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Physiologia

BEITRÄGE ZUR CHOLINESTERASE IN VIVO

I. BESTIMMUNG DER CHOLINESTERASEAKTIVITÄT IN VIVO

Von

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(Eingegangen am 23. Oktober 1959)

Es wurde eine Methode zur Bestimmung der Cholinesteraseaktivität des Ganglion cervicale superius von Katzen ausgearbeitet. Die Methode besteht darin, daß man das Ganglion von Locke-Lösung durchströmen läßt, welche Substrate der Cholinesterase (Azetylcholin, Azetyl-β-methylcholin, Äthylchlorazetat) und ihre Inhibitoren (Eserin, TEPP, Prostigmin) enthält und die Differenz zwischen der Substratkonzentration des ein- und ausströmenden Perfusats chemisch bestimmt. Diese Differenz ergibt sich aus der Cholinesteraseaktivität des Ganglions.

Über die Cholinesteraseaktivität des Ganglion cervicale superius der Katze stehen mehrere Angaben zur Verfügung. So werden laut GLICK [1] von der ganglionären Cholinesterase je Sekunde und Gramm 0,1 μg Azetylcholin, nach den Angaben von NACHMANSOHN [2] je Stunde und Gramm 400—600 mg Azetylcholin abgebaut. KOELLE und Mitarbeiter [4, 5, 6] haben die Lokalisation beider Cholinesterasearten in den sympathischen Ganglien unter Anwendung einer histochemischen Methode ziemlich genau festgestellt. Die unspezifische oder pseudo-Cholinesterase konzentriert sich im interstitiellen Bindegewebe und im Gliagewebe, die spezifische oder Azetylcholinesterase in den präganglionären Endigungen und in der Membran der Ganglienzellen.

Im Hinblick darauf, daß wir im Rahmen anderer Versuche [7, 8] die Rolle des Azetylcholin-Cholinesterasesystems im Prozeß der ganglionären Impulsübertragung untersuchten, ergab sich die Notwendigkeit, eine Aktivitätsmeßmethode auszuarbeiten, die uns im Verlauf der Impulsübertragung, der ganglionären Erregung, ein Bild der Cholinesterasefunktion vermittelt. Es mußte demnach eine Methode geschaffen werden, welche die Aktivität *in vivo* bestimmt.

Hierzu boten sich prinzipiell zwei Möglichkeiten. 1. Inkubation des herausgeschnittenen Ganglion cervicale superius in Substratlösung bei entsprechender Temperatur und Sicherung der Sauerstoff- und Nährstoffversorgung. 2. Perfusion des Ganglions mit der Substratlösung und Messung der Differenz zwischen der Konzentration der ein- und ausströmenden Substratlösung, d. h. der »arterio-venösen« Substratdifferenz.

Eine der im 1. Punkt erwähnten analogen Methode haben SCHLEYER u. Mitarb. [9] am elektrischen Organ des Torpedo electricus angewandt, SEIDLITZ [10] hingegen am Froschmuskel. Die Perfusionsmethode wurde von MARNAY

und NACHMANSOHN [11] am Hundemuskel, von BIKOW und PRAWDITSCH-NEMINSKAJA [12] am sympathischen Ganglion benutzt.

Im einzelnen untersuchten wir folgende Fragen:

1. Ist die Konzentrationssenkung, die bei der Perfusion der Azetylcholinlösung durch das Ganglion eintritt, der Cholinesteraseaktivität zuzuschreiben?

2. Ist der gewonnene Aktivitätswert von den Perfusionsbedingungen (Strömungsgeschwindigkeit, Diffusion und Konzentration des Substrats) abhängig?

3. Wie groß ist die Fraktion der Cholinesteraseaktivität des Ganglions, die sich bei der Perfusion messen läßt?

Methode

Das Ganglion cervicale superius von Katzen, die mit 80 mg/kg Chloralose i. p. narkotisiert worden waren, perfundierten wir nach der Methode von KIBJAKOW [3] mit Locke-Lösung, welche die verschiedenen Substrate der Cholinesterase in unterschiedlicher Menge enthielt. Die Substratkonzetration der durch die Arteria carotis communis einströmenden und durch die Vena jugularis interna ausströmenden Lösung wurde biologisch [13] und nach der chemischen Methode von HESTRIN [14] bestimmt.

Im Verlauf der Versuche kamen folgende Substrate zur Anwendung: Azetylcholin, Azetyl-β-methylcholin, Äthylchlorazetat. Als Inhibitor benutzten wir Eserin, Tetraäthylpyrophosphat (TEPP) und Prostigmin.

In einigen Versuchen bestimmten wir die Cholinesteraseaktivität des Ganglion cervicale superius auch *in vitro*. Die Methode dieser Bestimmung haben wir in einer früheren Mitteilung [7] beschrieben.

Ergebnisse

1. *Ist die Konzentrationssenkung, die bei der Perfusion der Azetylcholinlösung durch das Ganglion eintritt, der Cholinesteraseaktivität zuzuschreiben?*

Zur Beantwortung dieser Frage wandten wir zwei Methoden an: *a)* Im Falle der Cholinester reazetylierten wir zwecks der Bestimmung des Cholingehalts das ausströmende Perfusat. *b)* Zusammen mit den Substratlösungen führten wir die spezifischen Cholinesteraseinhibitoren in das Ganglion ein.

a) Als Beispiel der ersten Methode sei ein Versuch demonstriert: das Ganglion cervicale superius wurde mit Azetylcholinlösung von 540 μg/ml nomineller Konzentration perfundiert. Der Versuch ergab folgende Resultate:

Tabelle I

	AchBr. μg/ml	a—v Diff. μg/ml	Nach Reazetylierung Ach μg/ml
Einströmung	534	—	598
Ausströmung I	496	38	604
„ II	500	34	588
„ III	496	38	592

Wie aus den Angaben in Tabelle I ersichtlich, ist der Cholingehalt des ein- und ausströmenden Perfusats innerhalb der Fehlergrenzen des Reazetylierungsverfahrens [15] als konstant zu betrachten, die im Verlauf der Perfusion auftretende arterio-venöse Differenz demnach nicht einer im Cholingehalt eingetretenen Veränderung zuzuschreiben. Da aber bei der HESTRINschen Methode die Menge der C-O-C-Bindungen gemessen wird, läßt sich die Konzentrationssenkung im ausströmenden Perfusat auf die Azetylcholinhydrolyse zurückführen.

b) Hiernach ergibt sich die Frage, ob die im Verlauf der Perfusion eintretende Azetylcholinhydrolyse nicht auf einer von der Cholinesteraseaktivität unabhängigen Wirkung beruht. Es war daher nötig, neben dem Substrat auch die spezifischen Cholinesteraseinhibitoren in das Ganglion einzuführen und zu untersuchen, ob diese eine Senkung oder Hemmung der Hydrolyse bewirken. Einen derartigen Versuch zeigen die Tabellen II und III.

Tabelle II

	Ach µg/ml	Hydrolyse µg/ml
Locke		
Einströmung	504	—
Ausströmung	464	40
$10^{-6}M$ TEPP		
Einströmung	478	—
Ausströmung I	480	∅
Ausströmung II	478	∅

Tabelle III

	Ach µg/ml	Hydrolyse µg/ml
Lokce		
Einströmung	522	—
Ausströmung I	464	58
Ausströmung II	472	50
$10^{-2}M$ Prostigmin		
Ausströmung	522	∅

Im folgenden Versuch wurde als Substrat Äthylchlorazetat (ÄCA), als Inhibitor Eserin in der Konzentration $10^{-5}M$ benutzt.

Tabelle IV

Konzentration des einströmenden Perfusats

	300 µg/ml ÄCA	300 µg/ml ÄCA + 10^{-5} Eserin
Hydrolyse I	7,56 µg/min	3,51 µg/min
„	7,56 µg/min	3,51 µg/min

Von Eserin wurde die Äthylchlorazetathydrolyse auf weniger als die Hälfte herabgesetzt, aber nicht vollständig gehemmt, was mit den Resultaten SCHLEYERS [9] am elektrischen Organ übereinstimmt. Seiner Ansicht nach beruht dies darauf, daß im elektrischen Organ auch eine andere Esterase anwesend ist, welche Äthylchlorazetat abzubauen vermag.

Aus den unter *a)* und *b)* beschriebenen Versuchen wurde gefolgert, daß die Konzentrationssenkung der durch das Ganglion cervicale superius perfundierten Azetylcholin-Locke-Lösung auf die Cholinesteraseaktivität des Ganglions zurückgeführt werden muß.

KOELLE u. Mitarb. haben nachgewiesen, daß beide Cholinesterasearten in den sympatischen Ganglien vorkommen. Das spezifische Substrat der pseudo-Cholinesterase ist Butyrylcholin, das der Azetylcholinesterase Azetyl-β-methylcholin. Bei der Perfusion beider Substrate durch das Ganglion war Hydrolyse zu beobachten, woraus geschlossen werden darf, daß beide Formen der Cholinesterase auch im intakten Ganglion aktiv sind.

Als Beispiel für die Hydrolyse von Azetyl-β-methylcholin sei folgender Versuch angeführt:

Tabelle V

	Ac-β-methylcholin-Konzentration µg/ml	a—v Differenz µg/ml
Einströmung	107,2	—
Ausströmung	104,8	2,4
Einströmung	1308	—
Ausströmung	1188	120
Einströmung	12160	—
Ausströmung	10800	1360

2. *Einfluß der physikalischen Perfusionsbedingungen auf das Ausmaß der Hydrolyse*

Wenn das in der Locke-Lösung strömende Substrat aus den Gefäßen austritt und auf dem Wege der Diffusion die Ganglienzellen und präganglio-

nären Endigungen oder andere cholinesterasehaltige Strukturen erreicht, so ergibt sich die Frage, ob die physikalischen Bedingungen der Perfusion (Druck, Strömungsgeschwindigkeit, Konzentration) das Ausmaß der festgestellten Hydrolyse beeinflussen.

Bei dem Mechanismus der Substratkonzentrationssenkung im Perfusat dürfte es sich darum handeln, daß das in den interzellulären Raum, an die Oberfläche der Zellen gelangte Substrat von der Cholinesterase hydrolysiert wird. Dies zieht die Konzentrationssenkung des Substrats in der interzellulären Flüssigkeit nach sich, die durch die Diffusion aus den Gefäßen kompensiert wird. Folgende Gleichung bringt die Geschwindigkeit der Substratkonzentrationssenkung zum Ausdruck:

$$-\frac{dc}{dt} = k\ C_0 \tag{1}$$

in der dc die Konzentrationsveränderung, dt die Zeit, k die Konstante der Hydrolysengeschwindigkeit und C_0 die Ausgangskonzentration bedeutet. Die Substratkonzentration der interzellulären Flüssigkeit entspricht demgemäß Zeit t dem Beginn der Hydrolyse folgend

$$C_t = C_0\ e^{-kt} \tag{2}$$

wo C_t die Zeit t nach Beginn der Hydrolyse gemessene Substratkonzentration bezeichnet. Die in der interzellulären Flüssigkeit eintretende Konzentrationssenkung beträgt demnach auf Grund der Gleichungen (1) und (2)

$$\Delta C = C_0\ (1-e^{-kt}) \tag{3}$$

In Anbetracht dessen, daß Azetylcholin nach den Berechnungen OGSTONS [16] über sehr große Diffusionsfähigkeit verfügt, weiterhin der extra- und intravasale Raum des Ganglions auf großer Fläche miteinander im Kontakt stehen, können wir die Substratkonzentration der beiden Räume als identisch annehmen. Das Ausmaß der Hydrolyse haben wir in der je ml abgebauten Substratmenge gemessen. Die in der Gleichung (3) vorkommende Zeit t bezeichnet somit — in Minuten — diejenige Zeitdauer, in welcher 1 ml Flüssigkeit durch das Ganglion strömt, d. h. mit der Cholinesterase in Kontakt bleibt. Diese Zeitdauer ist naturgemäß von den verschiedenen Perfusionsgeschwindigkeiten abhängig. Der k-Wert läßt sich aus der Gleichung (3) errechnen, wenn wir sie folgendermaßen verändern:

$$k = \frac{1}{t} \ln \frac{C_0}{C_0 - \Delta C} \tag{4}$$

Wenn wir t mit der zur Durchströmung von 1 ml erforderlichen, in Minuten gemessenen Zeit substituieren, so gewinnen wir aus den Resultaten

eines Versuches bei den verschiedenen Perfusionsgeschwindigkeiten folgende k-Werte:

Tabelle VI

Perfusionsgeschwindigkeit ml/min (V)	0,05	0,24	0,45	0,51	0,60	0,84
Zur Durchströmung von 1 ml erforderliche Zeit in Min. (t)	20	4	2,22	1,95	1,66	1,18
Geschwindigkeitskonstante (k)	0,013	0,015	0,017	0,018	0,019	0,022

Die Substratkonzentration war in diesem Versuch 572 μg/ml. Wie Tabelle VI zeigt, ist der k-Wert bei mittleren und hohen Perfusionsgeschwindigkeiten nahezu konstant, und eine wesentliche Abweichung von diesem Wert besteht nur bei sehr niedriger Perfusionsgeschwindigkeit. Bei dem erwähnten Versuch war zwischen der arterio-venösen Azetylcholindifferenz, dem ΔC-Wert und der Perfusionsgeschwindigkeit folgende Korrelation Festzustellen (Abb. 1).

Wenn wir nunmehr den Wert k mit 0,017 annehmen und den Zusammenhang zwischen ΔC und der Perfusionsgeschwindigkeit aufzeichnen, so sehen

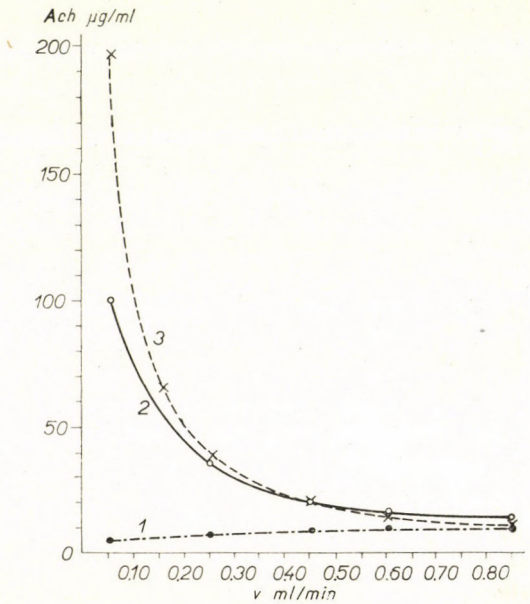

Abb. 1. Die Abhängigkeit der arterio-venösen Azetylcholinkonzentrationsdifferenz ($\triangle C$) und der je Minute hydrolysierten Substratmenge von der Perfusionsgeschwindigkeit. Ordinate: Arterio-venöse Substratkonzentrationsdifferenz in μg/ml; Abszisse: Perfusionsgeschwindigkeit ml/min. Kontinuierliche Linie: Versuchskurve; gestrichelte Linie: nach Gleichung (3) errechnete Kurve. $C_0 = 572$ μg/ml. Strichpunktierte Linie: je Minute hydrolysierte Substratmenge in μg/min. Der Wert von k beträgt für die errechnete Kurve 0,017

wir, daß die Versuchsergebnisse und die errechnete Kurve nur bei sehr niedriger Perfusionsgeschwindigkeit wesentlich voneinander abweichen, hingegen bei mittlerer und hoher Perfusionsgeschwindigkeit gut übereinstimmen. Bei niedrigen Perfusionsgeschwindigkeiten dürfte die Abweichung darauf beruhen, daß in einem Teil der Kapillaren bei niedrigem Druck keine Substratlösung strömt, so daß vom Enzym weniger Substrat erreicht und infolgedessen das Resultat der Hydrolyse niedriger wird. Aus der Übereinstimmung bei den mittleren und hohen Perfusionsgeschwindigkeiten darf aber geschlossen werden, daß die Gleichung (3) die Kinetik der im Verlauf der Perfusion vor sich gehenden Hydrolyse mit annähernder Genauigkeit widergibt. In Abb. 1 verbindet die kontinuierliche Linie die im Versuch ermittelten Werte, die gestrichelte Linie die errechneten Werte.

In anderen Versuchen stellten wir folgende Geschwindigkeitskonstanten der Azetylcholin- und Azetyl-β-methylcholinhydrolyse fest: Azetylcholin = 0,016, 0,013, 0,015; Azetyl-β-methylcholin = 0,0012, 0,0011.

Bei gleicher Untersuchung der Äthylchlorazetatspaltung ergaben sich auf Grund der Gleichung (3) folgende Geschwindigkeitskonstanten: 0,080, 0,076, 0,051, 0,088.

Die je Minute hydrolysierte Substratmenge ($\Delta C \cdot V$) muß nach der Gleichung (3) praktisch konstant sein, was jedoch nicht bei jedem Versuch der Fall war. Bei niedrigen Perfusionsgeschwindigkeiten war die je Minute hydrolysierte Substratmenge wiederholt geringer. Wie bereits erwähnt, dürfte das darauf zurückzuführen sein, daß der Kontakt zwischen Enzym und Substrat durch die bedeutende Senkung des Perfusionsdrucks infolge der Verringerung der durchströmten Kapillaren beeinträchtigt wird. Reale Hydrolysewerte treten daher laut Abb. 1 im Ganglion cervicale superius nur bei einer 0,25 ml/min übersteigenden Perfusionsgeschwindigkeit in Erscheinung. Die Veränderungen der je Minute hydrolysierten Substratmenge gibt in Abb. 1 die strichpunktierte Linie wieder.

MARNAY und NACHMANSOHN [11] haben nach der Perfusionsmethode die Cholinesteraseaktivität des Muskels untersucht und in bezug auf den Zusammenhang zwischen der Perfusionsgeschwindigkeit und arteriovenösen Konzentrationsdifferenz ein mit unserem Ergebnis übereinstimmendes Resultat festgestellt. Die Veränderung der in der Zeiteinheit abgebauten Substratmenge im Verhältnis zur Perfusionsgeschwindigkeit tritt bei ihnen noch ausgeprägter in Erscheinung, was darauf hindeutet, daß das, was wir über die Kapillardurchströmung bei verschiedenen Perfusionsdruckwerten oben gesagt haben, für den Muskel in noch höherem Maße gilt. MARNAY und NACHMANSOHN folgerten aus ihren Resultaten, daß der Muskel zwei Cholinesterasen enthält: ein rasch hydrolysierendes, in geringer Menge konzentriertes und ein langsam hydrolysierendes, in großer Menge diffus lokalisiertes Enzym. Die parallel zur Perfusionsgeschwindigkeit zutage tretende Hydrolysesteigerung führen sie

darauf zurück, daß bei rascher Perfusion genügend Substrat mit dem rasch hydrolysierenden Enzym in Kontakt kommt und die abgebaute Substratmenge größer sein wird, während die vom langsam hydrolysierenden Enzym abgebaute Menge wegen seiner diffusen Lokalisation von der Perfusionsgeschwindigkeit weniger beeinflußt wird. Diese Erklärung von MARNAY und NACHMANSOHN stimmt mit der unsrigen im wesentlichen überein, obwohl die genannten Autoren die quantitativen Zusammenhänge der Hydrolyse und Perfusionsgeschwindigkeit auf andere Weise festzustellen suchten.

3. *Vergleich der in vivo und in vitro gewonnenen Aktivitätsergebnisse*

Wird die Cholinesteraseaktivität bei der Perfusion gemessen, so stellt sich schließlich die Frage, wie groß die Fraktion der gesamten Cholinesteraseaktivität des Ganglions ist, die nach dieser Methode bestimmt werden kann. Zugleich aber besteht die Möglichkeit, die Permeabilitätsverhältnisse der verschiedenen Substrate und Inhibitoren zu untersuchen. Zuerst untersuchten wir die Frage mit dem gut diffundierenden Äthylchlorazetat, das zugleich ein gutes Substrat der Cholinesterase bildet. Im Verlauf der Versuche bestimmten wir nach der Perfusionsmethode die *in-vivo*-Aktivität des Ganglions, wonach wir es homogenisierten und auch die Hydrolyse *in vitro* feststellten. Einige Ergebnisse dieser Versuche sind in Tabelle VII zusammengefaßt.

Tabelle VII

ÄCA-Konz. µg/ml	Hydrolyse in vitro µg/min	Hydrolyse in vivo µg/min	In vivo im Prozentsatz von in vitro
270	26,6	18,9	71
353	40,0	33,1	83
357	32,5	15,1	49*

* Der Versuch erfolgte mit niedriger ausströmender Tropfenzahl.

In der Größenordnung stimmt das Verhältnis des Abbaus *in vivo* und *in vitro* mit den Angaben SCHLEYERS [9] überein, der am elektrischen Organ des *Torpedo electricus* ermittelte, daß von den in der Substratlösung inkubierten Schnitten des elektrischen Organs etwa 60—63% der nach Homogenisierung hydrolysierten Menge abgebaut worden waren. Aus Tabelle VII geht hervor, daß Äthylchlorazetat bei intakter Struktur nicht imstande ist, die gesamte, es hydrolysierende Enzymmenge durch Diffusion zu erreichen. Es ist möglich, daß das Ganglion eine Esterase enthält, die in ihrer Natur und Lokalisation mit der Cholinesterase nicht identisch ist. Auf diese Tatsache kann aus den Angaben SCHLEYERS [9] ebenso wie aus unserem in Tabelle IV mitgeteilten

Versuch geschlossen werden. Diese Esterase wird von Eserin wahrscheinlich nicht gehemmt.

Im Zusammenhang mit Azetylcholin und Azetyl-β-methylcholin haben wir das Verhältnis der Hydrolyse *in vivo* und *in vitro* gleichfalls untersucht. Diese Frage wollen wir indessen, da sie auch zu verschiedenen anderen Beobachtungen geführt hat, in einer späteren Mitteilung behandeln.

Die nach der Perfusionsmethode vorgenommenen Versuche erhellen in gewisser Hinsicht auch die Permeabilitätsverhältnisse der Cholinesterase-Inhibitoren. Bei der Besprechung des 1. Punktes erwähnten wir bereits die Hemmungswirkung von Eserin auf die Hydrolyse von Äthylchlorazetat *in vivo*. Tabelle IV ist zu entnehmen, daß Eserin *in vivo* nur wenig mehr als zu 50% imstande ist, die Äthylchlorazetathydrolyse zu hemmen. Diese Tatsache läßt den Schluß zu, daß es nicht die gesamte Äthylchlorazetat hydrolysierende Enzymmenge zu erreichen vermag. Dies ist möglicherweise darauf zurückzuführen, daß auch die sympathischen Ganglien ein Enzym enthalten, das zum Abbau von Äthylchlorazetat imstande, aber mit der Cholinesterase weder in seiner Struktur noch Lokalisation identisch ist.

Aus der Tatsache, daß die Azetylcholinhydrolyse mit 10^{-6} *M* TEPP 100%ig gehemmt werden kann, ziehen wir den Schluß, daß die Permeabilitätsverhältnisse von Azetylcholin und TEPP im großen ganzen übereinstimmen.

LITERATUR

1. Glick, D.: J. gen. Physiol. **107**, 370 (1938).
2. Nachmansohn, D.: C. R. Soc. Biol. (Paris) **129**, 830 (1938).
3. Kibjakow, A.: Pflügers Arch. ges. Physiol. **232**, 432 (1933).
4. Koelle, G. B.: J. Pharmacol. **103**, 153 (1951).
5. Holaday, D. A., Kamijo, K., Koelle, G. B.: J. Pharmacol. **111**, 241, (1954).
6. Koelle, G. B.: J. Pharmacol. **114**, 167 (1955).
7. Fehér, O., Bokri, E.: Pflügers Arch. ges. Physiol. **269**, 55 (1959).
8. Fehér, O., Bokri, E.: Pflügers Arch. ges. Physiol. **269**, 67 (1959).
9. Schleyer, W. L.: Biochim. biophys. Acta **16**, 396 (1955).
10. *Сейдлиц О.:* Доклады Акад. Наук СССР 6, 170 (1938).
11. Marnay, A., Minz, B., Nachmansohn, D.: C. R. Soc. Biol. (Paris) **125**, 43 (1937).
12. Prawditsch-Neminskaja, T.: Szovjet Orvostud. Beszámoló, Sebészet, **5** (Okt. 1951).
13. Fehér, O.: Acta physiol. hung. **11**, 291 (1957).
14. Hestrin, S.: J. biol. Chem. **180**, 249 (1949).
15. MacIntosh, F. C., Emmelin, N.: J. Physiol. **131**, 477 (1956).
16. Ogston, A. G.: J. Physiol. **129**, 222 (1955).

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BEITRÄGE ZUR CHOLINESTERASEKINETIK IN VIVO

II. VERGLEICH DER AZETYLCHOLIN- UND AZETYL-β-METHYLCHOLINHYDROLYSE AM OBEREN HALSGANGLION DER KATZE IN VIVO UND IN VITRO

Von

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Die Azetylcholin- und Azetyl-β-methylcholinhydrolyse durch die Cholinesterase des *in vivo* perfundierten und dann homogenisierten Ganglion cervicale superius der Katze wurde untersucht. Azetylcholin ergab *in vitro* eine glockenförmige Substratkurve, woraus hervorgeht, daß das Ganglion hauptsächlich Azetylcholinesterase enthält. *In vivo* zeigten die Werte große Streuungen. Azetyl-β-methylcholin ergab *in vitro* eine glockenförmige Substratkurve, *in vivo* jedoch die für Pseudocholinesterase charakteristische S-förmige Substratkurve. Es darf daher angenommen werden, daß die Kinetik der Azetylcholinesterase unter diesen Umständen mit der *in vivo* festgestellten Kinetik nicht übereinstimmt.

Nachdem wir in der vorigen Mitteilung [1] geklärt haben, welche Faktoren die Azetylcholinhydrolyse im Perfusionsversuch *in vivo* regulieren, untersuchten wir die Frage, ob besondere kinetische Gesetzmäßigkeiten zur Geltung kommen, wenn die Cholinesterase ihre Aktivität an die Struktur gebunden, an ihrer Entstehungsstelle ausübt. Zu diesem Zweck untersuchten wir die Azetylcholin- und Azetyl-β-methylcholinhydrolyse in Perfusionsversuchen *in vivo* und suchten diese mit der Cholinesteraseaktivität von Ganglionhomogenaten zu vergleichen. Aus den Versuchsergebnissen errechneten wir auch die Dissoziationskonstanten der Ganglioncholinesterase nach der graphischen Methode von LINEWEAVER und BURK [2].

