells of E. coli K 12 and B were grown on mineral salts medium A containing 0.2% glucose (Davis, Mingioli, 1950). Acetylornithinase was prepared as described previously (Vogel, Bonner, 1956). The "standard" reaction mixture for determination of enzyme activity contains 50 μmoles of potassium phosphate buffer (pH 7.0), 3 μmoles of α-N-acetylornithine, 0.5 μmoles of reduced glutathione, 0.1 μmoles of $CoCl_2$ in a total volume of 0.5 ml. The reaction mixtures were incubated at 37°C. The reaction was arrested by the addition of 1.5 ml of ninhydrine reagent and the ornithine formed was determined by the alkaline ninhydrine method (Vogel, Bonner, 1956). One unit is defined as the amount of enzyme catalyzing the formation of 1.0 μmole of ornithine in 1 min at 37°C. Specific activity is expressed as units per mg of protein.

As Fig. 1a shows, acetylornithinase hydrolyzes the α-N-acylornithines tested but the rate of the reactions is different. The rate of hydrolysis of α-N-butyrylornithine is only 1.9 per cent of that of α-N-acetylornithine as shown in Table 1. The K_m values of the enzyme for different α-N-acylornithines are similar and only decrease slightly with increasing the number of carbon atoms of the acyl group, as shown in Table 1.

Since the growth rate of the arginine-less mutant strain on different α-N-acylornithines is the same and the "in vitro" rate of hydrolysis of the same compounds is different, we have studied the possible reasons of these discrepancies. First of all it is possible that some enzyme other, than acetylornithinase is responsible for the hydrolysis of acylornithines. We could not detect any other enzyme

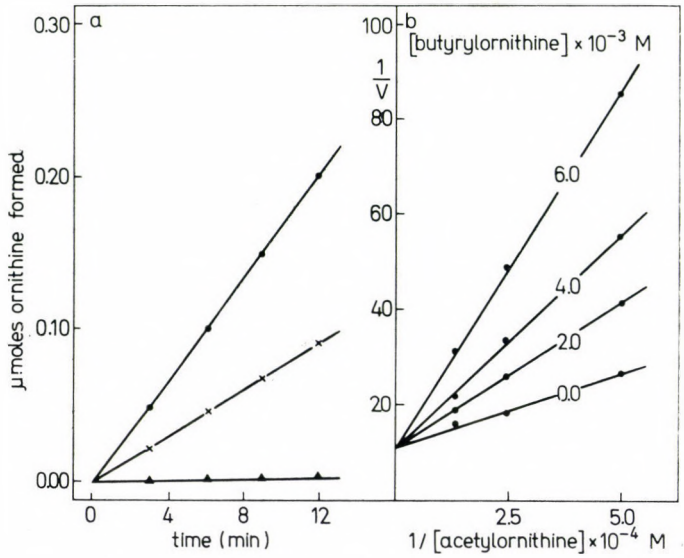

Fig. 1. The activity of acetylornithinase in the presence of α-N-acetylornithine (●—●), α-N-propionylornithine (x—x), α-N-butyrylornithine (▲—▲) (a); the effect of α-N-butyrylornithine on the rate of hydrolysis of α-N-acetylornithine (b). Standard reaction mixtures were used as described in the text, containing 7.3 µg of enzyme purified from E. coli K 12 and 3 µmoles of α-N-acylornithine tested (a); 5.8 µg of enzyme, α-N-acetylornithine and α-N-butyrylornithine as indicated (b)

in the cell-free extract of E. coli K 12 and B but acetylornithinase and the ratio of specific activities of the enzyme for the different α-N-acylornithines did not change

Table I

A comparison of the specific activities and of Michaelis constants of acetylornithinase purified from E. coli K 12 for the different α-N-acylornithines

Standard reaction mixtures were used as described in the text containing 3 µmoles of α-N-acylornithine tested. The Michaelis constants were determined graphically from the plots of reciprocal of initial velocity *vs* the reciprocal of substrate concentration

Substrate	Specific activity	Relative specific activity	K_m
Acetylornithine	2.24	100	4.5×10^{-3}M
Propionylornithine	0.98	44	3.0×10^{-3}M
Butyrylornithine	0.042	1.9	1.2×10^{-3}M

during the purification procedure. The experiment shown in Fig. 1b seems to support the assumption that a single enzyme is responsible for the hydrolysis of α-N-acylornithines studied, since α-N-butyrylornithine competitively inhibits the hydrolysis of α-N-acetylornithine because of the difference in the rate of hydrolysis of the compounds.

Another possible reason of the discrepancy observed is that in vivo the mechanism of action of the enzyme is different from that in vitro. It was found previously that in some organisms, instead of acetylornithinase, there is another enzyme, ornithine acetyltransferase, which transfers the acetyl group of acetylornithine to glutamate (Udaka, Kinoshita, 1958; De Deken, 1963; Staub, Dénes, 1966). We have found that the activity of enzyme increases threefold in the presence of 3 mM of glutamate but we could not detect any transfer of acetyl group to ^{14}C-labeled glutamate using a previously described method (Staub, Dénes, 1966). Aspartate activates the enzyme like glutamate, but succinate does not have any activating effect on the enzyme. We could not detect any difference in the specificity of acetylornithinase purified from K 12 or B strain of E. coli.