Methode

Die Bestimmung der Cholinesteraseaktivität des Ganglions durch Perfusion *in vivo* haben wir früher beschrieben [3].

Die Aktivität der Homogenate wurde nach folgender Methode festgestellt: Die beidseitigen oberen Halsganglien von 5 mit 0,1 g/kg Hexobarbital narkotisierten Katzen beiderlei Geschlechts wurden herausgeschnitten, gewogen und mit Locke-Lösung im Porzellanmörser homogenisiert. 1 ml Homogenat enthielt 10 mg Feuchtgewicht Ganglion. Die Inkubationsgemische hatten folgende Zusammensetzung:

Homogenat	0,500 ml
Veronal-HCl-Puffer pH 7,2	1,000 ml
Azetylcholin-HCl (10 mg/ml)	0,543 ml ($3 \cdot 10^{-3} M$)
Destilliertes Wasser ad	3,000 ml

Vom Inkubationsgemisch wurden in der 0. Minute der Inkubation 0,2—1,0 ml entnommen; die Hydrolyse wurde durch Zugabe von 1 ml 10%iger Trichloressigsäure abgestellt. Nach Filtration bestimmten wir die Azetylcholinkonzentration des Gemisches nach der HESTRINschen Methode [4]. Ähnlich gingen wir mit dem in der 30. Minute der Inkubation im Wasserbad bei 37° C entnommenen Inkubat vor.

In anderen Versuchen wurde die Hydrolyse von Azetyl-β-methylcholinbromid nach diesem Verfahren untersucht.

Wir verwendeten bei den Versuchen Azetylcholin HCl *(Hoffmann-La Roche)* und Azetyl-β-methylcholin HBr *(Light & Co.)*.

Ergebnisse

Azetylcholinhydrolyse in vivo und in vitro. Die Untersuchung der Cholinesterase mit der Perfusionsmethode *in vivo* wird durch den Umstand erschwert, daß an einem Ganglion die Hydrolyse nur bei einer, höchstens bei zwei Konzentrationen untersucht werden kann, weil die Enzymaktivität nach einer gewissen Zeit abnimmt. Da die Aktivität der Ganglien individuell ziemlich verschieden ist, zeigen die *in vivo* bei verschiedenen Konzentrationen gewonnenen Azetylcholinhydrolysewerte der einzelnen Ganglien große Streuungen. Zur Feststellung kinetischer Gesetzmäßigkeiten sind sie daher wenig geeignet.

Die bei der Hydrolyse von Azetylcholin *in vivo* gewonnenen Resultate enthält Tabelle I.

Tabelle I

Datum	Substratkonzentration in *M*	Hydrolysiertes Ach µg/g/30 min
20. XI.	$1{,}04 \cdot 10^{-3}$	85,7
25. XI.	$1{,}04 \cdot 10^{-3}$	117,5
9. II.	$2{,}03 \cdot 10^{-2}$	136,0
27. XI.	$2{,}29 \cdot 10^{-2}$	73,0
10. II.	$3{,}68 \cdot 10^{-2}$	76,0
17. XII.	$3{,}86 \cdot 10^{-2}$	202,0
30. III.	$3{,}94 \cdot 10^{-4}$	7,1

Aus den Angaben der Tabelle I kann nicht auf genaue kinetische Gesetzmäßigkeiten geschlossen werden; bei derartiger Streuung ist es nicht möglich, die Dissoziationskonstanten festzustellen. Die in Tabelle I enthaltenen Werte sind in Abb. 1 mit Kreuzen bezeichnet.

Abb. 1 zeigt die nach der in dieser Arbeit mitgeteilten Methode errechnete Substratkurve *in vitro*, welche den aus der Literatur bekannten glockenförmigen Verlauf aufweist und auch die Bestimmung der Dissoziationskonstanten gestattete. Von 2 Präparaten ergaben diese nach der Methode von LINEWEAVER und BURK [2] folgende Werte:

1. K_1: $7{,}70 \cdot 10^{-4}$ *M*
   K_2: $1{,}47 \cdot 10^{-2}$ *M*
2. K_1: $1{,}47 \cdot 10^{-3}$ *M*
   K_2: $1{,}30 \cdot 10^{-2}$ *M*

In Abb. 1 ist die mit dem Präparat Nr. 1 aufgenommene Substratkurve dargestellt. Von den beiden Präparaten werden am Substratoptimum stündlich auf 1 g Feuchtgewicht gerechnet 280 bzw. 352 mg Azetylcholin abgebaut. NACHMANSOHN [5] fand die Hydrolysegeschwindigkeit am Ganglion cervicale superius 18,2 bzw. 21,4 mg in 100 mg frischem Gewebe binnen 60 Sekunden.

Das Ganglion cervicale superius der Katze enthält sowohl die pseudo- wie die spezifische Cholinesterase. Die Substratkurve *in vitro* hat dennoch charakteristische Glockenform und zeigt nicht an, daß das Homogenat auch eine Esterase enthält, die mit zunehmender Konzentration immer größere Sub-

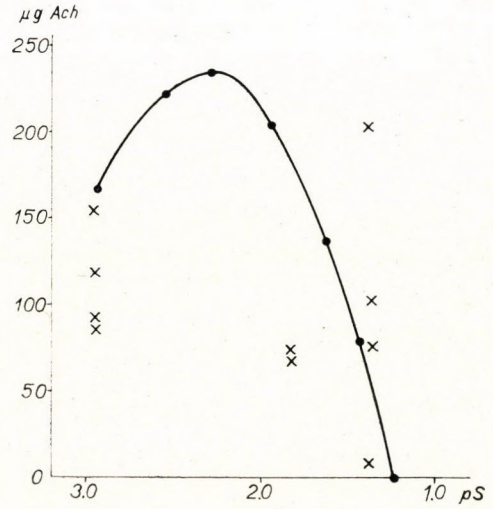

Abb. 1. Azetylcholinhydrolyse durch das obere Halsganglion der Katze *in vitro* und *in vivo*. Ordinate: In 30 Minuten hydrolysierte Ach-Menge in µg; Abszisse: Logarithmus der Substratkonzentration. Fortlaufende Linie: Substratkurve des Ganglionhomogenats. Kreuze: *In vivo* festgestellte Aktivität einzelner Ganglien in µg Ach/mg Gewebe/30 min

stratmengen abbaut. Wir führen dies darauf zurück, daß die pseudo-Cholinesterase, die nach KOELLE *und Mitarbeitern* im Interstitium des Ganglions anwesend ist, verglichen mit der Azetylcholinesterase geringe Aktivität besitzt und eine untergeordnete Rolle spielt.

Azetyl-β-methylcholinhydrolyse in vivo und in vitro. In einer anderen Versuchsreihe untersuchten wir die Azetyl-β-methylcholinhydrolyse *in vivo* und *in vitro*. Diese Substanz ist bekanntlich als spezifisches Substrat der Azetylcholinesterase zu betrachten.

Die Versuche *in vitro* ergaben die in Abb. 2 sichtbare Substratkurve (gestrichelte Linie). Aus der Kurve ließ sich die Konstante K_1 bestimmen, die $3{,}21 \cdot 10^{-3}$ *M* ausmachte, was innerhalb der in der Literatur angegeben Werte liegt. Die *in vivo* mit der Perfusionsmethode gewonnene Substratkurve

ist gleichfalls in Abb. 2 wiedergegeben. Im Gegensatz zu den Verhältnissen *in vitro* trat bis zu der höchsten unserseits angewandten Konzentration von $4{,}8 \cdot 10^{-2}$ *M* keine Substrathemmung ein, und der Verlauf der Substratkurve erinnert an die der pseudo-Cholinesterase. Die Dissoziationskonstante ist wesentlich höher als die *in vitro* festgestellte: $3{,}8 \cdot 10^{-2}$ *M* (Abb. 2).

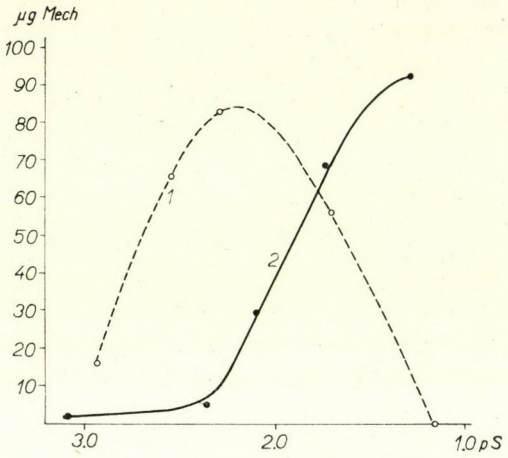

Abb. 2. Azetyl-β-methylcholinhydrolyse durch das obere Halsganglion der Katze *in vitro* und *in vivo*. Ordinate: In 30 Minuten hydrolysiertes Substrat in µg; Abszisse: Logarithmus der Substratkonzetration. Fortlaufende Linie: Azetyl-β-methylcholinhydrolyse *in vivo* in µg Mech/mg Gewebe/30 min. Gestrichelte Linie: Substrakturve des Ganglienhomogenats

Besprechung

Vergleichen wir die im höheren Konzentrationsbereich festgestellten Hydrolysegeschwindigkeiten *in vivo* und *in vitro*, so finden wir, daß *in vitro* die Hemmungswirkung des Substrats bereits stark zur Geltung kommt, während die Hydrolyse *in vivo* beinahe linear zur Konzentrationserhöhung zunimmt. Unseres Erachtens beruht die Erscheinung darauf, daß sich das Substratoptimum der Azetylcholinesterase *in vivo* stark zum höheren Konzentrationsbereich verschiebt. Auf die Deutung der Erscheinung werden wir später zurückkommen.

Wenn wir die *in vivo* ermittelten Azetylcholin- und Azetyl-β-methylcholinhydrolysewerte vergleichen, so sehen wir, daß die Azetylcholinhydrolyse starke Streuung zeigt und ihr keinerlei Gesetzmäßigkeit entnommen werden kann, während der an verschiedenen Ganglien festgestellte Azetyl-β-methylcholinabbau kinetisch verwertbare Gesetzmäßigkeiten aufweist. Der Unterschied dürfte darauf zurückzuführen sein, daß zwar der Azetylcholinesterasegehalt einzelner Ganglien im großen ganzen konstant ist, aber die pseudo-Cholinesteraseaktivität große Veränderungen zeigt, die zur Inkonstanz der Azetylcholinhydrolyse führen. Für diese Annahme zeugt ferner, daß die Sub-

stratkurve, wenn wir die azetylcholinspaltende Fähigkeit mehrerer Ganglionhomogenate untersuchen, bei verschiedenen Substratkonzentrationen den erwarteten regelmäßigen Verlauf aufweist. Das auf diese Weise hergestellte Präparat hat nämlich einen konstanten pseudo-Cholinesterasegehalt.

Bei der Untersuchung der Azetylcholin- und Azetyl-β-methylcholinhydrolyse *in vivo* ergibt sich zunächst die Frage, ob sich nicht die Messung der an und für sich vorhandenen Aktivität durch die Strukturgebundenheit der Cholinesterase, durch ihre Lokalisation an der Membranoberfläche oder innerhalb der Membran infolge Permeabilitätsschwierigkeiten komplizierter gestaltet. Zur Klarstellung dieser Frage haben wir die vergleichende Untersuchung der Hydrolyse dieser beiden Substrate *in vivo* und *in vitro* vorgenommen. SCHLEYER [6] hat gleichfalls versucht, diese Frage zu klären, und festgestellt, daß *in vivo* an intakten elektrischen Lamellen, wenn Äthylchlorazetat als Substrat benutzt wird, nur etwa 2/3 der Aktivität *in vitro* gemessen werden kann.

Unsere Versuche geben von dieser Frage kein derart einheitliches Bild. Bei der Untersuchung der Azetylcholinhydrolyse *in vivo* und *in vitro* sahen wir, daß *in vitro* die bezeichnende glockenförmige Substratkurve in Erscheinung trat, während die Azetylcholinhydrolyseergebnisse *in vivo* große Streuung zeigen.

Ein einheitliches und die Bedingungen der kinetischen Behandlung gewährleistendes Bild ergibt die Untersuchung der Azetyl-β-methylcholinhydrolyse. Hier läßt sich ebenfalls beobachten, daß die Kurve *in vitro* den erwarteten glockenförmigen Verlauf aufweist, während die Kurve *in vivo* bei niedrigeren Konzentrationen nach langsamer Erhöhung steil aufwärts steigt und die *in-vitro*-Werte weit hinter sich läßt. Eine Substrathemmung konnten wir im untersuchten Konzentrationsbereich nicht verzeichnen.

Es stellt sich die Frage, worauf diese unterschiedliche Hydrolyse *in vivo* und *in vitro* beruht. Wie einleitend erwähnt, sind wir der Meinung, daß die Abweichung hauptsächlich auf Permeabilitätsschwierigkeiten zurückgeführt werden muß. Es ist denkbar, daß das Substrat infolge der Hemmungswirkung der Membranstruktur nicht imstande ist, einen Teil der Cholinesterase zu erreichen oder zu dieser in einer Konzentration zu gelangen, wie dies in einer homogenen Lösung möglich wäre. Hiermit ließe sich das Verhalten der niedrigen Substratkonzentrationen im Falle beider Substanzen erklären. Aus welchem Grunde kommt es aber bei der Azetyl-β-methylcholinhydrolyse *in vivo* zur jähen Knickung der Kurve und dazu, daß die Hydrolyse *in vivo* bei gewissen Konzentrationen das Mehrfache des Wertes *in vitro* ausmacht? Man könnte sich vorstellen, daß infolge Permeabilitätsschwierigkeiten in der Perfusionsflüssigkeit eine wesentlich höhere Substratkonzentration angewendet werden muß, um an der Enzymoberfläche eine lokale Konzentration zu erreichen, die eine rasche Hydrolyse ermöglicht. Offen bleibt aber die Frage, worauf es zurück-

geführt werden kann, daß die *in vivo* festgestellten Hydrolysewerte an einem Punkt den Wert der Hydrolyse *in vitro* auch im absoluten Wert übersteigen. Wenn lediglich Permeabilitätsschwierigkeiten vorliegen würden, so könnten diese Werte höchstens bis zu *in-vitro*-Niveau steigen.

Die Antwort auf diese Frage gibt in bezug auf Azetyl-β-methylcholin auch die Form der *in-vivo*-Kurve, an der keine Substrathemmung festgestellt werden kann. Die Ursache der Abweichung wird demnach bei Azetyl-β-methylcholin sicher, bei Azetylcholin möglicherweise der Umstand sein, daß die Hydrolyse *in vivo* und *in vitro* jeweils eine andere Kinetik hat. Die Cholinesterase befindet sich scheinbar an ihrer natürlichen Stelle, in der Zellmembran, unter Verhältnissen, die die Entwicklung einer Substrathemmung nicht begünstigen.

ZELLER [7] stellt sich die Substrathemmung so vor, daß sich zu einer einzigen hydrolysierenden Cholinesteraseeinheit, an eine Anion- und eine Estergruppe, 2 Azetylcholinmoleküle binden und die wechselseitige Hydrolyse gegenseitig verhindern. Nach BERGMANNS Modell [8] hingegen, laut welchem zur Bindung des Kations am Azetylcholinmolekül an der Azetylcholinesteraseoberfläche zwei negative Gruppen erforderlich sind, kann man sich die Substrathemmung so vorstellen, daß sich an beide negative Ladungen je ein Azetylcholinmolekül bindet, die wechselseitig verhindern, daß ihr Azyl-Sauerstoff in die Nähe der Estergruppe gelangt.

Welche Konzeption wir auch annehmen, das Ausbleiben der Substrathemmung *in vivo* läßt sich nur so erklären, daß die Cholinesterase in der Membran in eine sieb- oder porenartige Struktur eingebaut ist, welche es infolge der Beschränkung der Porengröße einfach unmöglich macht, daß gleichzeitig zwei Azetylcholin- oder Azetyl-β-methylcholinmoleküle in die Nähe der reaktiven Gruppen gelangen. Bei niedrigeren Konzentrationen wird die Hydrolyse durch diesen Umstand eingeschränkt, bei höheren die Entwicklung der Substrathemmung unmöglich gemacht. In diesen Fällen wird die Hydrolyse ähnlich vor sich gehen wie die der pseudo-Cholinesterase.

Eine andere beachtenswerte Tatsache, welche die Erklärung des Fehlens der Substrathemmung erleichtern wird, ist folgende: Sowohl MENDEL und RUDNEY [9] wie MYERS [10] haben festgestellt, daß sich das Substratoptimum der Cholinesterase in Anwesenheit von monovalenten Alkalimetall-, hauptsächlich von K-Ionen in hohen Konzentrationen, zu höherer Konzentration verschiebt. Bei niedrigen Konzentrationen wird somit der Hydrolysewert in Anwesenheit von K-Ionen niedriger, bei hohen Konzentrationen jedoch höher sein als ohne K. Es läßt sich denken, daß wenn die K-Ionen-Konzentration in der Zelle und Membran das Mehrfache der im Ganglienhomogenat ausmacht, dadurch das Substratoptimum der Cholinesterase zu höheren Konzentrationen verschoben wird, so daß sich die Substrathemmung nicht zu manifestieren vermag.

Der Mechanismus dieser Wirkung der K-Ionen ist heute noch unbekannt, doch darf angenommen werden, daß dieser Effekt nicht nur *in vitro*, sondern auch *in vivo* in Erscheinung tritt.

Wie immer wir die oben beschriebenen Tatsachen auch zu erklären suchen, muß aus dem Beispiel der Cholinesterase geschlossen werden, daß die Untersuchung der Enzyme in Homogenaten kein vollständiges Bild von ihrer Aktivität in der natürlichen Umgebung, ja vielleicht nicht einmal von ihrer physiologischen Funktion vermittelt.

Schließlich bleibt noch die Frage, warum die Substratkurve von Azetyl-β-methylcholin an der Grenze der niedrigen und mittleren Konzentrationen eine Knickung erleidet. Nach unseren an anderer Stelle mitgeteilten Versuchsergebnissen [3] wird durch die Erregung, Depolarisation der Nervenelemente die abgebaute Substratmenge *in vivo* ausgeprägt erhöht, entweder dadurch, daß im Anschluß an den Impuls eine größere Zahl reaktiver Gruppen frei wird, oder indem durch den Impuls infolge der Permeabilitätssteigerung einfach der Kontakt zwischen Enzym und Substrat erleichtert wird. In bezug auf die erwähnte Knickung der Substratkurve nehmen wir an, daß an diesem Punkt die depolarisierende Wirkung der Substratlösung auf die Ganglienzellen zur Geltung kommt und die Cholinesterase aus einem der beiden oder aus beiden erwähnten Gründen instand gesetzt wird, die Hydrolysegeschwindigkeit zu beschleunigen.

LITERATUR

1. Fehér, O., Bokri, E.: Acta physiol. hung. **18,** 1 (1960)
2. Lineweaver, H., Burk, D.: J. Amer. chem. Soc. **56,** 658 (1934).
3. Fehér, O.: Acta physiol. hung. **11,** 291 (1957).
4. Hestrin, S.: J. biol. Chem. **180,** 249 (1949).
5. Nachmansohn, D.: C. R. Soc. Biol. (Paris) **129,** 830 (1938).
6. Schleyer, W. L.: Biochim. biophys. Acta **16,** 396 (1955).
7. Zeller, E. A.: Advanc. Enzymol. **8,** 464 (1948).
8. Bergmann, F.: Faraday Society Discussions, **20,** 126 (1952).
9. Mendel, B., Rudney, H.: Science, **102,** 616 (1945).
10. Myers, D. K.: Arch. Biochem. Biophys. **37,** 469 (1952).

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CONNECTION BETWEEN VAGAL AFFERENTATION AND HIGHER NERVOUS ACTIVITY

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The influence on the higher nervous activity of vagotomy performed at various levels has been studied in the dog. As compared to the preoperative behaviour, conditioned reflexes after vagotomy exhibited quantitative and qualitative changes; inactive inhibitory states as well as active states changed in characteristic periodicity. In addition some other alterations such as repetitive paradoxical reactions and phasic states also occurred.

As to the explanation of the phenomena described, an indirect mechanism has been suggested by our observations: the periodical postvagotomic changes in the conditioned reflex activity were namely found to be closely connected with changes in the serum calcium level. Disappearance of the conditioned reflexes was associated with a high serum calcium level, and even the gradual extinction and restitution of the reflex activity went parallel with the change of the serum calcium level.

The cortical representation of the autonomic nervous system has long been recognized. HUGHLINGS JACKSON [1] already stated that visceral functions possessed cortical representation, as they were distributed in epilepsy. BECHTEREW [2] observed that a lesion in the neighbourhood of the central gyrus was followed by temperature elevation in the contralateral half of the body.

The problem of the physiological role and significance of vegetative, and, in general, of visceral, interoceptors has received a decisive impulse through the investigations of BYKOW and his school [3]. These authors dealing with the cortical analysis of the internal organs have supplemented and completed PAVLOV's conception on the conditioned reflexes. BYKOW namely stated that the cerebral cortex is regulating the behaviour of the organism by temporary connections, and alters the functional state of the internal organs by the same mechanism. At the same time there exists an inverse connection as the interoceptors of the internal organs are capable of reacting on the central nervous system and of altering thereby behaviour of the animal.

It is remarkable that the pertaining works of BYKOW, in the first line those dealing with the cortical analysis of internal organ functions, should have appeared in 1928 [4], at a time when the morphology of the cortical representation of visceral interoceptors was still unknown and no anatomical data permitted to suppose direct impulses to go from the interoceptors to the cortex.

The first proof of that process was made known as late as 1938. BAILEY

and BREMER [5], investigating the cerebral cortex in the cat with electrophysiological methods, have observed local changes in the cortical potentials after repeated stimulation of the vagal nerve. The area determined in this manner corresponded to the lower and posterior parts of the composite gyrus lying at the orbital surface of the frontal lobe.

Among the more recent findings, those of BONVALLET, DELL and HUGELIN [6] are of special interest. These authors extended the experiments of BAILEY and BREMER to an analysis of the subcortical and cortical projections of the vagal nerve and verified that visceral vagal projections did in fact reach the cortex, to the most part at the upper area of the olfactory cortex. Beside the cortical projections, direct subcortical vagal projections could be demonstrated at the level of the temporal region, localized in the lateral and basal magnocellular nuclei of the amygdale complex.

GERYABIN [7] observed a marked diminution in the alimentary conditioned reflex activity after the destruction of the thalamus and hypothalamus, with the relative preponderance of inhibitory processes. GRASTYÁN, LISSÁK and KÉKESI [8] investigated the alimentary conditioned reflexes as well as the conditioned avoidance behaviour in the after electrical stimulation of the hypothalamus and the reticular formation. They concluded that stimulation of these areas was facilitatory in effect and influenced conditioned responses in direction of an inhibition. ÁDÁM *et al.* [14] demonstrated that bilateral denervation of the carotid sinus altered the previously elaborated conditioned exteroceptive motor reflexes. This alteration manifested itself with the dominance of excitatory processes over inhibitory ones.

Although the connections between visceral afferentation and higher nervous activity have been widely investigated with both conditioned reflex and electrophysiological methods, the functional significance of the cortical connection of vagal afferentation itself has hardly been studied. Our earlier investigations into the vagal afferentation and vagal efferentation [9—11] led us to extend our studies to the fields of higher nervous activity. The present report deals with the effect on conditioned reflexes of the interruption of the vagal nerve, and the results gained on three animals will be described in detail.

Methods

The experiments were performed in a sound-proof Pavlovian chamber. A conditioned reflex with feeding as unconditioned stimulus was elaborated in the dogs according to the stereotypes to be described. A saliva fistula was prepared the animals; saliva drops were counted by means of a modified electrical drop-counter. In three dogs stereotypes were elaborated as follows,

Dog "Nero": sound stimulus 6/2*
bell
projected light
sound stimulus 6/2*
differentiating sound stimulus 600/2*
bell

Dog "Griff": bell
sound stimulus 6/2*
differentiating sound stimulus 60/5*
sound stimulus 6/2*
bell

Dog "Bogár": bell
projected light
sound stimulus 60/2*
differentiating sound stimulus 600/6*
sound stimulus 60/5*

The first number refers to the frequency of the sound generator, while the second number to the intensity.

The experiments were made daily between 3 to 5 p. m. Intervals between the stimuli lasted 5 minutes, while the conditioned stimulus was left acting for 20 seconds. The preoperative experimental period lasted several months with each dog. After fixation of the conditioned responses the animals were subjected to subdiaphragmatic or thoracocervical vagotomy (thoracal on the right side and cervical on the left), and investigation of the conditioned reflexes was continued.

Results

Dog "Nero"

This dog was subjected to subdiaphragmatic vagotomy following the normal period of conditioned reflexes. On the effect of the vagotomy, the conditioned responses underwent a marked change of which the main characteristics were a gradual decrease in the responses so that the animal at the 6th to 7th days failed to excrete even one drop of saliva after the conditioned stimulus; it did not take attention to stimuli and did not eat. During the experiment lasting 30 minutes the number of conditioned and unconditioned drops failed to attain 15. In addition, marked somnolence was observed.

Conditioned reflexes changed in the following way. Differentiation became absolute as soon as 3 days following vagotomy. On the fourth day, the light stimulus became ineffective. On the fifth day, only one bell caused one drop of conditioned saliva to be excreted, while stimulus 6/2 was completely ineffective. The total of the conditioned saliva drops underwent a gradual decrease.

After this period demonstrating the diminution of the conditioned reflex activity, a gradual restitution occurred from the 10th to 12th days. At the 10th day, the animal produced one drop saliva after the first bell and 8 drops after sound 6/2. Reaction to light returned by the 11th day and by the 12th day, this stimulus was even more effective than sound 6/2.

At the 13th to 14th days, a new decrease was observed, even though to lesser extent than in the previous inhibitory state. Characteristic of this stage was also a shift in the relation of individual stimuli, as demonstrated in Fig. 1.

Fluctuation occurred also on further days, but the drop count of conditioned saliva in the inhibitory period failed to reach the abscissa. At the time of restitution, conditioned salivary secretion was higher than in the normal period. The changes described were daily observed during the normal period lasting 1.5 months.

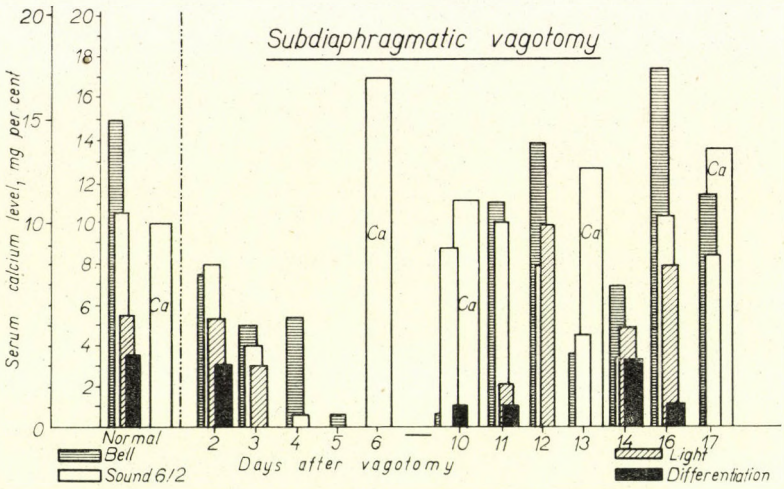

Fig. 1. Dog N e r o. Quantitative changes in conditioned salivation and serum calcium level before and after subdiaphragmatic vagotomy

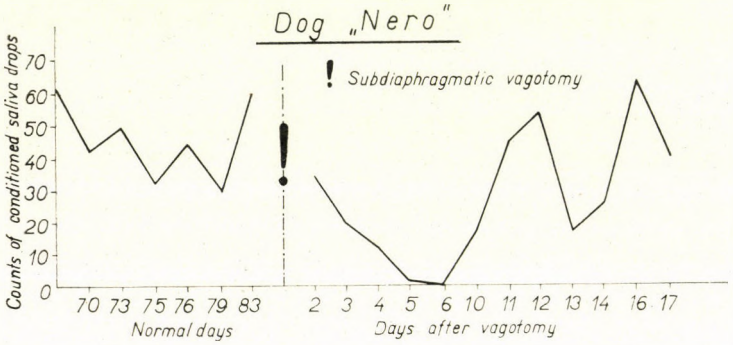

Fig. 2. Dog N e r o. Variations in total conditioned saliva drop count before and after vagotomy

Fig. 2 shows the fluctuations in the total conditioned drop count before and after vagotomy.

Dog "Griff"

This animal was subjected to thoracocervical vagotomy. The findings essentially corresponded to those described above. All conditioned reflexes disappeared as soon as two days after operation, with the exception of differentiating inhibition, which gave one drop of saliva. The animal on the third and fourth day did not react to the stimuli, did not eat, conditioned motor reactions were absent.

Restitution began on the fifth day, when the first bell, the first sound 6/2 and the differentiating sound stimulus 60/5 produced conditioned salivation, one drop each. Conditioned responses were completely normalized by the 10th day and, just as in the case of dog "Nero", the conditioned saliva drop

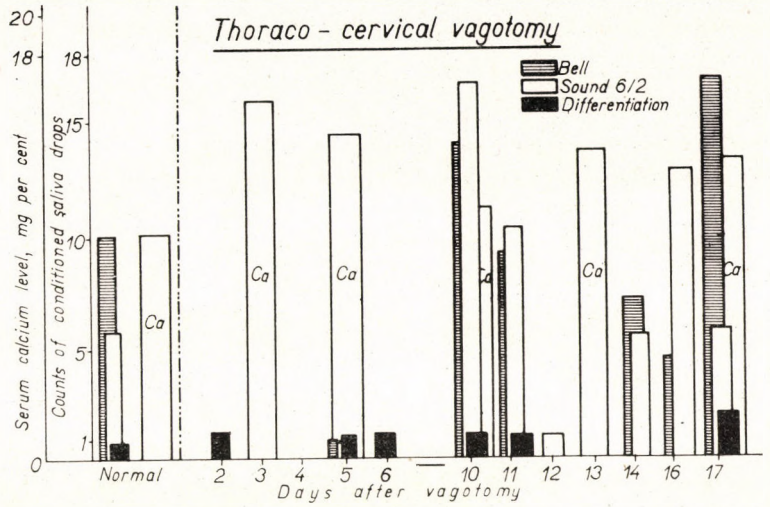

Fig. 3. Dog G r i f f. Quantitative changes in conditioned salivation and serum calcium level before and after thoracocervical vagotomy

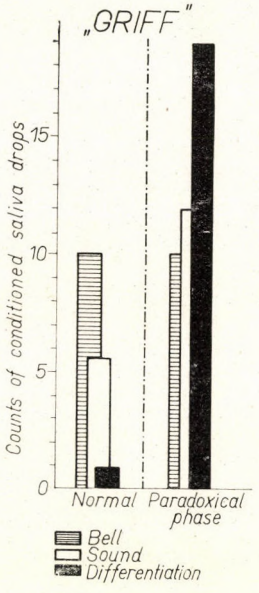

Fig. 4. Dog G r i f f. Typical form of paradoxical reaction 20 days after vagotomy

count was higher than in the normal period. Subsequently, fluctuations were stronger than in the first dog. In the last week a characteristic paradoxical reaction even occurred. The dog died 28 days after the vagotomy, a time 7 to 10 days longer than the usual survival after an intervention of this type *(Fig. 3 and 4)*.

Dog "Bogár"

After subdiaphragmatic vagotomy, periodicity was observed also in this dog, although less extreme than in the other animals. Extinction and restitution of the reflexes were also more gradual.

This dog was subsequently subjected to thoracocervical vagotomy. Postoperative fluctuations in the conditioned reflexes were extreme, differentiation markedly determinated, especially in the second part of the period, when paradoxical reactions were also repeatedly noted.

As vagotomy was performed in two stages, survival time was considerably lengthened. The animal was in perfect condition and had lost no weight 4 months after starting the experiments.

Then the right cervical vagal stump was also transected. Soon thereafter characteristic signs of bilateral cervical vagotomy developed, the conditioned reflexes gradually disappeared and the animal died 7 days after the last operation (Fig. 5).

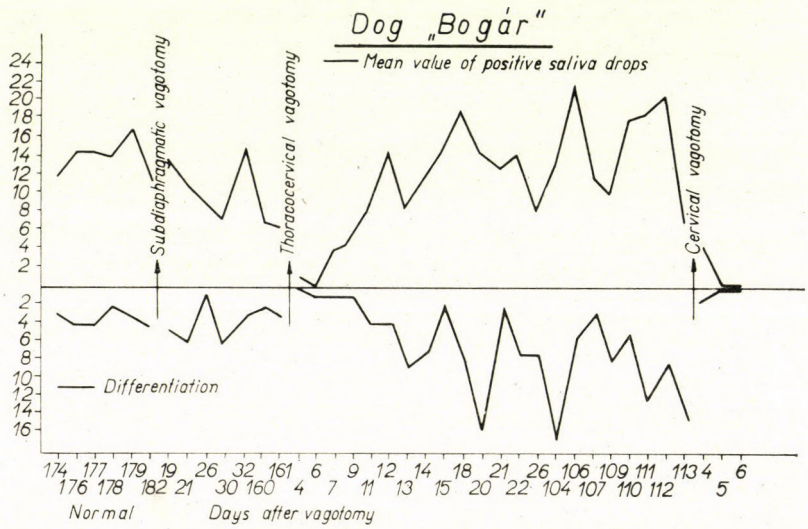

Fig. 5. Dog B o g á r. Changes in the mean positive and negative saliva drop count at normal state, and after subdiaphragmatic, thoracocervical as well as cervical vagotomy.

Discussion

In our experiments vagotomy was followed by quantitative and qualitative changes in the conditioned reflex reactions. Inactive inhibitory states and active states alternated with each other in characteristic periodicity. In addition, other manifestations were observed, such as repetitive paradoxical reactions and phasic states.

These results clearly demonstrate that an interruption of the afferen t impulses mediated through the vagal nerve brings about a change in th e elaborated conditioned reflexes. The question to be answered is, of course,

how this effect came into play: whether the interruption of the connection between vagus and cerebral cortex, or another indirect mechanism was responsible for the changes observed.

Prior to the detailed analysis of the role played by vagal afferentation, an observation made in previous experiments should be mentioned. Investigations into the serum components of vagotomized dogs had namely revealed marked changes in the calcium level. This surprising finding was then controlled during the present conditioned reflex studies, and a close relationship was found between the periodical alterations of the conditioned reflex activity and the calcium level. Disappearance of the conditioned reflexes was associated with high serum calcium values and even the gradual extinction and restitution of the reflexes was accompained by corresponding shifts in the serum calcium level, as demonstrated in Figs. 1 and 3.

Data in the litarature concerning the relationship between parathyroid gland and higher nervous activity support our observation. In the experiments of ANDREYEV and PEGSLEY [12], administration of parathyroid extract decreased the alimentary conditioned reflexes, enhanced the consecutive inhibition, accelerated the extinction and produced catalepsy: in other words a marked dominance of inhibitory process over excitatory ones was brought about. This effect on the conditioned reflexes was mediated through the elevation of the serum calcium level. The authors cited found a direct relationship between the shifts of the serum calcium level and the functional state of the cerebral cortex.

The influence of calcium administration (infusion of $CaCl_2$) on the conditioned reflexes was controlled also in our experiments. Conditioned reflex activity was considerably diminished at a serum calcium level of 13 mg per 100 ml (Table I).

Table I

Dog "Griff"

Effect of calcium infusion on conditioned reflexes. Differentiation became absolute, conditioned salivation decreased and inhibition was extended also to the positive sound stimulus following differentiation

Normal			Calcium infusion		
Stimulus	Duration of stimulus in sec.	Count of conditioned saliva drops	Stimulus	Duration of stimulus in sec.	Count of conditioned saliva drops
Bell I	20	15	Bell I	20	4
Sound 6/2	20	5	Sound 6/2	20	7
Sound 60/5	20	1	Sound 60/5	20	—
Sound 6/2	20	9	Sound 6/2	20	—
Bell I	20	11	Bell I	20	13

The findings just discussed raise the hitherto scarcely studied problem of the relationship between vagus and parathyroid, *viz.* calcium household. According to ROBY and SMITH [13], subdiaphragmatic vagotomy counteracts the tetany-inducing effect of parathyroidectomy. This observation is in partial accordance with our own observations.

The remarkable shifts in the serum calcium level might of course represent only one of the factors involved in the postvagotomic regulation of conditioned reflex activity and some further mechanism will most probably be revealed by future studies.

A further problem to be investigated in that the alimentary condition of the animal is also modified by the interruption of the vagal nerve. Our further investigations into the higher nervous activity of vagotomized animals are therefore planned to be performed by making use of other conditioned reflexes too.

It is, however, to be noted that the correlation of conditioned and unconditioned saliva drop counts remained practically unchanged after vagotomy. Also noteworthy is the finding that thoracocervical vagotomy failed to induce a marked postoperative change in the alimentation of the the animals, when these were fed outside the chamber.

Although in our opinion the method employing alimentary conditioned reflexes which is so well situated for the analysis of higher nervous activity, has proved reliable in the present experiments, in our future studies we shall control and complete our investigations with other methods.

LITERATURE

1. JACKSON, J. H.: A study of convulsion. Trans. St. Andrews Med. Grad. Ass. **3**, 162—207 (1870).
2. BECHTEREW, W. M. (БЕХТЕРЕВ, Б. М.): Одозрение психиатрии Ass. 3, 162-207 (1870.) нр. 4-0 (1014).
3. BYKOW, K. M.: Az agykéreg és a belső szervek. Akadémiai Kiadó, Budapest, 1953.
4. BYKOW, K. M. (БЫКОВ, К, М.): Труды III. Всесоюзного съезда физиологов, Ленинград, 1928, стр. 263.
5. BAILEY, D., BREMER, F.: J. Neurophysiol. **1**, 405 (1938).
6. BONVALLET, M., DELL, P., HUGELIN, A.: J. physiol. (Paris) **44**, 222 (1952).
7. GERYABIN, (ГЕРЯБИН,): Физ. Ж. СССР 32/5, (1946).
8. GRASTYÁN, E., LISSÁK, K., KÉKESI, F.: Acta physiol. hung. **9**, 133 (1956).
9. TIGYI, A., LISSÁK, K.: Acta physiol. hung. **6**, 477 (1954).
10. TIGYI, A., LISSÁK, K.: Acta physiol. hung. **8**, 231 (1955).
11. TIGYI, A., MIRISZLAI, E., KISS, K., LISSÁK, K.: Acta physiol. hung. (In the press.)
12. ANDREYEW, L. A., PEGSLEY, L. J. (АНДРЕЕВ, Л. А., ПЕГСЛЕЙ, Л. И.): Физлов. Ж. СССР 18, 1 (1945).
13. ROBY, CH. C., SMITH, S., PFEIFFER, C.: Amer. J. Physiol. **129**, 766 (1940).
14. ÁDÁM, GY., BÉLA, A., FÖVÉNYI, J., SZÉKELY, J., NAGY. K.: 25th Meeting of the Hungarian Physiological Society, Szeged, 1959.

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THE INFLAMMATORY "LEUCOPENIC FACTOR" (MENKIN) AND THE PHAGOCYTOSIS OF LEUCOCYTES

By

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MENKIN's inflammatory leucopenic factor administered intravenously to dogs, inhibits the phagocytosis of bacteria by polymorphonucleat granulocytes. The effect is parallel with the changes in the blood count, and is observable also *in vitro*, in the concentration range of from 10^{-4} to 10^{-6}. Some clinical aspects are also mentioned.

It has repeatedly been pointed out that exudates, of whatever origine, stimulate the phagociting activity of leucocytes. The effect is much more marked than that of blood serum or transudates. We have shown this action of the exudate to be a combined resultant of several factors [5, 6.]. We demonstrated that in addition to histamine, serotonin and nucleic acids, from among MENKIN's inflammatory stimulators leucotaxine, exudine and the leucocytosis promoting factor stimulate the process [7, 8], while necrosin inhibits it [9]. This time we investigated the effect of MENKIN's leucopenic factor [13], a polypeptide, on the phagocytosis of bacteria by leucocytes. The experiments were made in dogs, under conditions *in vivo* and *in vitro*.

Methods

The measure of phagocytosis was determined by the method of PLATONOW—LUDÁNY—VAJDA, described in detail previously. In the phagocytic system 0.1 ml of blood, then 0.1 ml of the bacterium suspension were added to Ringer solution containing 7 mg per 100 ml of heparin. The bacterium suspension was lyophilized *Staphylococcus pyogenes aureus* stored in ampoules under refrigeration. The suspension contained 10 million germs per mm^3. The total volume of the system was 1 ml. Siliconized tubes were used. After 30 minutes in an incubator and subsequent centrifugation, smears were prepared and stained according to *Gram*. We determined the number of microorganism phagocyted by 200 leucocytes. The difference from the initial value was expressed in per cent. The limit of error was $\sigma < \pm 8$ per cent.

Leucopenic factor was prepared according to MENKIN. The material was obtained from 2—3 day old exudates produced in big dogs by treatment with terebenthine oil and fractionated precipitation with ammonium sulphate. In contrast to leucopenine, the leucopenic factor is heat-stable. In the experiments *in vivo* we determined the number of polymorphinuclear granulocytes and lymphocytes. After longer periods have been allowed for adaptation, the experiments were performed without anaesthesia. The leucopenic factor was administered intravenously, in doses of from 2 to 4 mg/kg. Blood samples were obtained from the saphena parva vein.

Results and discussion

The experiments *in vivo* are illustrated in Fig. 1 (four experiments). One hour following the injection, the granulocyte count was definitely decreased.

A similar change was noted in the lymphocyte count, and the phagocyting activity also markedly diminished. At 3 and 4 hours a moderate granulocytosis with a slight increase in phagocytosis was observed. During that period there was no change in the body temperature of the animals.

The results *in vitro* are summarized in Table I. In the concentration range of from 10^{-4} to 10^{-6} the leucopenic factor inhibited the phagociting activity of granulocytes. Thus, the inflammatory agent has a direct action on the cell itself. Further studies are needed to determine the processes in the

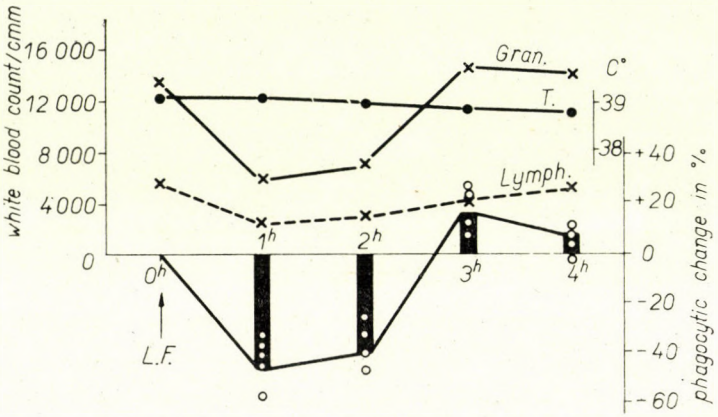

Fig. 1. Effect of leucopenic factor (LF) on the circulating leucocyte count and on the phagocyting activity of granulocytes

background of this; surface, and cell metabolic effects may be involved. Otherwise, the effect is comparable to that obtained with leucopenine, isolated from alkaline exudate [14].

Table 1

Count of staphylococci phagocyted and percentage change in phagocytosis

N°	Initial value Hep. Ringer sol.	Leucopenic factor		
		10^{-4}	10^{-5}	10^{-6}
1.	396	282 (—29)	329 (—17)	388 (—2)
2.	489	283 (—42)	367 (—26)	436 (—11)
3.	412	276 (—33)	317 (—23)	383 (—7)
4.	564	344 (—39)	428 (—24)	513 (—9)
5.	454	313 (—31)	386 (—15)	431 (—5)
6.	368	217 (—41)	400 (—15)	338 (—8)
	Mean :	*—35,8%*	*—20,0%*	*—7,0%*

Ample evidence is available as to the relation of inflammatory processes to leucopenia [16, 2, 3, 4, 1, 17, *etc.*]. Under certain conditions in inflammatory processes a marked leucopenia, or even agranulocytosis may result from the inhibition of leucocytopoiesis. MENKIN has demonstrated the important role of the two inflammatory agents, leucopenine and the leucopenic factor, present also in exudates. Our investigations have shown that in addition to the above morphological changes a reduced functional value of the leucocytes should also be taken into account; this is manifest in their osmotic resistance [11], and in their phagocyting activity.

The authors are indepted to Mrs. I. BOGNÁR and Mr. J. DOMONKOS for their technical help.

LITERATURE

1. DAMASHEK, W.: in CHRISTIAN, A, A.: Oxford Medicine, New York, Oxford Univ. Press, Vol. 3. 1944.
2. FRITZ-HUGH, T., KRUMBHAAR, E. B.: Amer. J. med. Sci. **183,** 104 (1932).
3. LAWRENCE, J. S.: J. Amer. med. Ass. **116,** 478 (1941).
4. LAWRENCE, J. S.: Amer. J. Pathol. **116,** 333 (1940).
5. LUDÁNY, G.: Verhandlungen der Deutschen Internistentagung, Leipzig, 1955.
6. LUDÁNY, G.: Proceedings of the XX. International Physiological Congress, Bruxelles, 1956.
7. LUDÁNY, G., VAJDA, GY.: Arch. int. pharmacodyn. **85,** 484 (1951); **100,** 339 (1955); **121,** 459 (1959).
8. LUDÁNY, G., VAJDA, GY., RIGÓ, J., HAN TO VU, Acta physiol. hung. **14,** 371 (1958).
9. LUDÁNY, G., VAJDA, GY., FEHÉR, I., HORVÁTH, G.: Experientia (Basel) **15,** 31 (1959).
10. LUDÁNY, G., VAJDA, GY., DÖKLEN, A., FEHÉR, I.: Naturwissenschaften **47,** 111 (1960).
11. LUDÁNY, G., RIGÓ, J., BUDAVÁRI, I.: In the press.
12. MENKIN, VALY.: Arch. Pathol. (Chicago) **41,** 50 (1946).
13. MENKIN, VALY: Arch. Pathol. (Chicago) **42,** 154 (1946).
14. MENKIN, VALY: Arch. Pathol. (Chicago) **46,** 145 (1948).
15. MENKIN, VALY: Biochemical Mechanisms in Inflammation. Thomas, Springfield, 1956.
16. SCHULTZ, W.: Dtsch. med. Wschr. **48,** 1495 (1922).
17. STURGIS, C. C.: Clinics **1,** 492 (1942).

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EFFECT OF HYPOTHERMIA ON PHOSPHORYLASE ACTIVITY IN THE LIVER

By

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The effect of deep hypothermia on the phosphorylase activity of rat liver has been studied in connection with investigations into the role played by adrenaline, a hormone figuring in the activation of liver phosphorylase. It has been found that

1. Phosphorylase activity decreases in deep hypothermia: the deeper and the more prolonged the hypothermia, the greater the diminution in the activity of the enzyme.

2. The hypothermic decrease of liver phosphorylase activity is not irreversible, as rewarmed animals exhibit enzyme activities identical with those found in normothermic rats.

3. Adrenaline decrease phosphorylase activity in the liver of normothermic animals. As a consequence of the lower initial activity, the percentage increase is even more marked in hypothermic than in normal rats.

4. The enzyme system which activates liver phosphorylase is not damaged by hypothermia.

5. The hypothermia-induced decrease in liver phosphorylase activity is not due to an impairment of the phosphorylase activating enzyme system, but to the lack of adrenaline and, presumably, to a lack of glucagon.

Although hypothermia was long ago recognized to influence the blood sugar level, the pertaining data in the literature are not unequivocal. Elevation of blood sugar level and diminution of liver glycogen content were observed at the initial stage of hypothermia, with the concomitant appearance of shivering [1]. On further cooling, the blood sugar level either remained unaltered or decreased, depending on the carbohydrate reserve, as well as on the shivering of the animals, or the chronic or acute way of cooling [2]. In other experiments [3], slow gradual cooling induced hypoglycaemia. There is, however, general agreement that if during the cooling no shivering occurs, in other words if there is no defence, the blood sugar level either remains unchanged or decreases.

The effect of hypothermia on the activity of muscle phosphorylase participating in the carbohydrate metabolism was investigated by Krebs and Fischer [4]. They found no significant difference betveen normothermic and hypothermic rabbits. Since liver phosphorylase, too, is involved in carbohydrate metabolism and as it differs from the muscle enzyme chemically, physiologically and immunologically [5], it seemed interesting to investigate the effect of hypothermia on its activity. In this connection, an attempt was made to clarify during hypothermia the action of adrenaline, a hormone equally

figuring in carbohydrate metabolism, in the activation of liver phosphorylase and in the regulation of blood sugar level [6].

Methods

Animals. The experiments were performed on albino rats ranging in weight from 200 to 300 g. As known, rats tolerate deep hypothermia very well. In addition, they are easily and quickly cooled, almost without any defence reaction. Cooling was made by means of cracked ice in nylon bag, as long as the rectal temperature fell to 18 to 20°C. Some of both the control and the hypothermic animals were then treated with 10 μg adrenaline intracardially.

Determination of phosphorylase and glucose-6-phosphatase activity. The animals in hypothermia were bled and 1 g liver was excised. This was immediately frozen in dry ice and extracted with a 20fold excess of 0.1 *M* NaF and 0.6 *M* sodium glycerolphosphate at pH 6.0. Phosphorylase activity was measured according to GREEN and CORI [7], determining the inorganic phosphate liberated from glucose-1-phosphate. Glucose-6-phosphatase activity was determined according to CORI [8], measuring the inorganic phosphate liberated from glucose-6-phosphate. Inorganic phosphate was determined with the method of TAUSSKY and SHORR [9]. The results refer to 100 mg wet liver weight.

Results and discussion

First, the liver phosphorylase activity was investigated under hypothermic conditions. As shown in Fig. 1 the activity of the enzyme decreased in proportion with the grade and duration of hypothermia. The height of the columns in Fig. 1 shows the average phosphorylase activity, while the points correspond to the individual values. In our opinion, the diminution of the phosphorylase activity was not due to stagnation of liver blood induced by hypothermia, as the activity of glucose-6-phosphatase, an enzyme similarly

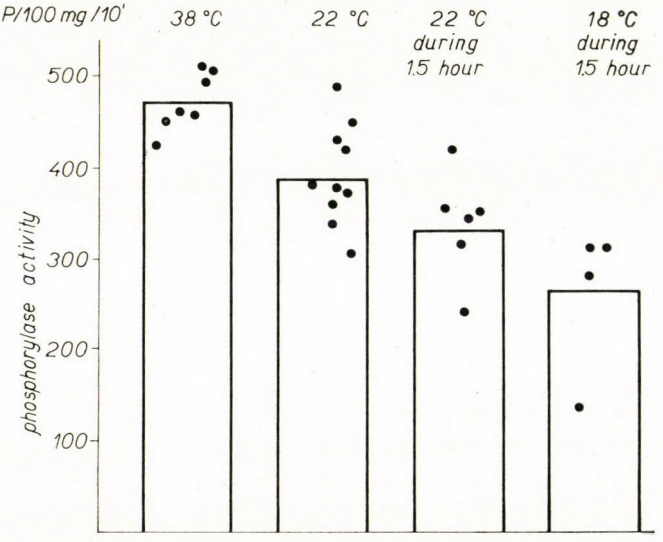

Fig. 1. Effect of hypothermia on phosphorylase activity in rat liver

involved into carbohydrate metabolism, failed to exhibit any marked change (Fig. 2).