The results presented indicate that the same enzyme is responsible for the in vitro hydrolysis of the three acylornithines studied. If we further assume that the in vivo catalytic mechanism of this enzyme is identical with that in vitro, it would follow that about 2 per cent of the total acetylornithinase activity present in cells of strain BC 28^{-1} is sufficient to meet the ornithine requirement of the arginine pathway.

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Spectrophotometric Determination of Histidine in Proteins with Diethylpyrocarbonate

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In the present paper a simple and specific method is described for the spectrophotometric determination of histidine content in proteins by the use of DEP.* DEP is widely used in the food industry for preserving fruits and wine. It was shown by Rosnati (1964) and Larroquére (1964) that DEP reacts with the amino groups of amino acids and primary amines at neutral and slightly alkaline pH. Mühlrad et al. (1967) have found that beside amino groups DEP also reacts with other side chains at slightly alkaline pH, such as the imino nitrogen of imidazol group in histidine, the guanidyl group of arginine, the ε-amino group of lysine, the phenolic hydroxyl of tyrosine and the sulfhydryl group of cysteine. These reactions lead to the formation of the corresponding carbetoxy derivates. Fedorcsák and his associates (Hullán et al. 1965, Fedorcsák, Ehrenberg 1966, Fedorcsák, Rosén 1966) have found that DEP reacts with proteins and inhibits the activity of ribonuclease and trypsin.

In the contrast to the other compounds reacting with DEP histidine reacts readily at pH 6, though the velocity of the reaction is lower than that at higher pH values. The formation of N-carbetoxy-histidyl side chains in proteins results in an increase of absorption between 220 and 260 mμ (Mühlrad et al. 1967).

In the present investigations the specificity of the carbetoxylation of protein histidyl side chains was studied. This paper describes the application of DEP for the spectrophotometric determination of histidine content in proteins.

The carbetoxylation reaction was studied both with N-acetylhistidine and proteins. DEP was a commercial product "Baycovin" produced by Bayer Co., Leverkusen, Germany. DEP is very labile in water and decomposes rapidly to ethanol and carbon dioxyde, therefore the stock solutions were prepared daily by dilution with 95 per cent ethanol. In the experiments with proteins DEP was added in 1×10^{-3} to 1×10^{-2} molar concentrations and care was taken to keep the ethanol concentration in the samples below 0.5 per cent.

Protein samples were prepared in 0.1 M phosphate buffer pH 6.0 and the reaction with DEP was carried out at 0°C. It was found that in a five to ten molar ratio of DEP to histidine or histidyl side chains the carbetoxylation reaction was completed after incubation for 15 to 30 minutes.

* *Abbreviations:* DEP — diethylpyrocarbonate, GAPD — glyceraldehyde-3-phosphate dehydrogenase (EC. 1. 2. 1. 12), LDH — lactic dehydrogenase (EC. 1. 1. 1. 12)

The absorption of the samples was measured between 220 and 260 mμ and was compared to that of the non-treated ones.

As it can be seen from Fig. 1 the formation of carbetoxy-histidine markedly increases the absorption between 220 and 260 mμ of both N-acetylhistidine and proteins. The difference spectra of carbetoxy-N-acetylhistidine and DEP-treated proteins are similar with a small but significant shift in the position of the maxima. These are located at 234, 239 and 241 mμ for carbetoxy-N-acetyl-histidine, denatured and native proteins, respectively.