We observed interesting seasonal fluctuations of the phosphorylase activity in autumn and winter. As seen from Fig. 3, the normothermic values

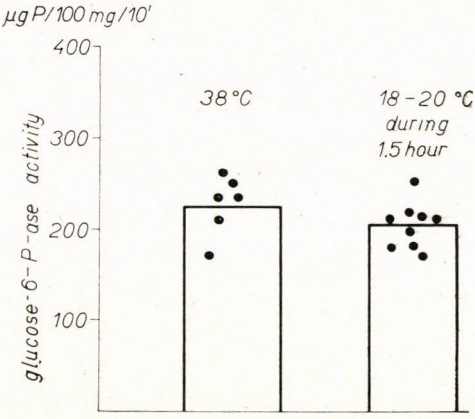

Fig. 2. Effect of hypothermia on glucose-6-phosphatase activity in rat liver

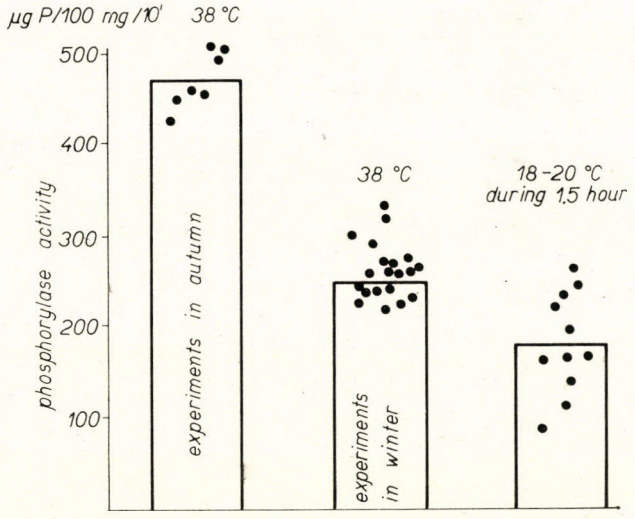

Fig. 3. Effect of hypothermia and seasonal influences on phosphorylase activity in rat liver

were lower in winter than in autumn. As an effect of hypothermia, winter animals reacted with a smaller decrease, meanwhile the deviation of the individual values from the arithmetic mean was greater than in autumn animals.

To ascertain whether the effect of hypothermia was reversible in character, some cooled animals were rewarmed to normal body temperature. Fig. 4 shows the liver phosphorylase activity in hypothermic and normothermic

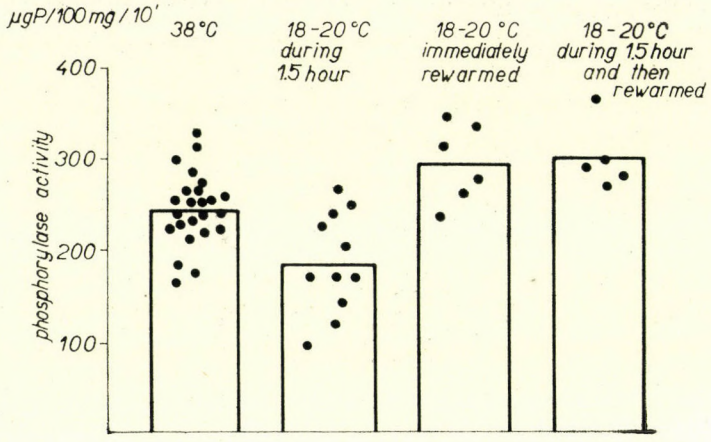

Fig. 4. Activity of liver phosphorylase after rewarming

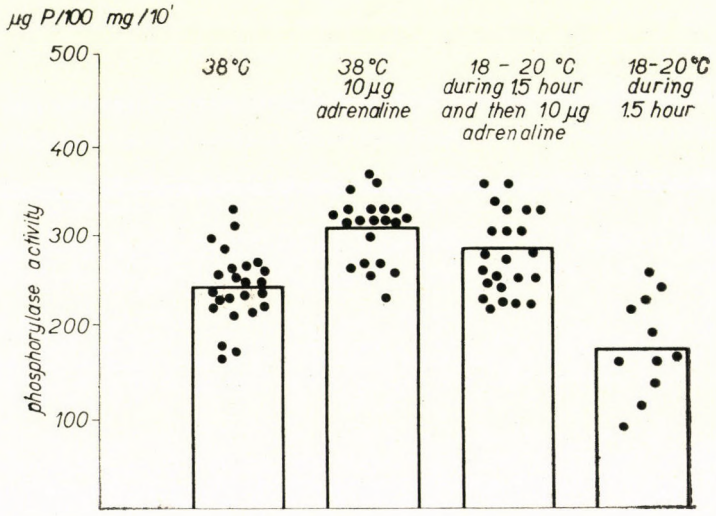

Fig. 5. Effect of adrenaline on liver phosphorylase activity in normal and hypothermic rats

animals, as immediately after rewarming, and 1 $^1/_2$ hours later. As seen, in the rewarmed animals phosphorylase activity reached, or even exceeded, the values found in normothermic rats.

In a search for the mechanism of the diminished phosphorylase activity in hypothermia, the effect of adrenaline figuring in the activation of the enzyme

was investigated under conditions *in vivo*. Fig. 5 shows the effect of adrenaline given intracardially to hypothermic and normothermic animals. In our previous experiments [6] in normothermic rats phosphorylase activity was increased by adrenaline. The adrenaline-induced activation was now found higher in hypothermic than in normothermic animals: the peak activity was identical in both cases, but the starting value was lower in the hypothermic rats.

From the experiments described above it may be seen that the hypothermia-induced decrease in liver phosphorylase activity is not due to an impairment of the enzyme system activating phosphorylase, but rather to the lack of adrenaline and, presumably, of glucagon. This statement is in accordance with the results of HUME *et al.* [10] according to which hypothermia of 26° and 21°C elicited a tenfold respectively hundredfold decrease in the adrenaline and noradrenaline secretion of the adrenal medulla.

Our results corroborate the observation that rapid cooling without defence is associated with hypoglycaemia, a finding easy to explain by the observed decrease in liver phosphorylase activity.

LITERATURE

1. FUHRMAN, F. A., CRISMON, J. M.: Amer. J. Physiol. **149,** 552 (1947).
2. HORVATH, S. M., SPURR, G. B.: The Physiology of Induced Hypothermia. Washington, 1956.
3. GROSSE-BROCKHOFF, F., SCHOEDEL, W.: Arch. exp. Pathol. Pharmakol. **201,** 417 (1943).
4. KREBS, E. G., FISCHER, E. H.: J. biol. Chem. **216,** 113 (1955).
5. JÓKAY, I., BOT, GY., SZILÁGYI, T.: Acta physiol. hung. **14,** 155 (1958).
6. BOT, GY., SZILÁGYI, T., SZABÓ, E.: Acta physiol. hung. **11,** 421 (1956).
7. GREEN, A. A., CORI, G. T.: J. biol. Chem. **151,** 21 (1943).
8. CORI, G. T., CORI, C. F.: J. biol. Chem. **199,** 661 (1952).
9. TAUSSKY, H. H., SHORR, E.: J. biol. Chem. **202,** 675 (1953).
10. HUME, D. M., EGDAHL, R. H., NELSON, D. H.: The Physiology of Induced Hypothermia, Washington, 1956.

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PRESSOR—DEPRESSOR COUNTERREGULATION IN EXPERIMENTAL HYPERTENSION INDUCED BY VARIOUS METHODS

By

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By means of the authors own method, the changes in the free, diffusible histamine content of the blood plasma during the infusion of 20 μg/kg/min. of adrenaline were studied in normal dogs, as well as in dogs with experimental hypertension.

(i) In the normal dogs the free histamine level of the blood plasma increased to 2 to 4 fold the initial value during adrenaline infusion. At the peak of histaminaemia the carotid pressure recorded on the Leersum loop decreased to below the initial level.

(ii) The free histamine level was unchanged during adrenaline infusion in dogs made hypertensive by depressor and carotid sinus denervation.

(iii) During the development of renal hypertension induced by the cellophane perinephritis method the free (diffusible) histamine content of the blood plasma increased parallel with the increase of blood pressure, remained at a maximum level for 6 to 8 months, subsequently to decrease to its preexperimental value.

(iv) In the first months of the hypertensive state induced by perirenal fibrosis the plasma histamine level increased to 2 to 4 fold on the effect of adrenaline. During the subsequent months this response to the adrenergic stimulation tended to decrease, then ceased altogether, in other words a state corresponding to the counterregulatory block immediately following carotid sinus and depressor denervation developed.

In recent years we have studied several aspects of that form of circulatory regulation which manifest itself with a mobilization of antagonists by vasoactive agents. In mammalian heart preparations we have examined the reversed, acetylcholine-like effects in response to adrenaline, as well as to the stimulation of the accelerator nerves [1, 2, 3, 4]; further, we have investigated the cause and mechanism of vasodilatation and acetylcholine mobilization in response to adrenaline and stimulation of the lumbosacral sympathetic chain [5, 6, 7, 8], in the artificially perfused hind limbs of dogs. In addition, we have observed in adapted unanaesthetized dogs kept under physiological conditions the phenomenon described earlier by other authors that during the intravenous infusion of adrenaline the blood histamine level increases to manifold the initial value [9].

The evidence obtained in these earlier experiments has induced us to conclude that the mobilization of antagonistic substances in response to cholinergic or adrenergic stimulation might be considered a compensatory phenomenon in physiological blood pressure control: in other words that the arterial pressure would arise as a result of a compensatory equilibrium between the pressor and the depressor neurohormonal activities.

To check the reliability of this hypothesis, we investigated in what measure the control mechanism providing for an equilibrium of pressor and depressor agents would take effect in the experimental hypertension of dogs.

Methods

Unanaesthetized normotensive and hypertensive dogs adapted to experimentation were used to study the changes in blood pressure and plasma histamine level in response to the infusion of adrenaline. Blood pressure was recorded continuously from the carotid artery, by means of a suitable apparatus applicable to the Leersum-loop. Adrenaline dissolved in physiological saline was infused into the saphenous vein and blood was obtained from the jugular vein. The rate of infusion was set at 20 μg/kg/min. of adrenaline, the constancy of the rate was ensured by means of a motor-driven syringe. Beside the continuous recording of blood pressure, samples of blood for histamine assay were obtained from the jugular vein before, during and after adrenaline infusion. It was realized that the histamine bound to protein and formed elements was present in inactive form and for this reason the free, diffusible, vasoactive histamine content of the blood plasma was determined. The method employed may be outlined as follows.

To prevent blood clotting, crystalline potassium oxalate (30 mg/10 ml of blood), to inhibit plasma histaminase semicarbazide (10^{-3} *M*), were used. The plasma was dialysed against an equal volume of physiological NaCl solution for 1 hour, then after adding to it 0.1 ml of normal hydrochloric acid, the dialysate was evaporated dry. The residue was extracted with 3×1 ml of absolute aethyl alcohol and after adding to it 0.1 ml of normal hydrochloric acid the extract vas evaporated dry in a water bath, taken up in 0.4 ml formic acid-acetic buffer and a determined amount of it was run in a horizontal paper electrophoretic apparatus. The buffer used in the electrophoresis consisted of twice distilled glacial acetic acid: twice distilled formic acid: distilled water 15 : 5 : 80; pH 1.8. *Macherey—Nagel* N° 214 paper strips measuring 4 by 40 cm each were used. The sample containing histamine was applied near the positive pole onto the strip wetted with buffer. The sample was run at 1.5 mA/strip, 0°C, for 30 minutes. To locate histamine, one of the extracts was taken up in 0.4 ml formic acid-acetic acid buffer containing 100 μg/ml of histamine and 0.1 ml of this was applied to the control paper strip. After running, the strips were dried in flowing hot air. Histamine on the control strip was developed with the diazo reagent. The control strip showed the location of histamine on the test strip; from the corresponding area histamine was eluted three times, with a total volume of 10 ml of 0.01 *N*HCl, then the eluate was dried in a water bath. The residue was taken up in a given volume of Tyrode's solution and was tested, after checking the pH, for histamine content on guinea pig intestine.

Considering that the dog plasma contains little histamine and that the quantities of histamine eluted from the strips were extremely slight, titration on the guinea pig intestine was done by the superfusion method of GADDUM [10], which is 5 to 12 times more sensitive than the bath method. Owing to the extreme sensitivity of this method, it was imperative that the optimum conditions should be created: the more sensitive the method, the greater the deviations caused by changes in the sensitivity of the organ used in biological assay. Stability of sensitivity was ensured by the constancy of pH, temperature and ion concentration, the exchange of washing, standard and test fluids at equal intervals, and equal dosage of these.

In the superfusion technique the isolated organ is suspended in a space under constant vapour pressure and temperature and is kept in an optimal milieu by a flow of Tyrode's solution on its surface. The flow of physiological saline is interrupted for a while and the test solution is applied onto the surface. The test solution is washed off with Tyrode's solution and after an interval of the same duration the standard solution is superfused. We had devised an apparatus for the chronological co-ordination of part-processes and for keeping temperature constant; the detailed description of this apparatus has been published [11].

In Table I are shown the data for the histamine content of three different dog plasmas and for the percentual recovery of known amounts of histamine, added to these plasmas. The data have proved the reliability of our method because nearly 100 per cent of the histamine added was recovered in every case.

Hypertension was induced in two ways: depressor and carotid sinus denervation, and by bringing about perirenal fibrosis according to PAGE [14] and RAU [15].

Table I

	Added H μg/l	estimated μg/l	Recovered %
I. Plasma			
a)		16	
b)		16	
Plasma + H.	20	36	100
	20	34,5	92
	40	53	92,5
	40	55	97,5
II. Plasma			
a)		24	
b)		24,5	
Plasma + H.	10	33,5	92,5
	10	34,5	102,5
	30	52	93,3
	30	53	97
III. Plasma			
a)		9,6	
b)		10,1	
Plasma + H.	20	29	95
	20	30,2	101,7

Results

1. Experiments on normotensive dogs

In the first series the changes of plasma histamine during adrenaline infusion were studied in normotensive, unanaesthetized dogs. Earlier experiments (WENT and VARGA [9]) showed that during the infusion of 20 μg/kg/min. of adrenaline the total blood histamine level was raised by 40 to 42 per cent. These experiments had to be reproduced, because the values obtained included also those for the inactive histamine bound to protein and formed elements and because at that time the methods employed were not sensitive enough to determine the low level of free, diffusible, vasoactive histamine in the dog plasma. It was therefore deemed desirable to assay the free histamine of blood plasma during adrenaline infusion by the new, sensitive and more reliable. method. The results of 4 such experiments are presented in Fig. 1.

The duration of adrenaline infusion and the times of blood sampling are shown on the abscissa. The free, diffusible histamine content (in μg/ml) of plasma is shown on the ordinate. The results presented were obtained in 4 different dogs. The solid line indicates the changes in response to physiological saline infusion, the broken, dotted and result lines indicate those obtained during adrenaline infusion. The free histamine level of plasma increased gradually during adrenaline infusion, reaching a maximum in some cases after the stnpping of infusion. After the infusion of adrenaline had been

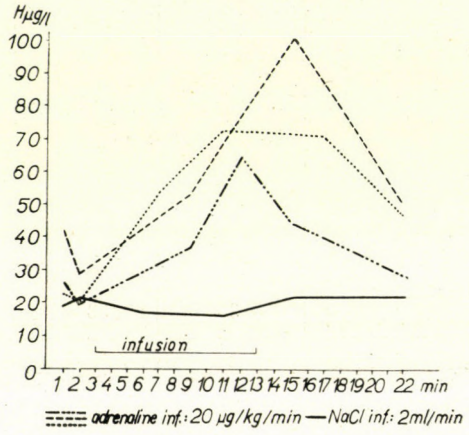

Fig. 1. Experiments in normotensive dogs. Changes of the free histamine level of plasma in response to adrenaline infusion (20 μg/kg/min). Abscissa: time in minutes. Ordinate: histamine content in μg/litre. Solid line: response to infusion of physiological saline; broken, dotted and result lines: responses to adrenaline infusion

brought to an end the level decreased, reaching the initial value in 10 to 13 minutes. The rise of level during infusion amounted to from 200 to 400 per cent.

In anaesthetized animals the increase of the histamine level in response to adrenaline manifested itself also in the carotid pressure recorded on the Leersum-loop.

Fig. 2 B shows that in the anaesthetized animals blood pressure steeply increased in response to adrenaline infusion, then decreased below the initial level. A comparison of Fig. 1 with Fig. 2 B reveals that this depression of carotid pressure took place at a time when the free histamine level of plasma was near or at the peak. This reversion of the pressor response during adrenaline infusion has not been observed in unanaesthetized animals, although in these, too, the free histamine level of plasma increased by 200 to 400 per cent in response to adrenaline. Further experiments should elucidate the cause of this difference in response between unanaesthetized and anaesthetized animals.

II. Experiments on hypertensive dogs

(i) To induce hypertension, carotid sinus and depressor denervation had been performed in 10 normotensive dogs. After operation their blood pressure rose to 220—250 mm Hg and remained permanently at that level.

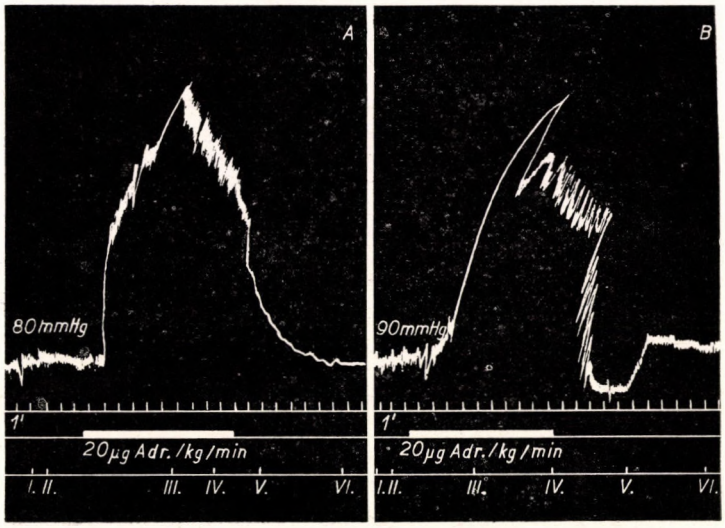

Fig. 2. Carotid pressure recorded on Leersum-loop during adrenaline infusion. *A* : unanaesthetized, *B* : anaesthetized dogs

When the postoperative period was over, the changes in the free, diffusible histamine level of the plasma of these hypertensive animals was studiedduring the infusion of 20 µg/kg/min. of adrenaline. The results are shown in Fig. 3.

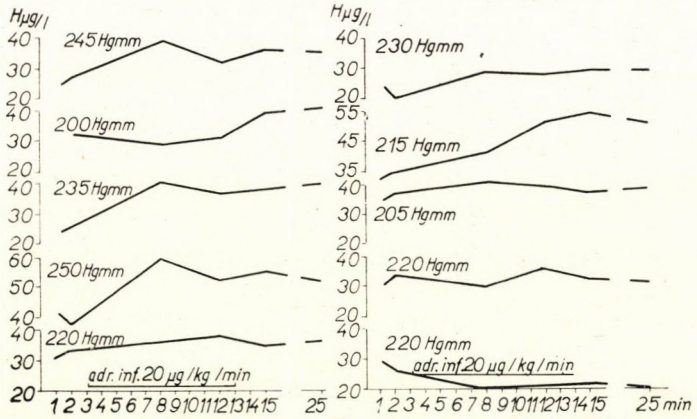

Fig. 3. Hypertension induced by carotid sinus and depressor denervation. Changes in the free histamine level of plasma during adrenaline infusion (20 µg/kg/min). Abscissa: time in minutes. Ordinate: histamine concentration µg/litre

In Fig. 3 the abscissa shows the time in minutes, and the ordinate the plasma histamine level in μg/l. It can be seen that as opposed to what occurred in the normal dogs (Fig. 1) this time there was practically no change in the plasma histamine level during adrenaline infusion. A similar result had been

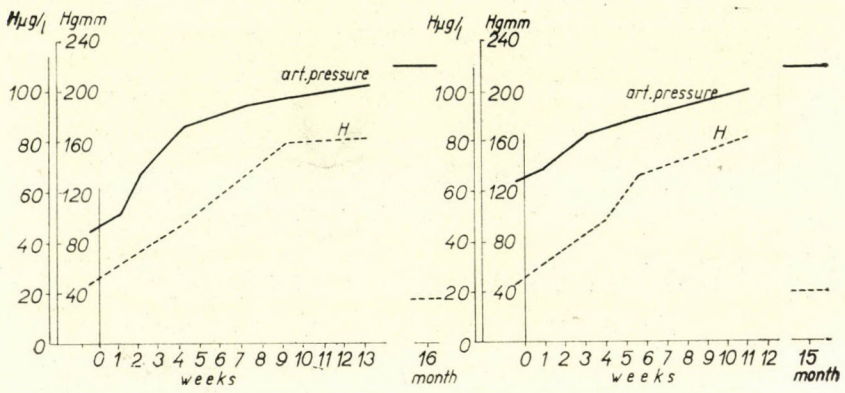

Fig. 4. During the development of hypertension induced by perirenal fibrosis the free histamine level of blood plasma increases parallel with the increase of blood pressure. Abscissa: time in weeks. Ordinate: blood pressure in mm Hg, histamine in μg/litre

obtained in our earlier experiments [11], but that time the uncertain method for histamine assay did not permit to draw conclusions.

(ii) In a second group (5 dogs) hypertension was induced by the modified method of RAU [13] resp. PAGE [12]. To induce perirenal fibrosis both kidneys were placed into cellophane sacs filled with acrylate shavings. Blood pressure began to increase within two weeks, reaching a peak during the 10th to 15th weeks. From then on blood pressure was established at a level around 200 mm Hg (Fig. 4).

Fig. 4 shows that during the development of hypertension induced by perirenal fibrosis the free plasma histamine level increased parallel with the increase of blood pressure. After hypertension had reached its peak, the plasma histamine level remained at a maximum for 6 to 8 months, decreasing to normal during the subsequent months. These results suggest that the counterregulatory mechanism comes into action not only in the adrenaline-histamine relation, but also in the relation of pressor substances formed as a result of renal lesion and histamine. Thus, the regulatory mechanism based on the mobilization of antagonists strives to compensate the action of pressor agents of renal origin. However, with protracted hypertension the possibility of this regulation gradually diminishes.

In the dogs rendered hypertensive by perirenal fibrosis the changes of the free, diffusible plasma histamine level were again studied in response to the infusion of 20 μg/kg/min. of adrenaline.

The solid lines in Fig. 5 represent the results obtained 3 to 6 months, the thin lines those 9 to 12 months, the broken ones those obtained 15 to 18 months after operation. During the first few months of hypertension the counterregulatory mechanism based on antagonist mobilization was undisturbed; the plasma histamine level increased to 2 to 3 fold its initial value in response to adrenaline. In the period of from 9 to 12 months this mechanism was markedly impaired: in spite of the lower initial values, the histamine

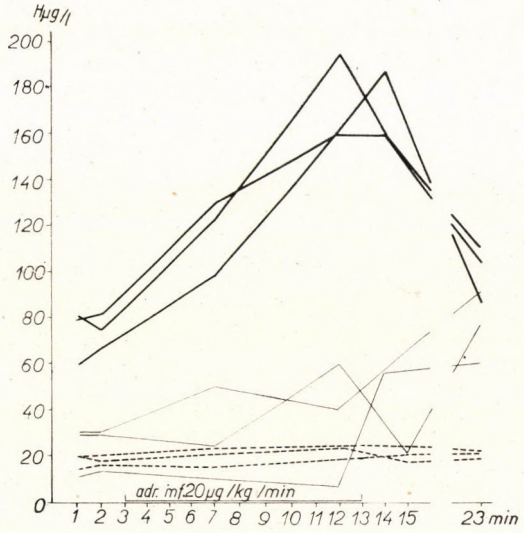

Fig. 5. Dogs rendered hypertensive by perirenal fibrosis. Changes in the free histamine level of plasma during adrenaline infusion. Abscissa: time in minutes. Ordinate: histamine concentration μg/l. The solid lines represent the results for the period of from 3 to 6 months, the thin ones those for 9 to 12 months and the broken lines those for 15 to 18 months after operation

level was less increased by the adrenaline infusion than before. Finally, after 12 months no counterregulation came into effect. In this late phase of renal hypertension there developed a condition identical with that immediately following carotid sinus and depressor denervation. This observation is in harmony with the evidence published by McCubbin, Green and Page [14] that in the late phase of renal hypertension a severe impairment of the baroceptor mechanism of the carotid sinus and aorta can be demonstrated by electrophysiological methods.

Discussion

Our recently employed methods have made it possible reliably to assay the quantity of free, diffusible, vasoactive histamine in the blood plasma of the dog. According to our results, in unanaesthetized dogs kept under

physiological conditions the diffusible histamine level of plasma increases by 200 to 400 per cent in response to the infusion of 20 μg/kg/min. adrenaline. In earlier experiments on the artificially perfused hind limbs of dogs [8] it was shown that the intraarterial administration of adrenaline and the electrical stimulation of the lower lumbar chain gives rise to a double — constrictor and dilator — effect, and that during the dilatatory response considerable quantities of acetylcholine are released from the perfused tissues [5]. The mobilization of histamine and acetylcholine in response to adrenergic stimulation is considered to be a compensatory mechanism in the service of physiological blood pressure control.

In the experiments described in this paper the mechanism providing for an equilibrium of pressor and depressor influences was studied in hypertensive dogs. The free, diffusible, vasoactive histamine content of the blood plasma was assayed before, during and after the intravenous infusion of adrenaline. It was found that in the hypertensive state induced by carotid sinus and depressor denervation this regulation is not functioning, as the free plasma histamine level remained unchanged during adrenaline infusion. During the first months of hypertension induced by the cellophane-perinephritis method of PAGE [12] the counterregulatory mechanism based on antagonist mobilization was still unimpaired in the adrenaline-histamine relation and was abolished only around the 15th month, when a condition appeared corresponding to the counterregulatory blockade seen immediately after carotid sinus and depressor denervation.

Thus, our experiments have confirmed the view that after a certain period renal hypertension interferes with neural regulation. They have also shown that in both kinds of experimental hypertension employed, *viz.* in hypertension following elimination of the baroceptor mechanism and in renal hypertension, the equilibration of vasoactive agents antagonizing each other is abolished. In other words, the counterregulatory control governed by the central nervous system does not any more come into play. It is believed that the mobilization of antagonistic agents in response to adrenergic stimulation is a compensatory phenomenon in the service of physiological blood pressure control, and its abolition leads to a preponderance of neurohormonal pressor activity.

LITERATURE

1. Went, I., Szücs, E., Kovács, T.: Acta physiol. hung. **6**, 47 (1954).
2. Szentiványi, M., Kövér, A.: Acta physiol. hung. **9**, 203 (1956).
3. Szentiványi, M., Kiss, E.: Acta physiol. hung. **10**, 337 (1956).
4. Szentiványi, M., Kiss, E.: Acta physiol. hung. **11**, 347 (1957).
5. Went, I., Szücs, E., HeténYi, E.: Acta physiol. hung. **9**, 193 (1956).
6. Szücs, E., HeténYi, E., Went, I.: Acta physiol. hung. **11**, 317 (1957).
7. Szücs, E., HeténYi, E., Went, I.: Acta physiol. hung. **11**, 327 (1957).
8. Went, I.: Ideggyógy. Szle. (Budapest) 149 (1957).
9. Went, I., Varga, E.: Acta physiol. hung. **3**, 377 (1952).
10. Gaddum, J. H.: Brit. J. Pharmacol. **8**, 321 (1953).
11. Kövér, A., Balla, L., Pusztai, L.: Acta physiol. hung. **11**, 363 (1957).
12. Page, J. H.: J. Amer. med. Ass. **113**, 2046 (1939).
13. Rau, G. C.: Science **111**, 229 (1950).
14. McCubbin, J. W., Green, J. H., Page, J. H.: Circulation Res. (N. Y.) **4**, 205 (1956).

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THE EFFECT OF SH-INHIBITORS ON GANGLIONIC TRANSMISSION IN THE SUPERIOR CERVICAL GANGLION OF THE CAT

By

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The changes in the electrical activity of the cat's superior cervical ganglion in response to SH-inhibitors have been studied *in situ* and in isolated ganglia, by recording the action potentials. At lower concentrations the SH-inhibitors blocked transmission reversibly and potentiated the ganglion stimulating action of acetylcholine injected into the blood stream. Higher concentrations inhibited the ganglionic excitation produced by acetylcholine and KCl injected into the blood stream. All the above effects of the SH-inhibitors were temporarily suspended by cysteine. The chief conclusions drawn from these results are as follows.

1. The two kinds of acetylcholine receptor system present in sympathetic ganglia are different in their sensitivity to SH-inhibitors.

2. The SH-groups play a significant role in the structure of the postsynaptic membrane and even in that of the acetylcholine receptors.

The problem of mediator-reception is one of the less known problems connected with the transmission of impulses. It is beyond doubt that in sympathetic ganglia acetylcholine acts as a mediator. Recently, increased attention has been focussed upon the analysis of the nature of acetylcholine reception [1, 6, 11].

We have undertaken to establish in which of the phases of ganglionic transmission have SH-groups a direct role, with special reference to the acetylcholine receptor function. For this purpose the superior cervical ganglion of the cat has been exposed to the action of sulphhydril inhibitors and changes in its function have been recorded.

The only pertaining report we have found in the literature is that by SMIRNOV *et al.* [10] who showed that the blocking of SH-groups by means of $CdCl_2$ results in the blocking of ganglionic transmission. As in their experiments the injected acetylcholine continued to stimulate the ganglion during the period of inhibition, the authors concluded that blocking was due to a diminution of acetylcholine release from the preganglionic endings, as a result of a paralysis of acetylcholine synthesis.

Methods

Fifty cats were used in the experiments. The animals were anaesthetized intraperitoneally by 0.10 g/kg chloralose. In most experiments the superior cervical ganglion was perfused *in vivo* by the method of KIBJAKOV [7], while in others the ganglion was excised to lead off action potentials.

The superior cervical ganglion, the cervical part of the sympathetic chain, the vagus, the common carotid artery and the internal jugular vein were exposed and the branches of the common carotid were ligated, except those supplying the ganglion. It was thus achieved that the perfusate administered through a cannula in the common carotid reached exclusively the superior cervical ganglion. On the other hand, ligation of the corresponding branches of the internal jugular vein made it possible to recover exclusively fluid that passed through the ganglion. Through the cannula in the common carotid Locke's solution filtered, oxygenated, and having a pH of from 7.2 to 7.4 was perfused at 140 mm Hg. The cannula in the common carotid served also for the injection of drugs without interrupting perfusion.

Ganglionic excitation was tested by the kymographic recording of the isotonic contractions of the nictitating membrane. The ganglion was stimulated electrically *via* the cervical sympathetic, as well as by giving injections of KCl and acetylcholine. Stimulation was done with 5 V, 200 μsec, 1—300/sec, for 5 sec, with a rectangular impulse generator.

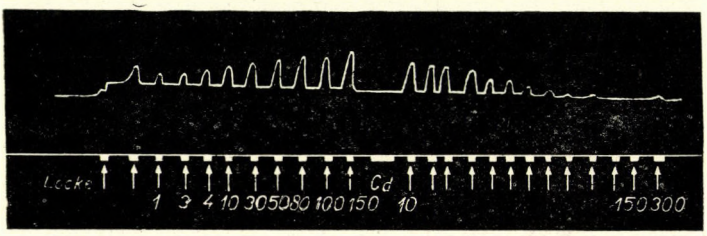

Fig. 1. Effect of $CdCl_2$ on ganglionic transmission. The figures beside the arrows indicate the frequency of stimulation in c/sec. Cd: Onset of $CdCl_2$ perfusion at a concentration of 5 μg/ml. ↑ :Preganglionic stimulation at 10/sec frequency, every minute

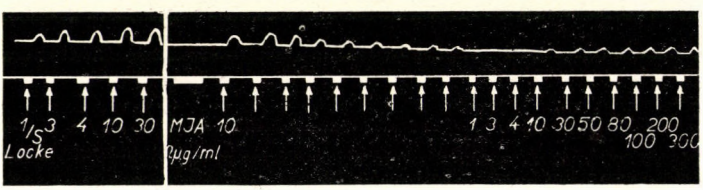

Fig. 2. Effect of monoiodoacetate perfusion on ganglionic transmission. Signs as in *Fig. 1*

In the second part of the experiments action potentials were led off from the superior cervical ganglion. The excised ganglion complete with pre- and post-ganglionic fibres was placed into a plexiglass chamber [5] allowing action potentials to be led off the ganglion fixed to stimulating and recording electrodes over periods of several hours. By changing the position of the chamber the preparation could be submerged into test solutions or into Locke's solution. Using a rectangular impulse generator, stimulation was made with 5 V, 200 μsec, amplifying with a battery-fed conventional RC amplifier. Recording from the oscillograph onto a photokymograph took place at a film speed of 9 cm/sec.

The test compounds used were acetylcholine bromide, monoiodoacetate (MIA), potassium chloride, p-chloromercuribenzoate (PCMB), zinc sulphate, cadmium chloride and cysteine, dissolved in Locke's solution, with the pH adjusted to 7.2—7.4.

Results

In the first series the effect of preganglionic stimulation was examined during perfusion with various SH-inhibitors. As Fig. 1 and Fig. 2 show, the preganglionic stimulation that had previously produced normal effects at every frequency was gradually losing potency during perfusion with $CdCl_2$

and MIA, respectively, and finally stimulation at a frequency of 10/sec became ineffective. At the same time, however, the blockade was suspended by stimulation at a higher frequency. Inhibition proved to be reversible in every case, *i.e.* perfusing the ganglion with Locke's solution restored transmission. There was no qualitative difference in the effect of the SH-inhibitors employed, they differed only in the effective concentration in the solution. The phenomenon shown in Figs. 1 and 2 could be elicited by $CdCl_2$ at concentrations of 8 to 10 μg/ml, MIA at 2 to 10 μg/ml, PCMB at 2 to 7 μg/ml, and $ZnSO_4$ at concentrations of 20 to 50 μg/ml. The effect was invariably proportionate to the concentration of the solution used, both as regards the final grade of inhibition and the rate of its development. At suitable concentrations, inhibition developed immediately following the onset of perfusion. With the same concentration of the SH-inhibitor and stimulation with the same intensity the measure of inhibition

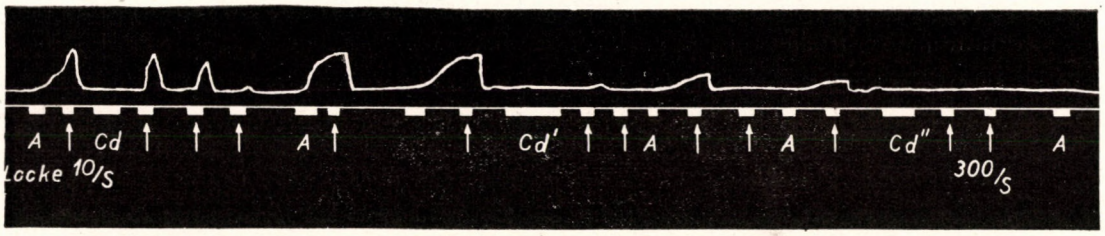

Fig. 3. Phase I and II of SH-inhibition in the case of $CdCl_2$ perfusion. Cd: Onset of $CdCl_2$ perfusion, 10 μg/ml. Cd′: Onset of $CdCl_2$ perfusion, 15 μg/ml. Cd″ : Onset of $CdCl_2$ perfusion, 20 μg/ml. A : Injection of 0.4 ml of 100μg/ml AchBr. At the peak of the ganglionic excitation caused by Ach, preganglionic stimulation was applied.↑ : preganglionic stimulation at a frequency of 10/sec

in a given case will be determined by the frequency of stimulation. After cessation of the effect of preganglionic stimulation direct stimulation of the ganglion with the same parameters proved to be effective in every case.

Next, it was examined whether the blockade brought about by the SH-inhibitors at the above concentrations would extend also to the ganglionic excitatory action of acetylcholine injected directly into the ganglion through the blood stream. As Fig. 3 reveals, we recorded first the ganglionic excitation in response to acetylcholine during perfusion with Locke's solution. In most cases preganglionic stimulation was employed at the peak of the ganglionic excitation thus produced, in order simultaneously to observe the two kinds of ganglionic excitation. As a result of perfusion with 10 μg/ml of $CdCl_2$, the subsequent stimulations were losing potency and finally transmission was blocked. If we then applied an identical dose of acetylcholine, its action was even enhanced in comparison with the former response. On increasing the concentration of $CdCl_2$ to 15 μg/ml and then to 20 μg/ml, the effect of that

dose of acetylcholine was first reduced and then ceased. In analogy to stimulations at higher frequency, higher doses of acetylcholine were still effective at a certain level of inhibition, *i.e.* they so-to-say broke through the block. The sequence of events in the development of the SH-blocking effect in the case of $CdCl_2$ and PCMB is illustrated in Figs. 3, 4 and 5. In the first phase of the effect the response to preganglionic stimulation decreased or ceased while the effect of acetylcholine injected at the same time was potentiated. If the concentration of the SH-inhibitor was low, inhibition was not intensified. On increasing the concentration after the first phase the formerly potentiated acetylcholine effect was also inhibited. We called this the 2nd phase of SH-inhibition. The average SH-inhibitor concentrations corresponding to the two phases were as follows.

1st phase	$CdCl_2$	8 to 10 μg/ml
	$ZnSO_4$	20 to 50 μg/ml
	MIA	2 to 10 μg/ml
	PCMB	2 to 7 μg/ml
2nd phase	$CdCl_2$	20 to 30 μg/ml
	$ZnSO_4$	45 to 60 μg/ml
	MIA	10 to 15 μg/ml
	PCMB	6 to 10 μg/ml

Subsequently, we examined the effect of $CdCl_2$ and of other SH-inhibitors on the ganglionic stimulation by KCl. In the 1st phase of inhibition the excitatory effect of KCl was unimpaired, while in the 2nd phase it decreased and ceased, like that of acetylcholine. There was no unequivocal evidence indicating that the action of KCl should have been potentiated (Figs. 4 and 5). It is

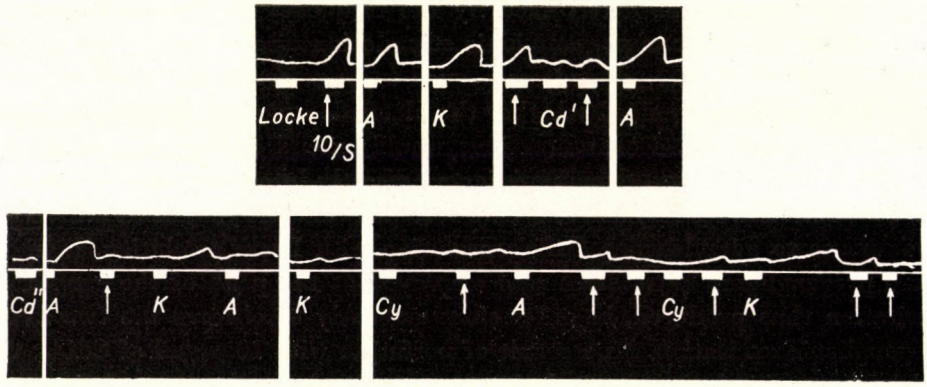

Fig. 4. Above: phase I, below: phase II of inhibition and reactivation in the case of CdC_2 perfusion. Cd′: Onset of 10 μg/ml $CdCl_2$ perfusion (phase I). Cd″: Onset of 30 μg/ml CdCl perfusion (phase II). A: Injection of 0.2 ml of 200 μg/ml Ach. K: Injection of 0.15 ml of 1 per cent KCl. Cy: Injection of 0.5 ml of 1 per cent cysteine.↑: Preganglionic stimulation, 10/sec

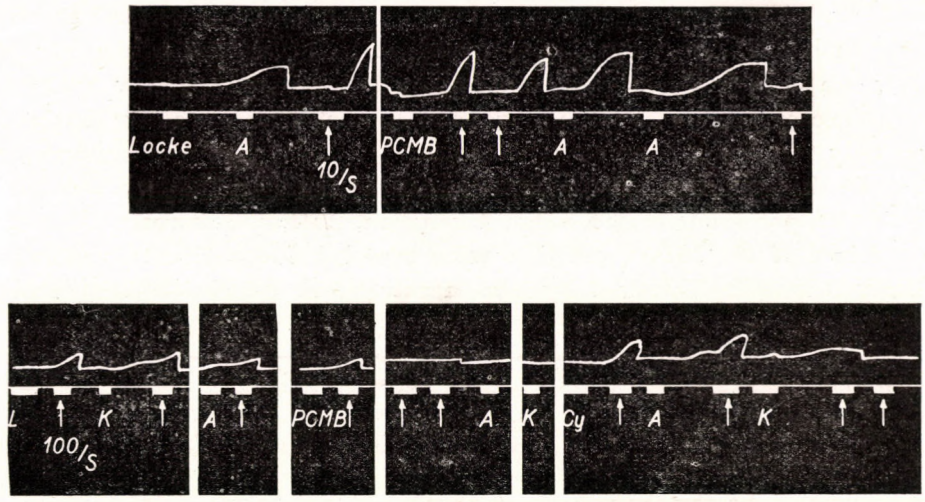

Fig. 5. Above: phase I of SH inhibition, below: phase II of inhibition and reactivation, using p-chloromercuribenzoate perfusion, at concentrations of 4.7 μg/ml and 7.4 μg/ml, respectively. (Signs at onset of perfusion.) L: Onset of perfusion with Locke's solution. A: 0.2 ml of 200 μg/ml AchBr injection. K: Injection of 0.1 ml of 1 per cent KCl. Cy: Injection of 1.0 ml of 1 per cent cysteine.↑: Preganglionic stimulation, 10/sec

noteworthy that in the 2nd phase the KCl effect was blocked before inhibition of the acetylcholine effect.

In our experiments all the above phenomena could be suspended with cysteine, an SH-reactivator. The 1 per cent solution of cysteine injected into the blood stream temporarily abolished the phenomena of inhibition in both the 1st and the 2nd phase (Figs. 4, 5 and 6). The degree of reactivation was directly proportional to the concentration of cysteine. (In the 2nd phase the reactivating effect of cysteine was weaker than anticipated. The cause of this is believed to be that at the concentration required for suspending inhibition

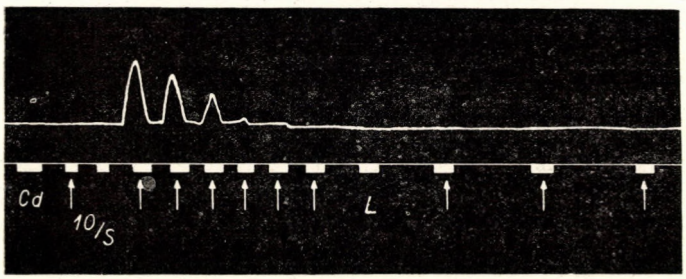

Fig. 6. Reactivation of ganglionic transmission by cysteine. Cd: Onset of 10 μg/ml $CdCl_2$ perfusion. Cy: Injection of 0.4 ml of 0.1 per cent cysteine. L: Injection of 0.4 ml of Locke's solution.↑: Preganglionic stimulation, 10/sec

in the 2nd phase, cysteine as an amino acid depressed by itself the action of acetylcholine. This property of cysteine and other amino acids has been described elsewhere [4, 2]). The sequence of events during reactivation was the same as in the case of inhibition: the preganglionic stimuli became active first and it was only then that the responses to acetylcholine and KCl were restored.

In the second part of our experiments the effect of $CdCl_2$ was studied on the action potentials of the isolated ganglion. As Fig. 7 reveals, the action potentials of the intact ganglion were recorded first. The tracings show clearly the spike followed by the negative and positive after-potentials,

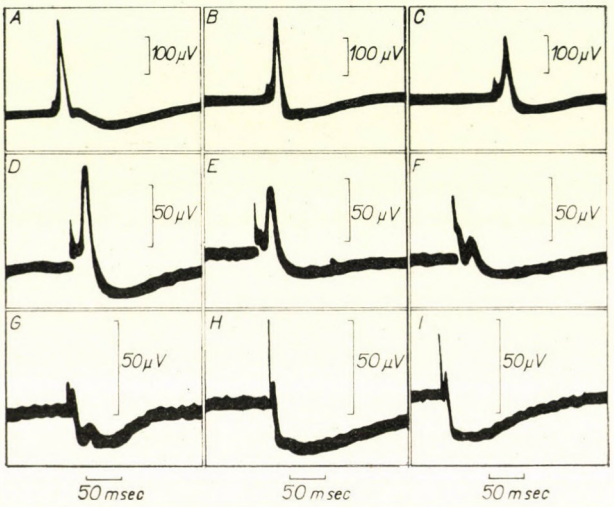

Fig. 7. Effect of 500 μg/ml $CdCl_2$ on the action potential of the isolated ganglion. Frequency of stimulation, 2/sec. Monopolar lead, platinum electrode. A: control. B: at 25 minutes of the $CdCl_2$ effect, C: at 35 min., D: at 45 min., E: at 50 min., F: at 55 min., G: at 60 min., H: at 90 min. and I: at 120 minutes

as described by ECCLES [3] (Fig. 7/*A*). In response to $CdCl_2$ the amplitude of the spike began to decrease, accompanied by changes in the after-potentials. The positive after-potential, showing a decreasing tendency itself, occurred with shorter and shorter latency periods, finally to merge with the negative after-potential preceding it (Fig. 7/*B*, *C*). After the disappearance of the spike a deep positive wave appeared in its place. Thus with the disappearance of the spike the recordable electrical activity did not cease, but became reciprocal (Fig. 7/*F*, *G*, *H*, *I*). At the same time intact action potentials could still be obtained from the preganglionic fibres. We were unable to observe a separation of the synaptic potential [5] in the course of the diminution of the spike potential.

Discussion

In studies on the effect of compounds used as enzyme-inhibitors it is of decisive significance to determine to what extent the effect might be ascribed to specific enzyme-inhibition by the compounds tested. In our experiments the ganglionic blocking action developed at extremely low concentrations of the active agents (in the case of $CdCl_2$ for example at $5.4 \cdot 10^{-5} M$) and was completely reversible. This rules out the possibility that the compounds tested would have acted as protoplasm poisons. This evidence together with the specific reactivation of SH groups (cysteine effect), as well as the identical action of the various SH-inhibitors greatly different in chemical structure has made it very probable that there was in fact a binding of SH-groups in the background of the phenomena examined.

The next question is that of the site of action of SH-inhibitors. At the same time when it blocked or substantially changed the action potential of the ganglion, $CdCl_2$ had no influence on the preganglionic action potential. After the development of the effect of SH-inhibitors the ganglionic cells could still be stimulated directly. From this we concluded that the site of action of $CdCl_2$ and of the other SH-inhibitors was in the synapse, in the first place. The possibilities for a synaptic site of action are the following.

1. Inhibition of acetylcholine synthesis.

2. Diminution of the amount of acetylcholine released from the presynaptic endings.

3. Diminution of the excitability of postsynaptic elements.

As to the first possibility according to PERRY [8], the acetylcholine depôt in the ganglion suffices for releasing effective amounts of acetylcholine in response to frequent stimulation, without resorting to synthesis. On the other hand, in our experiments the blockade caused by the SH-inhibitors had in most cases existed when the first test stimulation was applied. We are therefore unable to agree with SMIRNOV *et al.* [10] that an inhibition of acetylcholine synthesis would be responsible for the blocking of transmission.

The second possibility cannot be ruled out with absolute certainty. However, the fact that action potentials (even though in strongly altered form) can be recorded from the ganglion cells after transmission has been completely blocked (Fig. 7) indicates that the presynaptic impulses are still acting upon the ganglion cells. The mediator is in all probability released and its ineffectiveness must be due first of all to a change in the condition of the susceptible postsynaptic elements, the acetylcholine receptors.

This is supported also by the phenomena observed in the 2nd phase of inhibition. According to PERRY [9], acetylcholine injected into the blood stream acts exclusively on postsynaptic elements. Thus, if in the 2nd phase

of inhibition the response to acetylcholine is also suspended, this is undoubtedly due to a diminution of the excitability of postsynaptic elements, the third possibility for a synaptic site of action.

What then explains the different sensitivity to SH-inhibitors of the injected acetylcholine and preganglionic stimulation, although in both cases the cause of ganglionic excitation is acetylcholine?

In earlier experiments, FEHÉR and BOKRI [6] showed that the effect of preganglionic impulses could be blocked by penta- and decamethonium, without causing substantial changes in the response to acetylcholine. Higher concentrations of penta- and decamethonium, however, suspended the action of acetylcholine. On the basis of these and other experiments it is assumed that there are two receptor systems in the superior cervical ganglion, both of them cholinergic but differing in sensitivity to acetylcholine. One of them is a so-called "innervated receptor system" which is less sensitive to acetylcholine, is located to the innervated membrane areas of the ganglion cells and is directly involved in transmission. The other is the so-called "free receptor system" which is more sensitive to acetylcholine and is located mainly in the membrane areas free from preganglionic endings. Of these receptor systems the one designated "free" reacts first of all with the acetylcholine transported there by the blood stream, while the "innervated" one reacts with the acetylcholine released in the course of preganglionic stimulation. These two receptor systems differ in their sensitivity to the various biological agents. In the case of the SH-inhibitors in the first phase the more vulnerable innervated receptor function, while in the second phase the function of the less vulnerable free receptors seem to be paralysed. On the reactivation of SH-groups with cysteine the phase representing a milder grade of inhibition is reactivated earlier and more intensively, while phase II which develops in response to the stronger inhibition, is suspended later and in a lesser degree. It is believed that the difference in the behaviour to SH-inhibitors between the two assumed receptor systems is another proof justifying their separation.

On the basis of all the above we assume that the SH-inhibitors act by binding the SH-groups located in some postsynaptic structure. The question of course arises how far this structure might be identified with the acetylcholine receptors. In support of an identity is the competitive behaviour of the SH-inhibitors and the acetylcholine injected into the ganglion or released presynaptically. It appears as if the SH-inhibitors would occupy the place of acetylcholine and their action would be temporarily suspended as a result of an increased release of acetylcholine brought about by more frequent stimulation. It is known, however, that the classical competitive inhibitors of acetylcholine, such as for example d-tubocurarine, do not prevent the depolarizing action of KCl. In our experiments, however, in the second phase of their action the SH-inhibitors prevented also the depolarisation by KCl (Figs.

4 and 5). This fact suggests that the SH-inhibitors reduce the excitability of postsynaptic elements *in toto*. In addition, KCl seems to act first of all upon the free receptors, as supported by the fact that for the prevention of ganglionic excitəənpuı uoıtɐd by KCl such concentrations of SH-inhibitors were needed as for blocking the free receptors. Thus, there is a close parallelism between the effect of KCl and that of the injected acetylcholine, so that the blocking of ganglionic excitation by KCl would not mean an aspecific inhibition of depolarization, independent of the acetylcholine receptors.

A true competitive antagonism would, however, be conceivable only if there were SH-groups among those responsible for receptor function. There is no convincing evidence suggesting that this would be the case, although our studies did not rule out that possibility.

The potentiation of the acetylcholine effect in the first phase awaits explanation. In our opinion the inhibition of the innervated receptors might change the sensitivity of the free receptors to acetylcholine. This would mean that the two receptor systems mutually influence each other's sensitivity.

LITERATURE

1. Altamirano, M., Schleyer, W. L., Coates, C. W.: Biochim. biophys. Acta **16,** 268 (1955).
2. Damjanovich, S., Fehér, O., Halász, P., Mechler, F.: (In the press.)
3. Eccles, J. C.: J. Physiol. (Lond.) **85,** 179 (1935).
4. Eccles, J. C.: J. Physiol. (Lond.) **101,** 465 (1943).
5. Eccles, R. M.: J. Physiol. (Lond.) **117,** 181 (1952).
6. Fehér, O., Bokri, E.: Pflügers Arch. ges. Physiol. **269,** 68 (1959).
7. Kibjakov, A. W.: Pflügers Arch. ges. Physiol. **232,** 432 (1933).
8. Perry, W. L. M.: J. Physiol. (Lond.) **119,** 439 (1953).
9. Perry, W. L. M.: Brit med. Bull. **13,** 220 (1957).
10. Смирнов, Г. Д., Бызов, А. Е., Рампан, И. Ю.): Доклады Акад. Наук СССР **87** 155 (1952).
11. Wilson, I. B., Altamirano, M.: In Korey and Nurnberger: Neurochemistry London, 1956. p. 155.

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THE EFFECT OF ALPHA-AMINO ACIDS ON GANGLIONIC TRANSMISSION

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The effect of amino acids and related compounds on the ganglionic transmission has been studied.

The superior cervical ganglion of the cat was perfused according to KIBJAKOV and the contractions of the nictitating membrane elicited by preganglionic stimulation or by the intraarterial injection of acetylcholine or KCl were recorded. The alpha amino acids administered in perfusion blocked in proportion with their concentration the ganglion exciting action of acetylcholine and KCl, while they influenced preganglionic impulse transmission at high concentrations only.

As to the mode of action, it has been shown that the amino acids are suitable for differentiating the innervated receptors from the free acetylcholine receptors described by FEHÉR and BOKRI, and that the amino and the carboxyl groups must be present to elicit the above effects.

Conclusions have been drawn as to the possible physiological role of the amino acids.