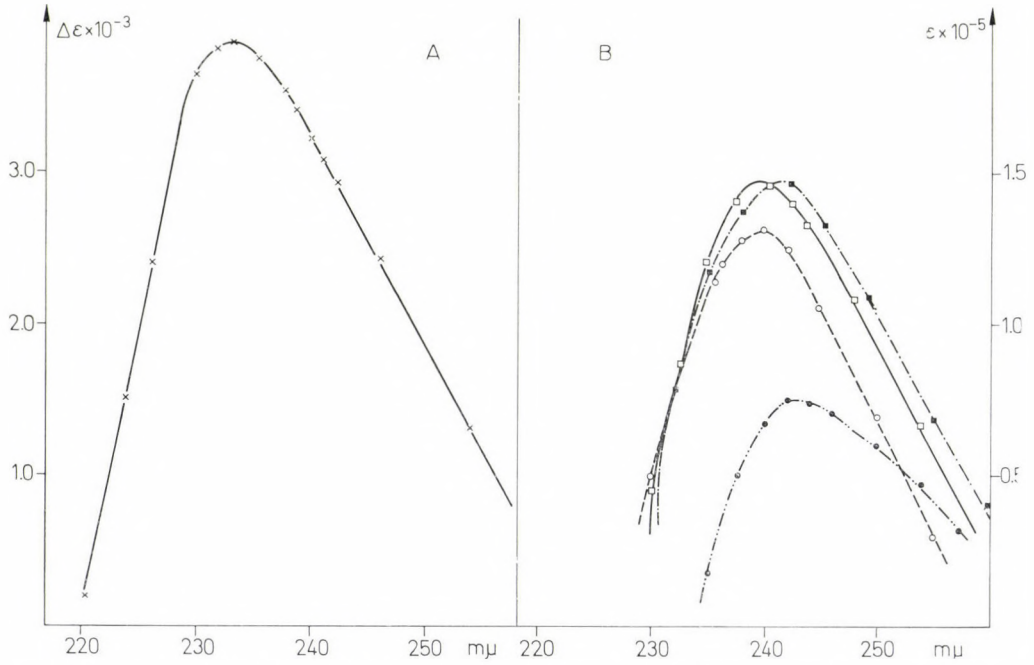

Fig. 1. Difference spectra of DEP-treated histidine and proteins *vs* untreated controls at pH 6.0 A — carbetoxy-N-acetylhistidine, B — proteins. ○ — GAPD, □ — LDH, solid symbols native proteins, open symbols proteins denatured with SDS in a 1 : 1 weight ratio

For the determination of histidine content proteins were previously denatured in 8M urea or in sodium dodecylsulfate. When the reaction of DEP with denatured proteins has been completed no free histidine was found in the sample with the modified Pauli-reaction (Sokolovsky et al. 1966).

The amount of amino acid side chains reacting with DEP under conditions different from those described in this paper was also controlled eg. with arginine (Oginsky, 1957), lysine (Moore, Stein 1948) tyrosine (Hughes, Streassle 1950) and cysteine (Boyer, 1954, Ellman 1959). It was found that in the proteins treated with DEP at pH 6.0 the amount of the above amino acid residues remained unchanged. The data suggest that the histidyl side chains of the proteins listed in Table I reacted completely and specifically with DEP.

The molar absorption difference for N-carbetoxy histidine at 240 mμ is $\Delta\varepsilon_{240} = 3.2 \times 10^3$. Using this extinction coefficient we determined the histidine content of some proteins. As shown in Table I the values obtained agree fairly well with the histidine content determined by amino acid analysis.

Table I

Number of histidine residues in proteins (moles per mole protein) determined spectrophotometrically upon treatment with DEP

Protein	Number of histidine residues found		
	Amino acid analysis	with DEP-reaction	
		Denatured protein	Native protein
Ribonuclease (pancreas)	4[1]	3.9++	0.9
Myoglobin §§ (sperm whale)	12[2]	11.8*	4.0
GAPD			
swine	44[3]	41+	20.9
rabbit	44[4]	43+	21
crayfish	—	25.3+	5.5
yeast	27[5]	25.5+	20.7
LDH			
swine skeletal	44[6]	44+	44
beef heart	33[7]	35+	17.4
Aldolase (rabbit)	42[8]	39 §	18.0

+ Denatured with sodium dodecylsulfate (1 : 1 w/w to protein)
++ Denatured in 8M urea + 0,1M cysteine
* Apomyoglobin, denatured in 8M urea at 50° C for 90 minutes
§ Denatured in 8M urea, calculated for a molecular weight of 141 000.
§§ Kindly gifted by Dr. H. C. Watson (Cambridge)

References to Table I: 1) Hirs et al, (1956), 2) Edmundson, Hirs (1962), 3) Harris, (1964), 4) Harris, Perham (1965), 5) Kaplan (1965), 6) Beney, Előd (1965), 7) Pesce et al (1964), 8) Shimizu, Ozawa (1967)

In the native proteins less histidine reacts with DEP than in the denatured ones (Fig. 1, Table I, column 4). This observation suggests that in native proteins the histidyl residues exist in two different states i.e. in a DEP-reactive and in a non-reactive state. The histidyl residues which did not react with DEP in the native state could be determined with the modified Pauli-reagent. It is interesting to note that practically all histidine residues of swine skeletal muscle LDH reacted with DEP in the native state (Előd, 1968).

The carbetoxylation of histidyl side chains in proteins with DEP at pH 6.0 seems to offer a rapid and specific method for the spectrophotometric determi-

nation of the histidine content in previously denatured proteins. The study of the incomplete reaction of histidyl residues with DEP in native proteins may be useful for the localization of the reactive and non-reactive histidyl residues the protein fabric. Investigations along this line are currently run in our laboratory.

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Errata

Vol. 2.

p. 55. Paragraph 2, row 13

instead of „...shifted towards longer wavelenghts." read „...shifted towards shorter wavelenghts."

P. 153. The right form of equation (3)

$$d_i = \frac{M_i - m}{\frac{M_i - M_{bi}}{d_b} - \frac{m - m_b}{d_b}} = \frac{d_b\,(M_i - m)}{(M_i - m) - (M_{bi} - m_b)} =$$

$$= \frac{d_b}{1 - \frac{M_{bi} - m_b}{M_i - m}} \qquad (3)$$

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