PERRY and REINERT [1] reported that the so-called denervation hypersensitivity (increased sensitivity to acetylcholine) [2] decreased when the previously denervated superior cervical ganglion of the cat was perfused with sodium glutamate dissolved in Locke's solution. BERGMANN, WILSON and NACHMANSOHN [3] studied the effect of amino acids on the highly purified acetylcholine esterase isolated from *Electrophorus electricus* and found that relatively low molar concentrations of amino acids inhibit the acetylcholine esterase. GERTNER and REINERT [4] investigated the effect of amino acids and the ionic milieu on the denervation hypersensitivity and found certain amino acids (*L*-glutamate and *L*-aspartate) to reverse the effect of denervation even in the absence of extracellular potassium.

We have mentioned in our paper on the effect of SH-inhibitors [5] that the amino acids cysteine and alanine definitely influenced ganglionic transmission in the superior cervical ganglion of the cat. This influence manifested itself with an inhibition of the ganglionic excitation produced by the injection of acetylcholine, while transmission was left practically unaffected. According to recent evidence [6], the omega amino acids, depending on the length of their chain, are selective inhibitors of the axodendritic and axosomatic synapses of the central nervous system. We have, accordingly, studied some alpha amino acids as to their action on the superior cervical ganglion of the cat, and drawn some conclusions as to their mode of action.

Methods

Fifty cats anaesthetized with 0.08 to 0.1 g/kg of chloralose intraperitoneally were used. The superior cervical ganglion was perfused according to KIBJAKOV [7], as described in detail in a previous report [5].

The effects of acetylcholine bromide, potassium chloride, cysteine, alanine, glycine, phenylalanine, lysine, methylamine, acetic acid, propionic acid, capric acid and tetraaethyl-pyrophosphate were studied. The test substances were dissolved in Locke's solution and administered in the form of continuous perfusion into the superior cervical ganglion. Recently prepared solutions were used in every case, with the pH adjusted to 7.2—7.4.

Results

The *L*- and racemic variants of the alpha amino acids of the simplest composition were used. The amino acids tested were the aliphatic monoamino-carbonic acids glycine, alanine and cysteine, the diaminocarbonic acid lysine, the monoaminodicarbonic acid glutamic acid, and the cyclic amino acid phenylalanine. The effect of perfusion with predetermined concentrations of these amino acids was examined on the ganglionic excitation elicited by preganglionic sympathetic stimulation, by the intraarterial injection of acetyl-choline, and by the intraarterial injection of KCl.

In the experiments with alpha-alanine (Fig. 1) it was found that perfusion with Locke's solution containing from 20 to 100 μg/ml of alanine, after a latency period proportionate to the concentration (2 to 8 minutes) strongly depressed or blocked the ganglionic excitation caused by 20 to 40 μg of Ach or 20 mg of KCl, leaving at the same time transmission, *i. e.* the nictitating membrane contraction in response to preganglionic sympathetic stimulation unaffected. The reversibility of the effect was proved by the fact that the de-pressor action of alanine ceased within a few minutes following the onset of perfusion with Locke's solution not containing alanine.

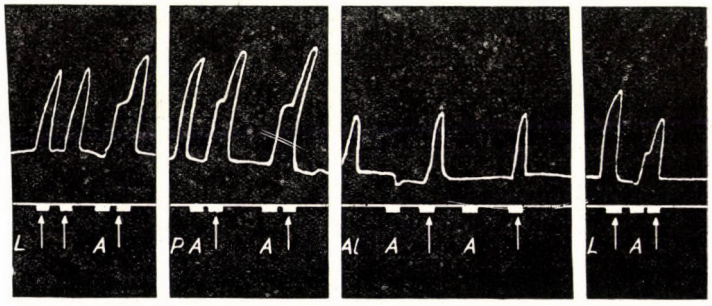

Fig. 1. Effect of alanine and propionic acid on the ganglionic excitation caused by preganglionic stimulation or by the injection of acetylcholine.↑: preganglionic stimulation (10/sec., 5 sec.). A: acetylcholine injection. L: perfusion with Locke's solution. P: perfusion with 100 μg/ml propionic acid. AL: perfusion with 100 μg/ml alanine. Following that, the responses to preganglionic stimulation and to the injection of acetylcholine are visible during perfusion with Locke's solution

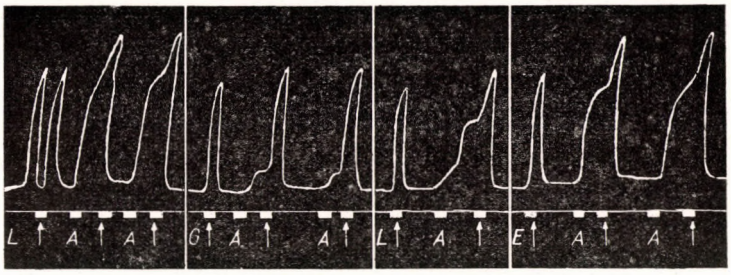

Fig. 2. Effect of glycine and acetic acid on the ganglionic excitation caused by preganglionic stimulation or by the injection of acetylcholine. ↑ : preganglionic stimulation (10/sec., 5 sec.), A: acetylcholine injection with Locke's perfusion, and the responses to the same during perfusion with glycine (100 μg/ml). Following that, the responses to indentical preganglionic stimulation and to acetylcholine injection during perfusion with Locke's solution, then with 100 μg/ml of acetic acid

In the concentration range of from 10 to 20 μg/ml of alpha alanine the humoral effects (injected Ach and KCl) were only slightly reduced even after prolonged perfusion, while concentrations of 100 μg/ml or higher depressed transmission, too (Fig. 1). On increasing the concentration of acetylcholine (to 80 μg/ml,) the drug was again causing ganglionic excitation, it so-to-say broke through the inhibition.

Perfusion with glycine (25 to 100 μg/ml) had a similar effect (Fig. 2). Perfusion with 25 μg/ml lysine diminished only slightly the excitation caused

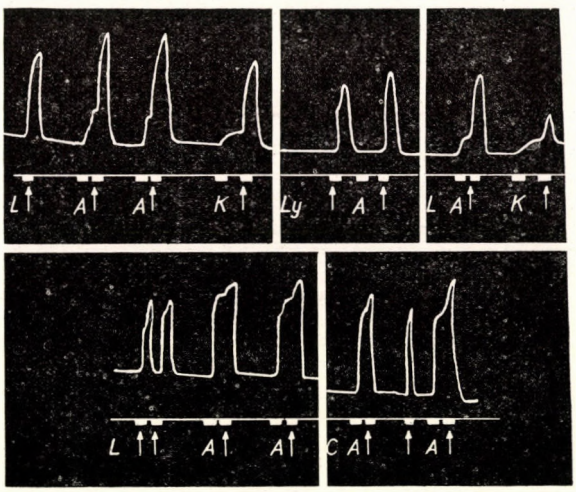

Fig. 3. Effect of lysine and capric acid on the ganglionic excitation caused by preganglionic stimulation or by the injection of acetylcholine. Responses to : ↑ preganglionic stimulation (10/sec., 5 sec.), A: 0.2 ml of 200 μg/ml of acetylcholine and to K: 0.2 ml of 1 per cent KCl injection during perfusion with Locke's solution, then during perfusion with 100 μg/ml of lysine (Ly), repeated perfusion with Locke's solution, then perfusion with capric acid (C) (100 μg/ml)

by acetylcholine, while perfusion with 100 μg/ml caused a marked depression or total inhibition, leaving transmission completely unaffected (Fig. 3). Cysteine and glutamic acid (50 to 100 μg/ml) acted in the same way as alanine (the effect of glutamic acid on the KCl-induced ganglionic excitation was not

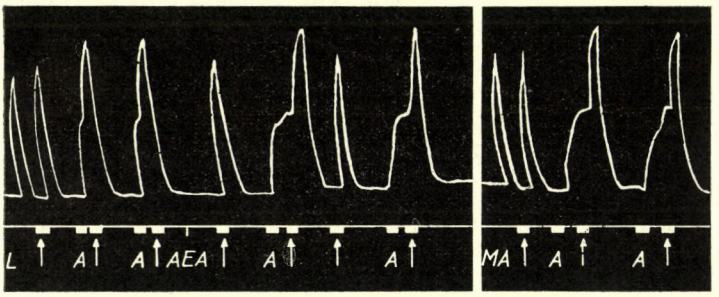

Fig. 4. Action of ethylamine and methylamine on the ganglionic excitation caused by preganglionic stimulation and by the injection of acetylcholine. : ↑ preganglionic stimulation (10/sec., 5 sec.), A: injection of acetylcholine (0.2 ml 200 μg/ml), L: perfusion with Locke's solution, AEA: with ethylamine (100 μg/ml) and with methylamine (MA, 100 μg/ml)

tested). Similarly to lysine, perfusion with 25 μg/ml of phenylalanine merely diminished the Ach and KCl effects, while these were abolished at a concentration of 100 μg/ml, leaving transmission intact.

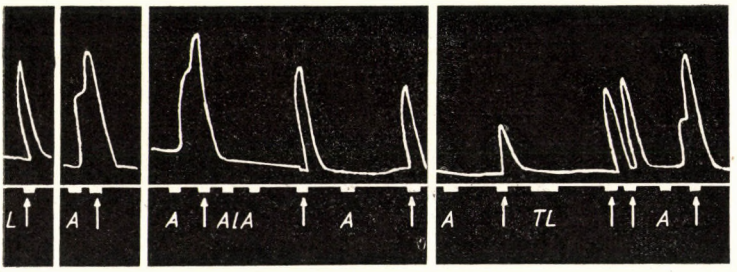

Fig. 5. TEPP has no influence upon the above effects. ↑: preganglionic stimulation (10/sec., 5 sec.), A: acetylcholine injection (0.2 ml 200 μg/ml), L: perfusion with Locke's solution, AL: perfusion with alanine (100 μg/ml), TL: perfusion with Locke's solution containing 10^{-6} *M* of TEPP

Next, we studied the role of the carboxyl and amino groups, so as to gain a deeper insight into the mechanism of action. We employed first compounds chemically corresponding to amino acids but not containing carboxyl groups, then compounds lacking both carboxyl and amino groups, viz. acetic acid (Fig. 2), propionic acid (Fig. 1) and capric acid (Fig. 3). Perfusion of these agents in the concentration range of from 50 to 100 μg/ml had no influence either on the ganglionic excitation elicited by Ach or KCl, or on the response

to preganglionic stimulation. Similar results were obtained with methylamine and ethylamine (Fig. 4).

BERGMANN, WILSON and NACHMANSOHN [3] suggested that the inhibition of choline esterase might play a role in the above actions of amino acids. To investigate this possibility we blocked the activity of ganglionic cholinesterase with 10^{-6} *M* TEPP. The effect of alanine perfusion (100 μg/ml) remained unchanged under these conditions, too (Fig. 5).

Thus, perfusion with amino acids at concentrations of 10^{-3} to 10^{-4} unequivocally depressed or completely inhibited the ganglionic excitation caused by Ach (20 to 40 μg) and KCl (20 mg), while the nictitating membrane contraction in response to preganglionic sympathetic stimulation was left unchanged. The reversibility of the action of amino acids follows from the fact that the original state was restored by perfusing the ganglion with pure Locke's solution. Ganglionic transmission was mostly depressed at concentrations of 100 μg/ml but in some cases even higher concentrations were required to attain this.

Discussion

The fact that within a certain range of concentration amino acids are capable of inhibiting the ganglion-excitatory action of acetylcholine while they leave the response to preganglionic stimulation unaffected, is another evidence contributing to our knowledge of the nature of acetylcholine receptors. FEHÉR and BOKRI [8] showed that the superior cervical ganglion of the cat contained two kinds of receptor system, the so-called "free receptor system" which is more sensitive to acetylcholine, but is less specific and can be readily blocked with atropine, and the so-called "innervated receptor system" which is less sensitive to acetylcholine, is more specific, and can be selectively blocked by penta- and decamethonium. According to the authors, the innervated receptors would be found under the end plates of the preganglionic fibres, while the free receptors occur in the directly not innervated areas of the postsynaptic membrane. The above described selective action of amino acids, notably that the alpha amino acids acting on the free receptors, prevent acetylcholine to combine with these receptors and to exert a biological action, can be interpreted on the basis of this theory to the validity of which it offers another proof. On increasing the concentration of amino acids the innervated receptors become also paralysed.

On the basis of the outlined findings we have attempted to elucidate the mechanism of combination with the receptor. As the amino acids used were different in chemical structure and yet similar in action, it has been concluded that with respect to the effect the amino acid property is of decisive importance. As the amino acid property is bound to the amino and carboxyl groups, the

question arose whether one or both of them are responsible. The experiments with carbonic acids and amines indicated that the presence of both groups is essential since neither carbonic acids, nor amines had inhibitory actions similar to those of amino acids. Considering that a similarity should be assumed to exist between the acetylcholine receptors and the amino groups of choline esterase, from the combination of amino acids with choline esterase, studied in more detail by BERGMANN, WILSON and NACHMANSOHN [3] conclusions may be drawn as to the mechanism of the combination of amino acids with the acetylcholine receptors. According to these authors, at concentrations two orders of magnitude higher than those employed by us the amino acids are blocking choline esterase in that the carboxyl group of amino acids combines with the ester, and the amino group with the anion group, of choline esterase. We therefore investigated whether the choline esterase activity or its inhibition had any role in the amino acid effect observed by us. Perfusion with $10^{-6}M$ TEPP, *i.e.* the complete inhibition of choline esterase, had no effect on the action of alpha alanine. This means that in the amino acid action the choline esterase played no role, it was only the combination with the acetylcholine receptors that proved to be essential. On the other hand, the studies of the above cited authors concerning the mechanism of linkage between amino acids and choline esterase offer a suitable basis for the explanation of the action of amino acids on the acetylcholine receptors.

According to our hypothesis the amino acids that have reached the surface of the postsynaptic membrane combine physico-chemically through the amino and carboxyl groups with the acetylcholine receptors, and by competitive inhibition prevent the mediator from exerting its action. After perfusion with the amino acids has been brought to an end, the amino acid molecules dissociate from the receptors and the original state is restored. The experiments of PERRY and REINERT fit well into this view; they found that in cats after cervical sympathicotomy and degeneration the response to injected acetylcholine decreased during the perfusion of sodium glutamate or of Locke's solution containing a high concentration of potassium. As we found that the amino acids act mainly on the free receptors, it was concluded that in the experiments of PERRY and REINERT [1] the diminution of the denervation hypersensitivity caused by amino acids involved an inhibition of the free receptors. On the basis of the experiments by FEHÉR and BOKRI [8] and of those outlined above it is assumed that the mechanism of the development of the denervation hypersensitivity takes place in the following steps: after the degeneration of the preganglionic fibre the innervated receptors also turn into free ones which are less specific but much more sensitive, and this condition will lead to the development of denervation hypersensitivity.

In the same test, GERTNER and REINERT [4] found that the denervation hypersensitivity is diminished or prevented by amino acids; however, with

the extracellular K concentration decreased, the above action of amino acids is not uniform. While L-aspartate and L-glutamate were effective, L-alanine, L-lysine and the D-amino acids were little or not effective. The authors cited regard the denervation effect to be due to a change in the ionic equilibrium: an outflow of intracellular K ions (cations) and amino acids (mainly glutamate and aspartate) from the ganglion cells. They interpret their experiments by claiming that by replacing the deficiency thus created the pre-denervation state, or the equilibrium, is restored. In our experiments the ganglionic excitation produced by KCl was blocked by the amino acids in a similar way they block the effect of the injected acetylcholine. We think that beside the changes in the ionic milieu changes in the receptor structure also play a significant role in the development of denervation hypersensitivity.

PURPURA, GIRADO, SMITH, CALLAN and GRUNDFEST [6] found that the omega amino acids, depending on their chain length, inhibited the superficial axodendritic synapses of the cortex, while they did not influence the activity of the axosomatic synapses. In their experiments the alpha amino acids were ineffective. They devoted great attention to gamma-amino-butyric acid, considering that this compound occurs in the central nervous system under physiological conditions. In the light of the evidence published by the above authors we see a further interest in our experiments in that the biologically active amino acids employed by us occur physiologically in the blood, just as the omega amino acids, especially the gamma-amino-butyric acid studied by PURPURA *et al.*, physiologically occur in the brain.

Our experiments, in addition to some recent data in the literature [6] thus suggest that the amino acids play the role of a regulator in nervous transmission.

LITERATURE

1. PERRY, W. L. M., REINERT, H.: J. Physiol. (Lond.) **126,** 101 (1954).
2. CANNON, W. B., ROSENBLUETH, A.: The supersensitivity of denervated structures.A law of denervation. Macmillan, New York.
3. BERGMANN, F., WILSON, I. B., NACHMANSOHN, D.: **186,** 693 (1950).
4. GERTNER, S. B., REINERT, H.: Arch. exp. Pathol. Pharmakol. **230,** 347 (1957).
5. MECHLER, F., DAMJANOVICH, S., HALÁSZ, P.: Acta physiol. hung. **16,** Suppl. 39 (1959).
6. PURPURA, D. P., GIRADO, M., SMITH, T. G., CALLAN, D. A., GRUNDFEST, H.: J. Neurochem. **3,** 238 (1959).
7. KIBJAKOV, A. W.: Pflügers Arch. ges. Physiol. **232,** 432 (1933).
8. FEHÉR, O., BOKRI, E.: Pflügers Arch. ges. Physiol. **269,** 68 (1959).

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AIR IODINE CONTENT AND ENERGY EXCHANGE OF THE RAT

By

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No correlation could be established between the metabolic rate of the rat and the iodine content of the air at a relative humidity of 25—75 per cent, whereas a close but complex correlation was found between basal metabolism and the apparent iodine content of air saturated at 37° C.

More than hundred years ago CHATIN [1, 2] concluded that goitre and cretinism were due to a deficiency of iodine in air and drinking water. Later the small amounts, about 0.4 $\mu g/m^3$, of iodine in the air were *a priori* thought to be ineffective as an antigoitrogenic factor [3, 4]. In areas with the air iodine as high as 2.0 $\mu g/m^3$ complete absence of goitre was observed even under goitrogenic conditions (lacks of iodine in drinking water, adverse social circumstances, *etc.*). Simultaneously the incidence of GRAVES' disease was found to be high [5, 6, 7].

Goitre *per se* does not permit any conclusions concerning the function of the thyroid gland, neither does the absence of goitre exclude functional disorders. Moreover, only amounts of air iodine 6—10 times above normal proved to be antigoitrogenic, and therefore the relation between increased air iodine content and thyroid function is still a problem.

The unexplained decrease in air iodine content to one tenth in Western Europe since 1933 has had no effect on the population [8]. On the other hand, the ashing of sea weeds in the production of iodine has greatly been increasing the iodine content of the air in Bretagne without any apparent damage to the population [9], although inhaled elementary iodine reaches immediately the blood stream [10, 11, 12] and the pulmonary uptake of 250—1000 μg of elementary iodine decreases minute and stroke volume, and mean arterial pressure for 25—30 minutes [13].

According to CURRY [14], air iodine plays a subordinate role and is not responsible for acute effects associated with weather changes. In his opinion, the increase in the oxidizing substances (aran) in the air induces a shift from sympathetic to parasympathetic tone. In order to demonstrate this the patients were questioned after spending a night in iodine and aran free air, and sleeplessness, nervosity and headache were assumed to prove an increase in sympathetic tone due to lack of aran. In other experiments aran

was added to the air and tiredness, yawning and somnolence were taken to indicate a prevalence of parasympathetic tone.

A study of the literature suggests that either the methods employed were inadequate or the amounts of iodine added to the air were far from physiological.

Methods

Our experiments were performed at an ambient temperature of 28°C under basal conditions on unanaesthetized sleeping male Wistar rats of our own breed. Heat production and heat loss were estimated in a rapidly responding respiratory calorimeter with time standards of 3 minutes and one and a half minute, respectively [15]. In all, 63 separate readings of O_2-consumption and CO_2-production, heat loss and water vapour loss were recorded during the experimental period of 40 minutes and the average of these served to calculate heat production and heat loss. Rectal temperature was measured only before and after determination, since prolonged insertion of a thermometer made the animals restless. The calorimeter permitted calculation of heat storage with satisfactory accuracy. The relative humidity of the calorimeter varied between 25 and 75 per cent and atmospheric pressure between 667 and 682 mm Hg.

Air iodine was determined by a procedure permitting the analysis of samples from short term experiments [16]. During a period of 40 minutes 60 litres of air were collected from the main tube just before this enters the metabolic chamber. The washing-tube containing alkaline sulphite was submerged into the water-bath of the calorimeter.

Results and discussion

No correlation could be established between air iodine content on the one hand and heat loss and heat production on the other (Fig. 1).

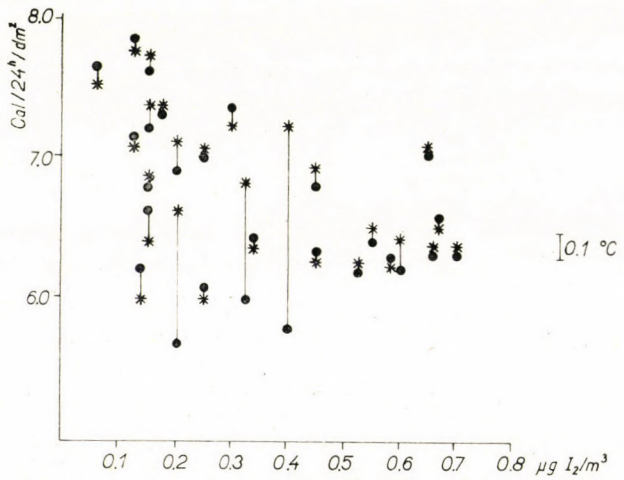

Fig. 1. Abscissa: energy exchange Cal/24^h /dm^2, ordinate: air iodine at a RH of 25—75%. Heat production = ●, heat loss =*, increase in body temperature = ⁂, decrease in body temperature = ⁂. All points represent the average of 63 determinations. Rat № 26. 250 g. Experiment: 20. Sept. — 3. Oct. 1957.

Next, the effect of varying the water vapour content on the results of iodine determination was investigated. It can be seen from Table I that after saturating the air with water vapour at 20° C the apparent iodine content was found significantly lower than before [17].

Table I

Iodine content at 20° C, and 35—70% relative humidity		Iodine content of the air saturated with water vapour at 20° C
$\mu g/m^3$		$\mu g/m^3$
0.47	n = 187 t = 8,40 $P > 0{,}001$	0.30

It was obvious to investigate the iodine content of the air saturated with water vapour at 37° C, since at the level of the laryx the temperature of inhaled air is about 34°C and its relative humidity more than 96% [18]. Table II demonstrates that the apparent iodine content of air with a relative humidity of 25—85 per cent at 20°C diminishes by as much as 43 per cent after saturation at 37°C.

Therefore, aliquot amounts of calorimeter air were saturated with water vapour at 37°C before iodine determination (Fig. 2).

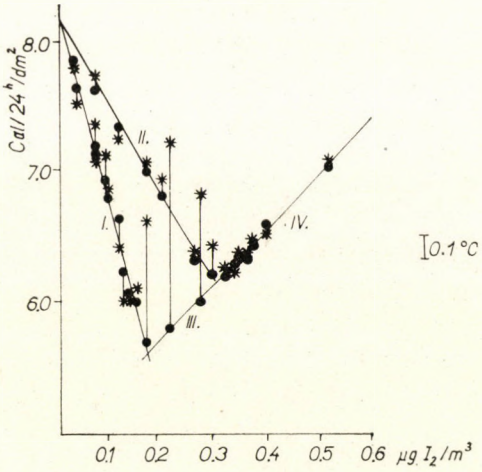

Fig. 2. Abscissa: energy exchange Cal/24h/dm², ordinate: iodine after saturating the air with water vapour at 37°C. Heat production = ●, heat loss = * increase in body temperature = ✱, decrease in body temperature = ✕, I, II, III, IV see text. All figures represent the average of 63 determinations. Rat № 26. 250 g. Experiment: 20. Sept. — 3. Oct. 1947.

Table II

Apparent iodine content at 20° C and 25—85% relative humidity $\mu g/m^3$		Apparent iodine content of air saturated with water vapour at 37° C. $\mu g/m^3$
0.44	n = 504 t = 25.3 $P > 0.001$	0.19

Fig. 2 clearly demonstrates that heat production approaches 8 Cal/day/dm^2/(0%) at an air iodine content of 0.00 $\mu g/m^3$. Up to 0.2—0.3 $\mu g/m^3$ the increase in air iodine is associated with a reduction in heat production. The slope of the curve is steeper in the autumn and in winter (Fig. 2. I) than in spring and summer (Fig. 2. II). Basal metabolism is lowest (—30%) at an iodine content of 0.2 $\mu g/m^3$ in winter and at about 0.3 $\mu g/m^3$ in summer. A further rise in air iodine is associated with a progressively increasing BMR (Fig. 2. III and IV). Heat loss agrees closely with heat production — changes of body temperature did not exceed 0.1°C during the experimental period — excepting range III in which disparity of heat production and heat loss may reduce body temperature by as much as 1°C.

Figures for O_2-consumption of normal rats were significantly lower than those obtained in closed circuit apparatuses and the generally accepted values were only approached in the case of a minimal air iodine content. At present no explanation can be given for this result; methodical errors, however, can be excluded by the simultaneous estimations of heat loss, since the latter rests on a different principle.

Factors other than iodine could also contribute to the results. Aran can, however, hardly play a role in this respect. CURRY demonstrated clearly that the water vapour content of a bathroom (27—30°C, 80 per cent relative humidity) promptly destroyed the aran in the air. This effect has to be taken into account especially at the temperature and humidity of the lungs.

LITERATURE

1. CHATIN, A.: C. R. **34,** 14 (1852).
2. CHATIN, A.: C. R. **34,** 409 (1852).
3. FELLENBERG, Th.: Biochem. Z. **139,** 371. (1923).
4. CAUER, H.: Veröffentlichungen aus dem Gebiete der Medizinalverwaltung **39,** 505 (1933).
5. CAUER, H.: Z. ges. phys. Ther. **37,** 203 (1929).
6. CAUER, H.: Z. ges. phys. Ther. **39,** 10 (1930).
7. WAGNER, H.: Wien. med. Wschr. **34,** 1084 (1929).

8. CAUER, H.: Z. Angewandte Chemie. Beiheft N° **34,** (1939).
9. CAUER, H.: Biochem. Z. **292,** 116 (1937).
10. LÖHR, H.: Arch. exp. Pathol. Pharmakol. **182,** (1936).
11. HOFMANN-CREDNER, D.: Wien. med. Wschr. **104,** 253 (1954).
12. HOFMANN-CREDNER, D., SPITZY, H.: Arch. phys. Ther. (Lpz.) **6,** 171 (1954).
13. SIEDEK, H.: Arch. phys. Ther. (Lpz.) 153 (1954).
14. CURRY, M.: Bioklimatik, München, 1946.
15. BALOGH, L.: Acta physiol. hung. **9,** 231 (1956).
16. BALOGH, L.: Acta physiol. hung. **14,** 7 (1958).
17. BALOGH, L.: Meteor. Rdsch. **12,** 180 (1959).
18. VERZÁR, F., KEITH, J., PARCHET, V.: Pflügers Arch. ges. Physiol. **257,** 400 (1953).

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Pharmacologia

ÉTUDE PHARMACOLOGIQUE DE LA 6-ACETYL-MÉTHOBROMIDE DE MORPHINE

Par

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(Reçu le 22 février, 1960)

Nous avons examiné l'action de la 6-acetyl-méthobromide de morphine en la comparant avec celle de la morphine, notamment en ce qui concerne son effet analgésique et son influence sur le volume respiratoire. Nous avons en plus étudié comment ces effets peuvent être influencés par les dérivés N-allyl. Nous avons étudié leurs effets béchiques, ganglioplégiques, leurs effets sur les plaques, motrices terminales et enfin l'accoutumance et la toxicité.

La constatation d'intéret théorique selon laquelle la 6-acetyl-méthobromide de morphine dérivé quaternaire de la morphine, tout en possédant un intense effet analgésique ne possède pas d'effet curarisant ni d'effet ganglioplégique présente aussi un intéret pratique. En effet ce composé possède, — chez la souris, — un effet analgésique 16,7 fois plus fort que celui de la morphine et diminue le volume respiratoire 1,9 fois moins fortement. Son action calmante sur la toux s'est montrée 1,8 fois plus forte que celle de la codéine. L'accoutumance chez la souris se développe moins rapidement qu'avec la morphine. La toxicité des deux produits est identique.

Dans nos travaux antérieurs [13, 15] nous nous sommes occupés des rapports entre la structure et les effets des dérivés de la morphine. L'un des buts de nos recherches était de nous rendre compte si l'on peut en modifiant la structure, obtenir des produits possédant les qualités thérapeutiques supérieures aux morphiniques utilisées jusqu'à maintenant. Le second but que nous nous sommes proposés c'était de savoir si les règles généralement valables concernant les rapports entre la structure et les effets des morphiniques sont ou ne sont pas applicables aux dérivés morphiniques que nous avons examinés.

Dans ce travail nous rendrons compte des effets de la 6-acetyl-méthobromide de morphine, l'un des composés mis au point dans l'Institut de Chimie Organique de Debrecen [1], dérivé quaternaire de la morphine, effets que nous avons comparés à ceux de la morphine. Nous représentons la structure de ce composé à la figure (No. 1). Dans les dérivés quaternaires de la morphine au cycle de piperidine contenant du N un groupe d'alcoyl s'attache au nitrogène, et si le composé de base adequat réagit avec un alcoyl-halogénide il se forme un sel quaternaire d'ammonium.

On a mis au point jusqu'à ce jour des dérivés quaternaires de nombreux alcaloides dont on a pratiqué l'étude pharmacologique [17]. Nous savons de ces études qu'en général les composés comportant de N quaternaire n'ont pas d'action sur le systême nerveux central ou n'ont pas d'action plus faible

que celui du composé de base tertiaire correspondant. Par contre presque tous ces dérivés paralysent la plaque motrice terminale (FÜHNER [6], MEYER [10]). Dans la littérature hongroise c'est Issekutz sen. qui a publié des données précieuses concernant la pharmacologie des composés quaternaires de l'ammonium [8].

La règle générale concernant les composés quaternaires est égalment valable pour les dérivés morphiniques quaternaires comme cela était démontré par TRENDELENBURG [17] en ce qui concerne la méthochloride de morphine, la méthobromide d'apomorphine, la méthochloride de codéine, la méthochloride d'acetylcodeine et la méthoïodide de thebaïne et par BRAENDEN, EDDY et HALBACH [2] en ce qui concerne la N-oxide de morphine, la méthochloride de morphine et la méthochloride de codéine.

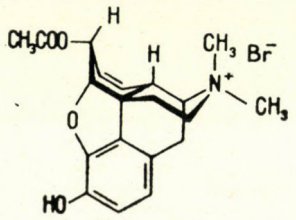

Fig. 1. 6-acetyl-méthobromide de morphine

En examinant les dérivés morphiniques nous avons constaté que la 6-acetyl-méthobromide de morphine--qui est une morphinique quaternaire—calme plus fortement la douleur des souris que la morphine. Partant de cette constatation nous nous sommes proposés de faire une étude plus poussée des effets de ce composé.

Méthode

a) Nous avons mesuré l'effet analgésique sur des souris par la méthode WOLFE-MACDONALD [18] modifiée par PÓRSZÁSZ-HERR [12].

b) Nous avons détérminé l'action exercée sur le centre respiratoire sur des lapins par la méthode de DRESER [4] modifiée par HERR-PÓRSZÁSZ [7].

c) L'accoutumance a été examinée sur des souris en administrant à des lots de 25 souris, quotidiennement, une dose déterminant un effet analgésique d'environ 100%. Nous avons mesuré l'accoutumance en précisant la diminution quotidienne de l'effet analgésique.

d) Nous avons examiné sur des souris dans quelle mesure l'administration de la N- allyl-normorphine et de la diacetyl-N-allyl-normorphine influencent l'effet analgésique 100% du composé. De ces deux antagonistes de la morphine nous avons administré par voie sous-cutanée le quart de la dose de la 6-acetyl-méthobromide de morphine.

e) Nous avons examiné sur des lots de 5 lapins dans quelle mesure ces deux antagonistes de la morphine influencent l'effet respiratoire dépressif de la 6-acetyl-méthobromide de morphine. Effet dépressif à 50% est obtenu par des doses de 8 mg/kg par voie sous-cutanée. Au cours de ces expériences nous avons administré une dose de 2 mg/kg de ces antagonistes, également par voie sous-cutanée après la mesure du volume respiratoire initial.

f) Nous avons mesuré l'activité béchique sur des cobayes par la méthode de EICHLER-SMIATEK [5] modifiée par nous-même [16]: nous avons déterminé la toux chez les cobayes

en leur faisant respirer de l'air contenant 0,6% de dioxyde de soufre, puis après administration sous-cutanée du composé, nous avons mesuré une demi-heure plus tard la diminution de la toux après administration d'un mélange de gaz identique. Nous avons examiné l'action de chaque dose sur des lots de dix cobayes.

g) Nous avons étudié l'action sur les ganglions de notre produit sur la membrane nictitante chez le chat anesthésié par administration d'uréthane i.p. à la dose de 1,0 g/kg. Nous avons excité la fibre préganglionnaire du ganglion cervical supérieur avec un coup de courant carré de 8 V, 2,5 milsec., et une fréquence de 4 à 5 au sec. administré toutes les minutes 3 fois de suite et répété à des intervalles de 10 minutes. Nous avons enregistré les contractures de la membrane nictitante sur du papier fumé. Les substances à examiner ont été diluées par de la solution physiologique et administrées par la veine fémorale.

h) Nous avons examiné l'action du produit sur la plaque motrice terminale sur des chats anesthésiés par voie i.p. par de l'uréthane à la dose de 1,0 g/kg. Nous avons excité le nerf sciatique mis à nu dans la région fessiaire par un coup de courant carré de 8 et 0,1 milsec, toutes les 6 secondes. En même temps que les contractures du gastrocnemius nous avons enregistré la pression dans l'artère carotide.

i) Afin d'examiner la toxicité nous avons administré la substance à des lots de 15 souris par voie sous-cutanée dans les solutions de 0,01 ml/g à des doses croissantes. Nous avons calculé la valeur du LD-50 selon la méthode de LITCHFIELD-WILCOXON [9.]

j) Nous avons interprété statistiquement tous nos résultats [3].

Les procédés techniques des points *a) b)* et *j)* ont été décrits dans une de nos publications antérieures [13].

Résultats

a) Les résultats obtenus concernant l'action analgésique de la 6-acetyl-méthobromide de morphine sont représentés sur la Fig. No. 2. Sur cette figure nous avons représenté également l'action analgésique de la morphine. Sur l'axe X sont représentées les doses par mg/kg et sur l'axe Y les valeurs en % de l'effet analgésique. Fig. 2 représente encore en dehors des courbes doses-effets leurs équations, l'erreur constante du coefficient et les résultats de l'épreuve.

La dose analgésique à 50% de la 6-acetyl-méthobromide de morphine est de 0,31 mg/kg, tandis que celle de la morphine est de 5,2 mg/kg. La 6-acetyl-méthobromide de morphine à donc une action 16,7 fois plus forte que celle de la morphine.

b) Nous représentons sur la Fig No. 3 de la même manière les effets obtenus sur le volume respiratoire des lapins. Il ressort de l'étude de cette figure qu'une dose de 2,7 mg/kg de morphine administrée par voie sous-cutanée diminue déjà de moitié le volume respiratoire des lapins tandis qu'il faut une dose de 5,2 mg/kg de la 6-acetyl méthobromide de morphine pour obtenir cet effet. Ce dernier produit possède donc une action dépressive respiratoire de 1,9 moins forte que celle de la morphine.

c) Nous représentons sur la Fig N° 4 l'accoutumance des souris à la 6-acetyl-méthobromide de morphine et à la morphine. Sur l'axe X figurent les jours, tandis que sur l'axe Y nous voyons l'effet analgésique en %.

Comme on peut constater, en administrant quotidiennement par voie sous-cutanée une dose ayant un effet analgésique à 100%, l'effet de la morphine n'est plus que de 33% au 26-ème jour, tandis que la 6-acetyl-méthobromide de morphine possède encore à cette date un effet analgésique de 68%.

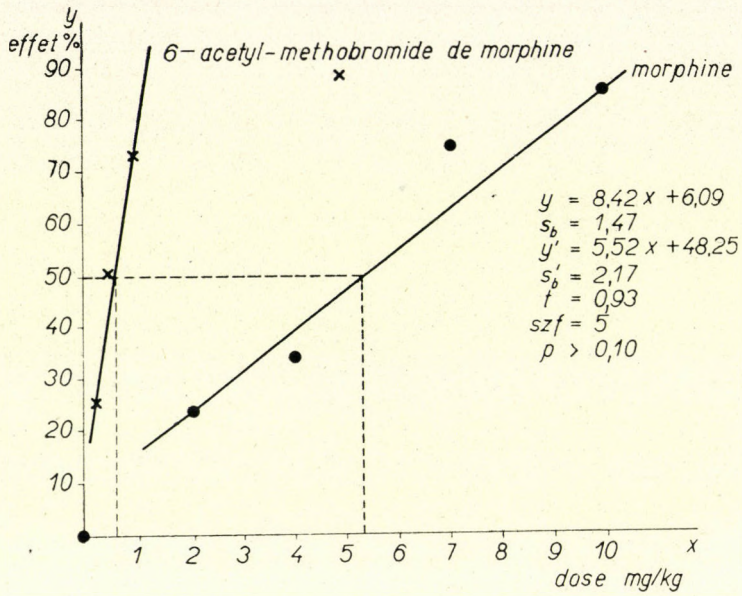

Fig. 2. Effet analgésique de la morphine et de la 6-acetyl-méthobromide de morphine

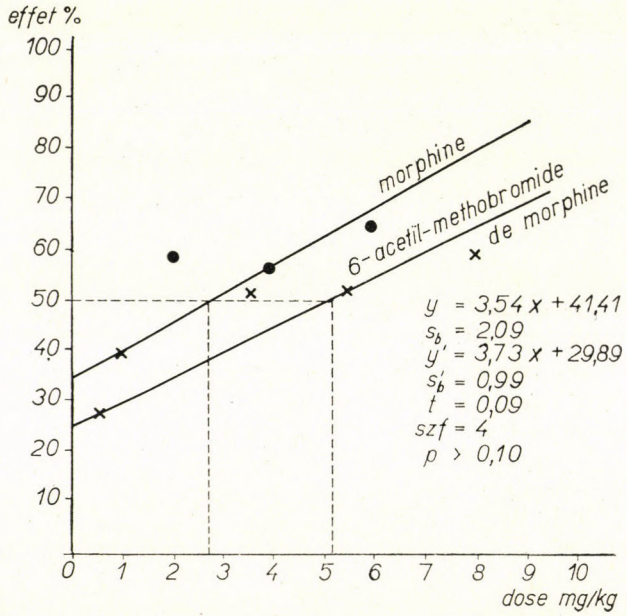

Fig. 3. Effet de la morphine et de la 6-acetyl-méthobromide de morphine sur la respiration des lapins

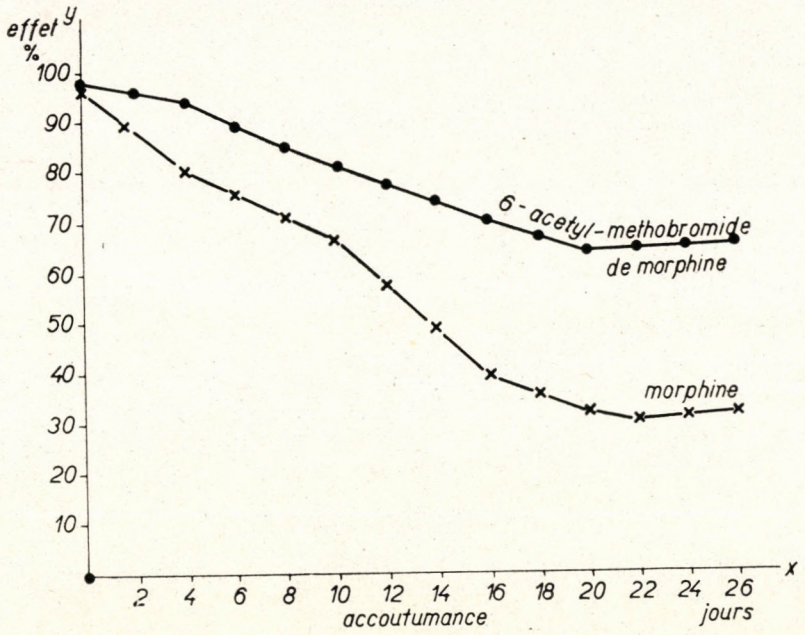

Fig. 4. Accoutumance

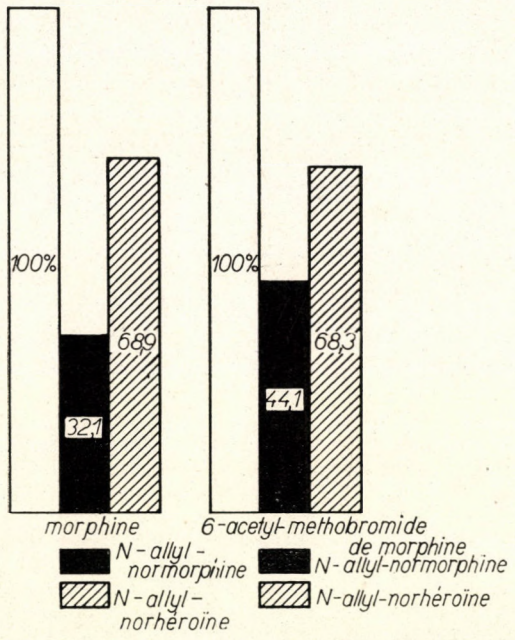

Fig. 5. **Effet analgésique de la 6-acetyl-méthobromide de morphine et de la morphine, administrée conjointement avec les deux N-allyl-antagonistes**

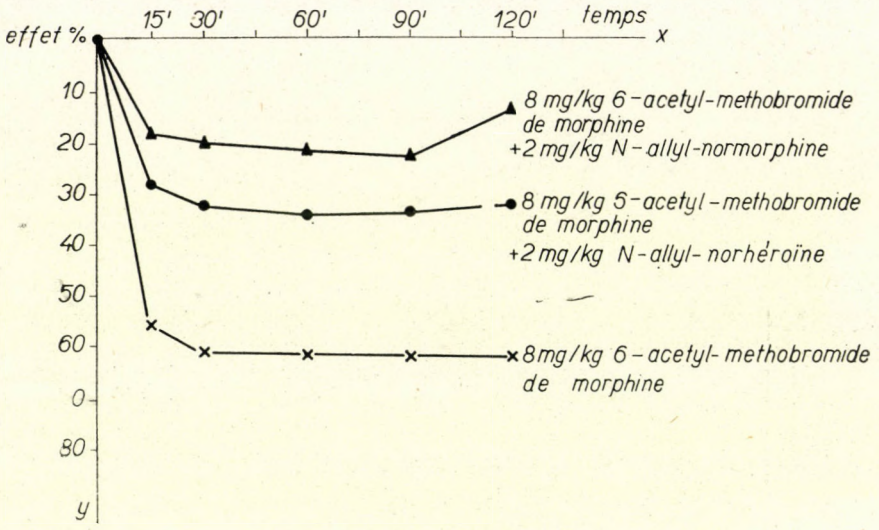

Fig. 6. Effet de la 6-acetyl-méthobromide de morphine et de ses antagonistes sur le volume respiratoire de lapin

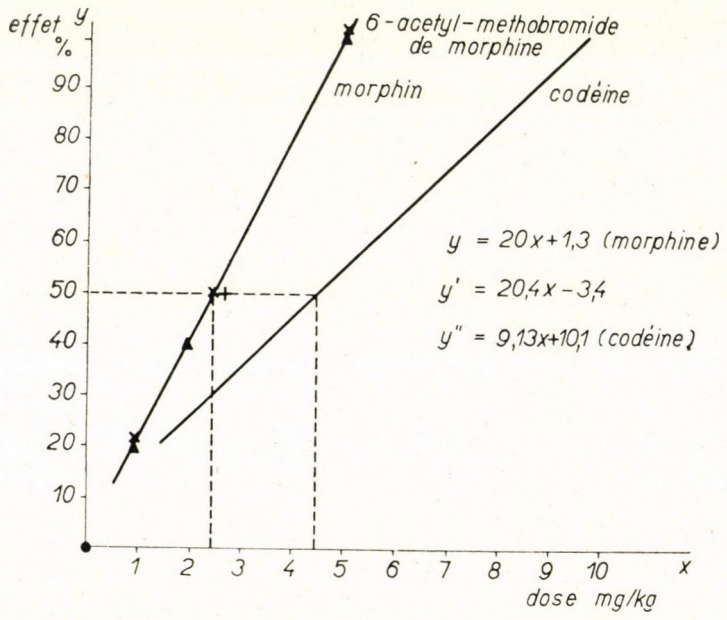

Fig. 7. Effets calmants sur la toux de la morphine, de la 6-acetyl-méthobromide de morphine et de la codéine

d) Nous représentons sur la Fig. N° 5 l'effet analgésique de la 6-acetyl-méthobromide de morphine administrée conjointement avec la N-allyl-normorphine et la diacetyl-N-allyl-normorphine ainsi que l'action de la morphine administrée en même temps que ces antagonistes. La colonne claire représente l'action analgésique 100% de la substance administrée seule, la colonne sombre l'effet obtenu par l'administration conjointe de la N-allyl-normorphine et la colonne hachurée l'administration conjointe de la diacétyl N-allyl-normorphine.

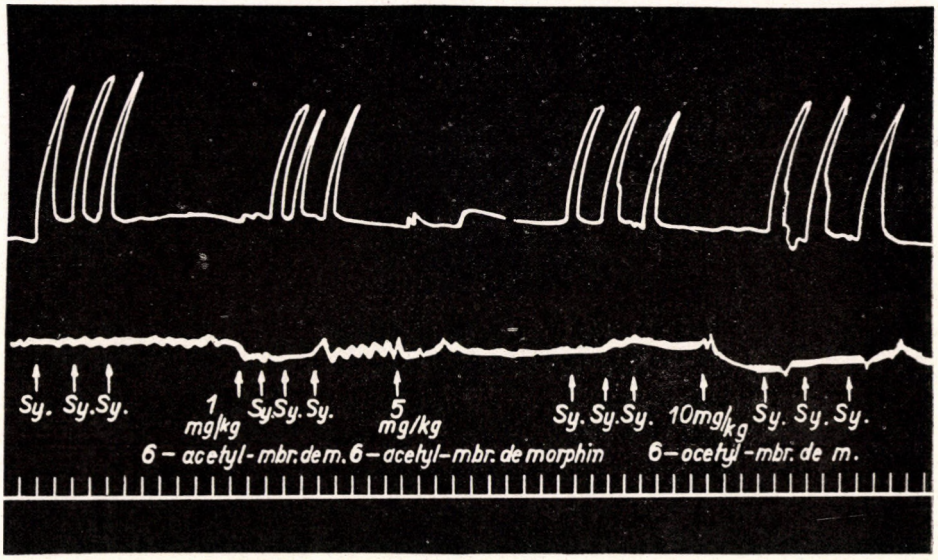

Fig. 8. Effet de la 6-acetyl-méthobromide de morphine sur le ganglion cervical supérieur du chat

Tandis que la diacetyl-N-allyl-normorphine diminue de la même manière l'action de la 6-acetyl-méthobromide de morphine et de la morphine (68,3% et 68,9%), la N-allyl-normorphine diminue plus fortement les effets de la morphine (32,1 contre 44,1%).

e) Nous représentons sur la Fig. N° 6 la diminution du volume respiratoire chez les lapins obtenue par administration isolée de la 6-acetyl-méthobromide de morphine et par son administration combinée avec la N-allyl-normorphine et la diacétyl-N-allyl-normorphine.

8 mg/kg de 6-acetyl-méthobromide de morphine diminue de 62% le volume respiratoire des lapins. Si nous administrons la même dose en même temps que 2 mg/kg de diacetyl-N-allyl-normorphine ou de 2 mg/kg de N-allyl-normorphine, la diminution du volume respiratoire n'est que de 34 %, respectivement de 20%. L'action antagoniste de la deuxième substance est donc plus forte que celle de la première.

f) Nous représentons sur la Fig N° 7 l'effet calmant sur la toux des cobayes de la morphine, de la 6-acetyl-méthobromide de morphine et de la codéine, (toux déclenchée en mélangeant du dioxyde de soufre à 0,6% à l'air inspiré.) Nous représentons également l'équation de régression de chaque courbe.

Comme on peut voir les courbes représentant l'effet-dose calmant de la toux de la morphine et de la 6-acetyl-méthobromide de morphine sont identiques. Leur ED-50 est également de 2,4 mg/kg tandis que lorsqu'on

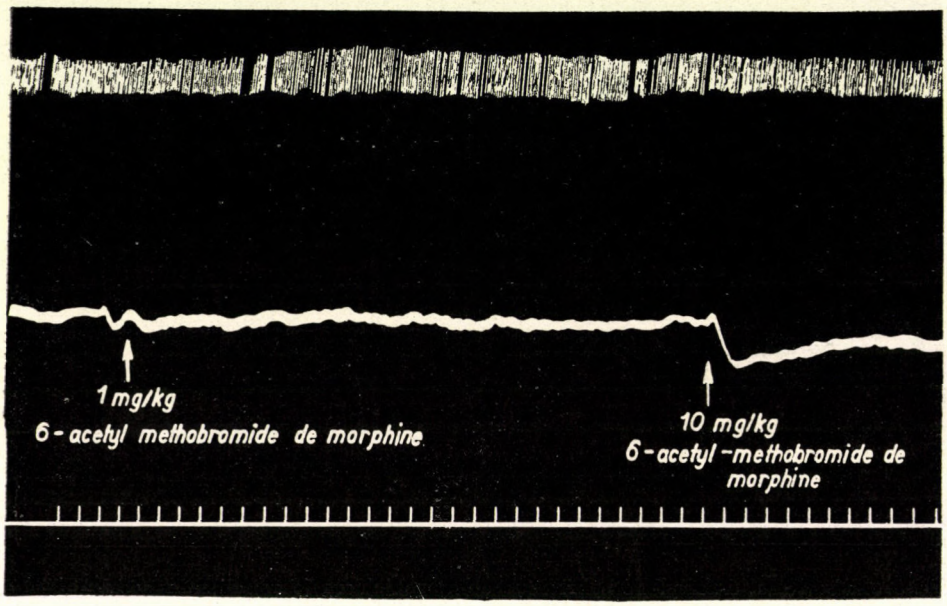

Fig. 9. Effet de la 6-acetyl-méthobromide de morphine sur la préparation nerf sciatique—m. gastrocnemius du chat

examine cet effet de la codéine on trouve que cette dose est de 4,3 mg/kg, donc les substances examinées ont une action calmante sur la toux de 1,8 fois plus forte que la codéine.

g) Sur la Fig. N° 8 on peut voir l'action de la 6-acetyl-méthobromide de morphine sur le ganglion cervical supérieur du chat. La courbe supérieure représente les contractions de la membrane nictitante après excitation de la fibre préganglionnaire de la sympathique cervicale, la courbe moyenne représente la pression mesurée dans l'artère carotide, tandis que la courbe inférieure représente le temps. Comme on peut voir l'administration de 1, de 5 et même de 10 mg/kg de 6 acetyl-méthobromide de morphine par voie intraveineuse n'influence pas les contractions de la membrane nictitante déterminées par l'excitation électrique de la sympathique cervicale. A ces doses ces composés

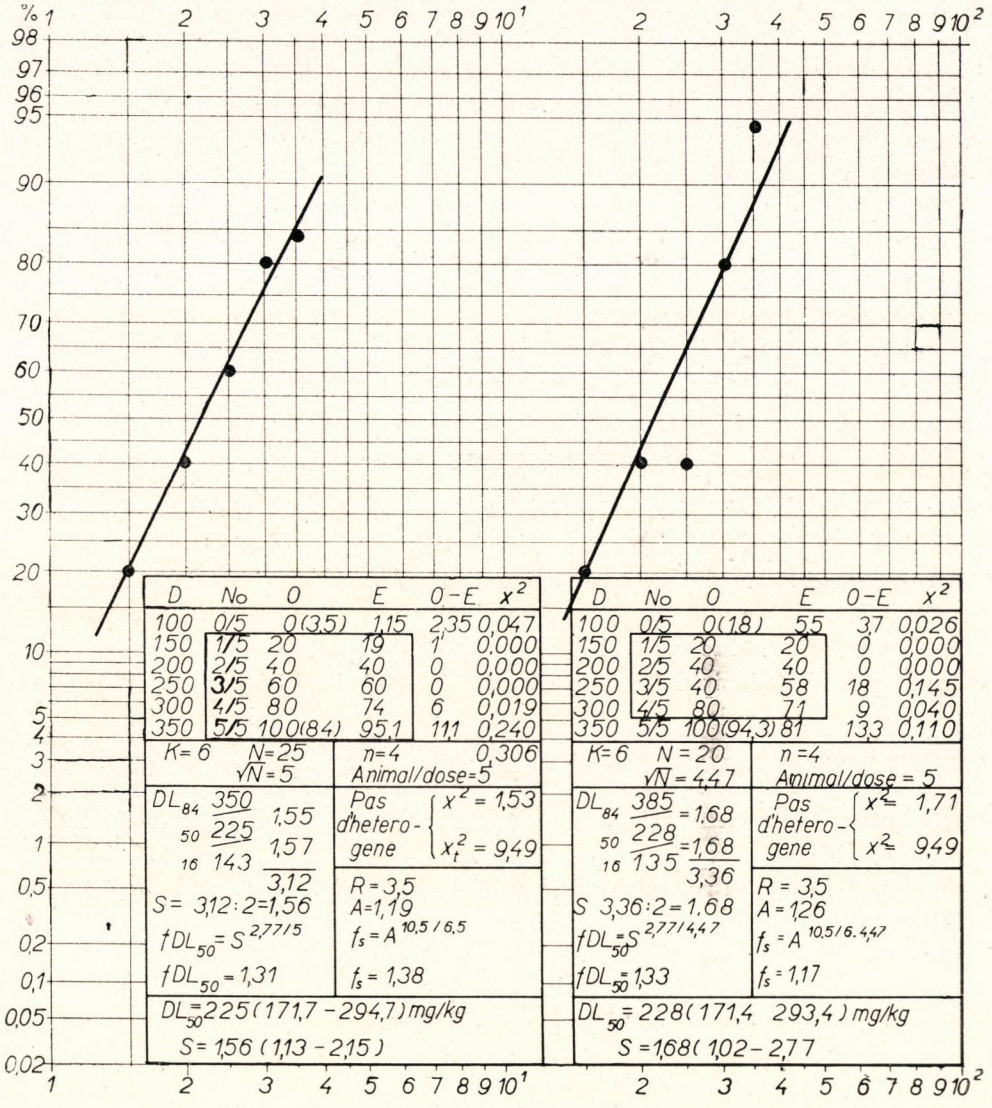

Fig. 10. Étude de la toxicité de la morphine et de la 6-acetyl-méthobromide de morphine chez la souris

n'ont donc pas d'effet ganglioplégique. 10/mg/kg déterminent une chute de la pression artérielle d'environ 20 Hgmm. La pression artérielle regagne progressivement en 10 minutes environ sa valeur initiale.

h) L'effet de la 6-acetyl-méthobromide de morphine sur la préparation nerf sciatique-m. gastrocnemius du chat est représenté sur la Fig. N° 9. La courbe supérieure représente les contractions du gastrocnemius obtenues

par l'excitation électrique du nerf sciatique. La courbe du milieu représente la pression mesurée à l'artère carotide, tandis que la courbe inférieure représente le temps en minutes. La 6-acetyl-méthobromide de morphine ne paralyse la jonction myo-neurale ni à la dose de 1 mg/kg, ni à la dose de 10 mg/kg. La dose de 10 mg/kg détermine ici aussi comme dans l'expérience représentée sur la figure précédente une chute de tension ayant une intensité et une évolution analogue.

i) La Fig. N° 10 représente les résultats de nos recherches concernant la toxicité de notre composé. La LD-50 par voie sous-cutanée chez la souris de la 6-acétyl-méthobromide de morphine est de 228 et celle de la morphine de 225 mg/kg. La toxicité des deux drogues est donc pratiquement identique.

Discussions des résultats

La 6-acétyl-méthobromide de morphine peut être obtenue comme sous-produit lors de la préparation de la morphine. En effet, lors de la déméthylisation par le CNBr de la diacétylmorphine, le CH_3Br produit réagit partiellement avec la diacétylmorphine qui n'a pas encore réagi. Nous séparons la diacétylméthobromide de morphine produit, de la diacétyl-cyano-morphine considérée comme produit principal en portant le mélange à ébullition avec une petite quantité d'eau: la diacetylméthobromide de morphine soluble dans l'eau se précipite. En même temps se précipite également la petite quantité superflue de CNBr laquelle par hydrolyse donne du HBr. En laissant se reposer dans une solution faiblement acide, le groupe 0_3-acetyl se détache, et la 6-acetyl-méthobromide de morphine se diluant mal dans l'eau froide se cristallise lorsqu'on l'a laissé au repos pendant un jour ou deux. On peut la purifier en recristallisant avec un peu d'eau bouillante. Il n' a pas de point de fusion net, à une température aux environs de 150 C° elle se précipite avec perte d'eau cristallique, puis se congèle en une masse vitreuse qui se défait avec développement de gaz et de brunissage.

$$[\alpha]_D^{20} = -131{,}3^0 \pm 0{,}6/\textit{eau}\ ;$$

Nous avons trouvé que la 6-acetyl-méthobromide de morphine possède chez la souris une action analgésique de 16,7 fois plus forte que la morphine. Cette constatation ne cadre pas avec les corrélations connues jusqu'à maintenant entre les effets et la structure des dérivés morphiniques.

Selon SCHAUMANN [14] il faut qu'il y ait dans chaque analgésique un groupe identique de méthyl-phenylpiperidine qui se rattache à un atome C quaternaire central. Il appelle cela «groupe analgiphore». La théorie du «groupe analgiphore» n'est pas valable pour les analgésiques du groupe des méthadones et dithyenylbutenylamine. Dans ces composés on ne retrouve pas de groupes méthyl-phenyl-piperidine et ils sont néanmoins des analgésiques

puissants. Selon BRAENDEN, EDDY et HALBACH [2] c'est la trivalence du N compris dans le cycle piperidine de la molécule de la morphine ainsi que le radical méthyl rattaché au N qui sont indispensables pour l'effet analgésique. L'absence de n'importe lequel de ses facteurs diminue notablement ou fait cesser l'effet analgésique. Avec la quaternarisation du N tertiaire (par exemple N-oxyde de morphine, méthochloride de morphine, méthochloride de codéine) nous obtenons des dérivés à effets analgésiques considérablement moins intenses qu'avec les composés de base correspondants. SCHAUMANN [14] a également constaté que le dérivé quaternaire de la pethidine manque d'efficacité.

TRENDELENBURG [17] explique l'activité moins forte sur le système nerveux central des composés quaternaires par rapport aux composés tertiaires correspondants par leur solubilité lipoïdique moins bonne. C'est pour cette

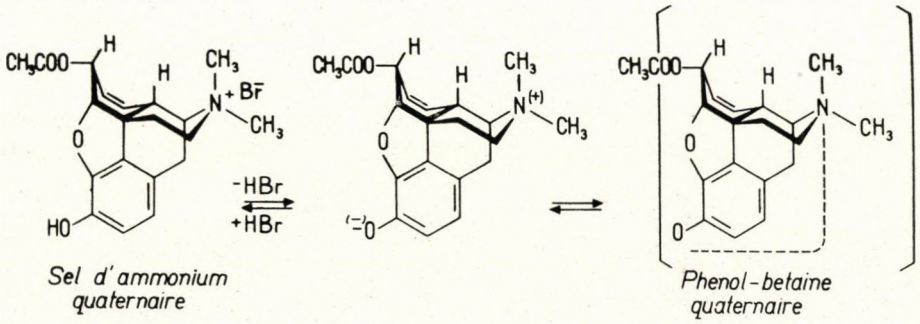

Fig. 11

raison qu'ils ne pourraient pénétrer dans les cellules nerveuses plus riches en lipoïdes. En dépit de ces faits notre dérivé quaternaire s'est avéré être un analgésique plus fort que la morphine. Il en résulte que la quaternarisation ne fait pas cesser dans tous les cas l'action sur le système nerveux central, c'est à dire elle n'entraine pas obligatoirement un effet curarisant. Le composé mis au point par la quaternarisation du N du cycle piperidine ne paralyse pas ou ne paralyse que très faiblement la plaque motrice terminale. POHL [11] a constaté que le couple quaternaire de la papaverine n'a pas d'action sur la plaque motrice terminale. TRENDELENBURG [17] a constaté sur la grenouille que l'acétobromide de morphine a un effet 50.000 fois plus faible que la curare.

MEYER [10] et FÜHNER [6] expliquent l'effet curarisant des composés quaternaires par leur alcalinité. Plus ces dérivés sont alcalins plus ils paralysent la plaque motrice terminale. Avec certaines substitutions, ainsi que par formation interne de sel les composés quaternaires peuvent perdre leur caractère alcalin et ainsi cessent leurs effets paralysants sur la plaque motrice terminale. Avec nôtre composé ce processus commence avec l'élimination du HBr d'une certaine quantité d'alcali (Fig. N° 11). Dans ce cas l'ion jumeau formé peut

lui-même agir comme tampon. La cause du changement de l'action peut être éventuellement expliquée par le fait que l'ion jumeau, c'est a dire la phenol-betaïne possède d'autres particularités pharmacologiques que le sel d'ammonium quaternaire, c'est à dire son cation. Il n'est pas non plus exclu que dans l'ion jumeau, c'est à dire dans la phenol-betaïne (formation interne de sel) la localisation dans l'espace des substituants du N sont éventuellement déformés par rapport à l'ion de l'ammonium quaternaire. L'alcalinité n'est cependant que l'une des conditions de cet effet. L'intensité de l'effet dépend de la libération potentielle des valences secondaires qui déterminent la position dans l'espace des radicaux s'y attachant.

Bien entendu nous n'avons nullement l'intention de mettre en doute — nous basant sur ces résultats — ni les corrélations entre la structure et les effets des dérivés quaternaires, ni la théorie du «groupe analgiphore» de SCHAUMANN. Nous voudrions seulement souligner que nous ne pouvons pas dans tous les cas prédire d'avance les effets possibles d'un composé, même en connaissant les corrélations générales de sa structure.

Il faut enfin parler des propriétés de notre composé qui sont plus avantageuses que celles de la morphine. La morphine détermine chez 60 à 80% des chiens des vomissements à la dose de 0,5—1,0 mg/kg. La 6-acetyl-méthobromide de morphine ne détermine pas de vomissement même à la dose de 4 mg/kg. Nous rendrons compte ailleurs de ces expériences actuellement en cours. A ce point de vue, notre composé ressemble au dérivé quaternaire de l'apomorphine qui n'a pas d'effet vomitif même à la dose de 6 mg/kg alors que la dose vomitive de l'apomorphine est de 100 μg/kg.

Un autre avantage de notre composé c'est qu'il présente chez la souris un spectre d'action plus large que celui de la morphine. 50% des souris sont tués par 43 fois la ED-50 de la morphine, tandis que pour obtenir le même résultat il faut 735,4 fois la ED-50 de la 6-acetyl-méthobromide de morphine. Si cette proportion existe également chez l'homme on pourrait utiliser la 6-acetyl-méthobromide de morphine avec moins de risques que la morphine

LITERATURE

1. BOGNÁR, R.: MTA Kémiai Tud. Oszt. Közl. (Budapest) **5,** 57 (1954).
2. BRAENDEN, O. J., EDDY, N. B., HALBACH, H.: Bull. Wld. Hlth. Org. **13,** 937 (1955).
3. BURN, I. H.: Biological Standardization. Oxford Medical Publications London, 1950.
4. DRESER, H.: Arch. exp. Pathol. Pharmakol. **26,** 237 (1889).
5. EICHLER, G., SMIATEK, A.: Arch. exp. Pathol. Pharmakol. **194,** 621 (1940).
6. FÜHNER, H.: Arch. exp. Pathol. Pharmakol. **58,**-1-1907.
7. HERR, F., PÓRSZÁSZ, J.: Arch. exp. Pathol. Pharmakol. **210,** 294 (1950).
8. ISSEKUTZ, B.: MTA Orv. Tud. Oszt. Közl. (Budapest). **3,** 61 (1951).
9. LITCHFIELD, J. R., jr., WILCOXON, F.: J. Pharmacol. **96,** 99 (1949).
10. MEYER, H.: Ergebn. Physiol. **1,** II, 197 (1902).
11. POHL, J.: Arch. int. Pharmacodyn. **13,** 479 (1904).

12. Pórszász, J., Herr. F.: Kísérl. Orvostud. (Budapest). **2**, 295 (1950).
13. Rausch, J., Szegi, J., Szlamka, I., Nagy, J.: Acta physiol. hung. **15**, 329 (1959).
14. Schaumann, O.: Arch, exp. Pathol. Pharmakol. **196**, 109 (1940).
15. Szegi, J., Rausch, J., Magda, K., Nagy, J.: Acta physiol. hung. **16**, 325 (1959).
16. Szegi, J., Rausch, J.: A kisérleti orvostudomány vizsgáló módszerei. Akadémiai Kiadó, Budapest.
17. Trendelenburg, P.: Handbuch der experimentellen Pharmakologie: 564. Springer, Berlin, 1923. Vol. .I, p. 564.
18. Wolfe, G., MacDonald, A. D.: J. Pharmacol. **80**, 300 (1944).

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РЕЗЮМЕ

ДАННЫЕ К КИНЕТИКЕ ХОЛИНЭСТЕРАЗЫ IN VIVO I. ИЗМЕРЕНИЕ АКТИВНОСТИ ХОЛИНЭСТЕРАЗЫ В ОПЫТАХ IN VIVO

О. ФЕХЕР и Э. БОКРИ

Авторы разработали метод для измерения активности холинэстеразы верхнего шейного узла у кошки. При этом методе проводится перфузия узла раствором Локка, содержащем субстраты (ацетилхолин, ацетил-β-метилхолин, этилхлорацетат) и ингибиторы (эзерин, простигмин) холинэстеразы, и измеряется химическим способом разница между концентрацией втекающего и вытекающего перфузатов. Эта разница обусловливается активностью холинэстеразы узла.

Авторы устанавливают, каким образом условия перфузии (концентрация субстрата, скорость течения) определяют размер изменения измеренной концентрации субстрата.

Сообщаются данные относительно условий пермеабильности отдельных субстратов и ингибиторов.

ДАННЫЕ К КИНЕТИКЕ ХОЛИНЭСТЕРАЗЫ IN VIVO II. СРАВНЕНИЕ ГИДРОЛИЗА АЦЕТИЛХОЛИНА И АЦЕТИЛ-β-МЕТИЛХОЛИНА В ВЕРХНЕМ ШЕЙНОМ УЗЛЕ КОШЕК IN VIVO И IN VITRO

О. ФЕХЕР и Э. БОКРИ

Авторы исследовали гидролиз ацетилхолина и ацетил-β-метилхолина холинэстеразой верхнего шейного узла кошки в случае перфузии in vivo и гомогенизации.

В случае ацетилхолина они получили in vitro колоколообразную кривую субстрата. Это указывает на то, что узел содержит главным образом ацетилхолинэстеразу. Полученные in vivo величины показали весьма большое рассеяние.

В случае ацетил-β-метилхолина они получили in vitro колоколообразную кривую а in vivo характерную для псевдо-холинэстеразы S-образную кривую субстрата. Ввиду этого можно предполагать, что кинетика ацетилхолинэстеразы при таких условиях не идентична с наблюдаемой in vitro кинетикой.

СВЯЗЬ МЕЖДУ ВАГУСНОЙ АФФЕРЕНТАЦИЕЙ И ВЫСШЕЙ НЕРВНОЙ ДЕЯТЕЛЬНОСТЬЮ

Я. МОЛЬНАР, А. ТИДЬИ и К. ЛИШАК

В опытах на собаках исследовалось действие проведенной на различных уровнях ваготомии на условные рефлексы. У животных с перерезанным блуждающим нервом возникают количественные и качественные изменения в выработанных до операции условных рефлексах; неактивные тормозные и активные фазы чередуются с характерной периодичностью. Удалось выявить также прочие проявления, так напр. повторно появляющиеся парадоксальные реакции и фазовые состояния:

Для объяснения возникновения вышеописанного явления авторам удалось опознать в ходе проведенных исследований косвенный механизм: периодическое изменение условных рефлексов после ваготомии находится в тесной связи с изменениями содержания кальция в сыворотке. При исчезновении условных рефлексов наблюдается высокий

уровень кальция в сыворотке, однако, постепенное прекращение и возвращение рефлексов также можно проследить параллельно с изменениями содержания кальция в сыворотке.

ВОСПАЛИТЕЛЬНЫЙ „LEUKOPENIC FACTOR" (МЕНКИН) И ФАГОЦИТОЗ ЛЕЙКОЦИТОВ

Г. ЛУДАНЬ, Д. ВАЙДА, А. ДЭКЛЕН и И. ФЕХЕР

Воспалительный „leukopenic factor" (Менкин), при внутривенном введении собакам, тормозит фагоцитоз бактерий гранулоцитами. Этот эффект развивается параллельно с изменением картины крови. При концентрации 10^{-4}—10^{-6}, эффект наблюдается также in vitro. Приводятся ссылки на клинические наблюдения.

ДЕЙСТВИЕ ГИПОТЕРМИИ НА АКТИВНОСТЬ ФОСФОРИЛАЗЫ ПЕЧЕНИ

В. ЧАБА, Т. СИЛАДЬИ, Э. САБО и ДЬ. БОТ

Исследовалась на белых крысах активность фосфорилазы печени при глубокой гипотермии, и в связи с этим была выяснена роль адреналина, участвующего в активации фосфорилазы печени.

Было установлено, что

при глубокой гипотермии активность фосфорилазы печени снижается. Снижение тем значительнее, чем более глубока и длительна гипотермия.

При гипотермии уменьшение активности фосфорилазы печени не представляет из себя необратимого изменения, ибо снова подогретые животные показывают такие же величины ферментативной активности, как и активность фосфорилазы печени нормотермических животных.

Адреналин повышает активность фосфорилазы печени нормотермических животных. У гипотермических крыс активирующее действие адреналина большее ибо он повышает низкие величины фосфорилазы гипотермических животных до почти одинакового с нормотермическими животными уровня.

При гипотермии активирующая фосфорилазу печени ферментативная система способна к функции.

Наблюдаемое при гипотермии снижение активности фосфорилазы печени обусловливается не поражением активирующей фосфорилазу ферментативной системы, а отсутствием не только участвующего в активации фосфорилазы адреналина, а, предположительно, и глукагона.

ИССЛЕДОВАНИЕ ПРЕССОРНО-ДЕПРЕССОРНОЙ ПРОТИВОРЕГУЛЯЦИИ ПРИ ЭКСПЕРИМЕНТАЛЬНОМ ПОВЫШЕННОМ КРОВЯНОМ ДАВЛЕНИИ, ВЫЗВАННОМ РАЗЛИЧНЫМИ СПОСОБАМИ

А. КЁВЕР, ДЬ. БЕРЕГСАСИ. ДЬ. МОЛЬНАР и И. ВЕНТ

Прежде чем приступить к своим экспериментам, авторы разработали точный и надежный метод для определения содержания свободного, способного диффундировать гистамина в плазме собак. При помощи этого метода исследовалось, с одной стороны, на нормальных собаках, а с другой на таких, у которых экспериментально было вызвано повышенное кровяное давление, изменение свободного, способного диффундировать содержания гистамина в кровяной плазме во время вливания 20 микрограмм кг/мин. адреналина.

1. У нормальных собак содержание свободного гистамина в плазме крови во время вливания адреналина повышается до 2—4-кратных значений исходной величины. При достижении максимума гистаминемии регистрированное на лоскуте Лерсуме давление в сонной артерии падает ниже исходной величины.

2. Содержание свободного гистамина в плазме собак, у которых вызывалось повышенное кровяное давление путем денервации депрессора и пазухи сонной артерии, во время вливания адреналина остается неизменным.

3. Во время развития ренальной гипертонии, вызванной методом целофанового перинефрита, содержание свободного гистамина в плазме повышается параллельно с кровяным давлением, в течение 6—8 месяцев оно остается на достигнутом наивысшем уровне, а затем в последующих месяцах падает до прибл. нормальной величины.

4. В первые месяцы состояния повышенного кровяного давления, вызванного перириальным фиброзом, содержание гистамина в кровяной плазме во время адреналинового вливания повышается до 2—4-кратных значений исходной величины. В последующие месяцы этот ответ на адренегрическое раздражение все больше уменьшается и наконец совершенно прекращается, т. е. наступает такое состояние, которое соответствует противорегуляционной блокаде, состоящейся немедленно после денервации пазухи сонной артерии и депрессоров.

ДЕЙСТВИЕ SH-ИНГИБИТОРОВ НА ГАНГЛИОНАРНУЮ ПЕРЕДАЧУ ВОЗБУЖДЕНИЯ В ВЕРХНЕМ ШЕЙНОМ УЗЛЕ КОШЕК

П. ХАЛАС, Ф. МЕХЛЕР, О. ФЕХЕР и Ш. ДАМЬЯНОВИЧ

Авторы исследовали изменение функции верхнего шейного узла кошек на действие SH-ингибиторов в опытах in situ и на изолированных узлах путем регистрации потенциалов действия. При меньшей концентрации SH-ингибиторы обратимо парализируют передачу возбуждения, и усиливают раздражающее узел действие инъицированного через кровяное русло в узел ацетилхолина, а в большей концентрации отражают раздражение узла, вызванное внутриартериальным введением ацетилхолина и KCl. Вышеуказанный суммарный эффект SH-ингибиторов временно может быть прекращен с помощью цистеина. Важнейшие заключения, сделанные из результатов опытов следующие:

1. Имеющиеся в симпатических узлах рецепторные системы ацетилхолина показывают также в отношении SH-ингибиторов различную чувствительность.

2. SH-группы играют важную роль в построении постсинаптической мембраны, да даже в построении ацетилхолиновых рецепторов.

ДЕЙСТВИЕ АЛЬФА-АМИНОКИСЛОТ НА ГАНГЛИОНАРНУЮ ПЕРЕДАЧУ ВОЗБУЖДЕНИЯ

Ш. ДАМЬЯНОВИЧ, О. ФЕХЕР, П. ХАЛАС и Ф. МЕХЛЕР

Авторы исследовали действие различных аминокислот и их родственных соединений на ганглионарную передачу возбуждения.

Они провели перфузию верхнего шейного узла кошек по методу Кибьякова, и регистрировали сокращения мигательной перепонки, вызванные преганглионарным раздражением или же внутриартериальной инъекцией ацетилхолина и KCl. Они установили, что растворенные в перфузате альфа-аминокислоты парализуют пропорционально своей концентрации раздражающее узел действие ацетилхолина и KCl в то время как на передачу преганглионарных импульсов они воздействуют только при большей концентрации.

Исследуя механизм действия авторы выявили, что аминокислоты пригодны для дифференциации описанных Фехерем и Бокри свободных и иннервированных ацетилхолиновых рецепторов, далее, что для вызывания вышеописанных действий необходимо совместное присутствие амино- и карбоксилрадикалов.

На основании литературных данных и своих собственных результатов, авторы выдвигают заключения о возможной физиологической роли аминокислот.

СВЯЗЬ МЕЖДУ СОДЕРЖАНИЕМ ИОДА В ВОЗДУХЕ И ЭНЕРГЕТИЧЕСКИМ ОБМЕНОМ КРЫС

Л. БАЛОГ и А. ПАЛЬФИ

При 25—75%-ой относительной влажности воздуха не удалось выявить связи между содержанием иода в воздухе и обменом веществ крыс. В противоположность этому наблюдалась тесная, но сложная связь между содержанием иода, выявляемым в насыщенном парами воздухе при температуре 37 °C, и основным обменом.

ФАРМАКОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ 6-АЦЕТИЛ-МОРФИН-МЕТОБРОМИДА

Я. РАУШ, Ё. СЕГИ, К. МАГДА, Ю. НАДЬ, Р. БОГНАР и Ш. САБО

Авторы исследовали и сравнивали с морфином обезболивающее действие 6-ацетил-морфин-метобромида, так и его действие на дыхание, возможность повлиять на эти действия производными N-аллила, далее его уменьшающее кашель действие, парализирующее ганглии действие, действие на двигательные пластинки, как и привыкание к нему и токсичность этого препарата.

Из результатов выдвигалось то теоретическое установление, что 6-ацетил-морфин-метобромид представляет четвертичное производное морфина, которое при значительном болеутоляющем действии не оказывается кураreподобного, парализирующего ганглии действия. Данное соединение заслуживает внимания также и с точки зрения практики, ибо его болеутоляющее действие на мышах в 16,7 раз больше чем то морфина, причем оно снижает объем дыхания в 1,9 раз меньше морфина. Смягчающее кашель действие в 1,8 раз сильнее действия кодеина. У мышей привыкание меньшее, чем в случае морфина, а их токсичность одинакова.

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ERRATA

In the Acta Physiologica Academiae Scientiarum Hungaricae **17**, 465 (1960), a paper was published by J. Biró, entitled: Action of Rauwolfia preparations on the bladder muscle. On page 467 the two Figures are inverted and the text below the Figure is missing.

In the right order, the lower Figure is Fig. 1. Its text is as follows:

Fig. 1.

Experiment No 59. Cat, weight 1.80 kg.

A — Upper line: bladder curve. Middle line: blood pressure curve, on the left mm Hg. Lower line: Ground line with time-signal each 30″. — 1 : 0.10 ml Rausedyl intravenously.

B — Marks as at A. The kymogram illustrates the 31—38′ after Rausedyl administration.

In the right order the upper Figure is Fig. 2. Its text is as follows:

Fig. 2.

Experiment No 50. Cat, weight 2.60 kg.

A — Upper line: bladder curve. Middle line: blood pressure curve, on the left mm Hg. Lower line: Ground line with time-signal each 30″. — ↓ : Lavage of the bladder with physiological saline of 38° C. 2 : 0.65 ml Rauwopur intravenously.

B — Marks as at A. The kymogram illustrates the 25—32′ after Rauwopur administration.

Biochemia

STUDIES ON THE FUNCTIONAL ROLE OF THE MYOFIBRIL-BOUND NUCLEOTIDE

I. PHOSPHORYLATION OF THE MYOFIBRIL-BOUND NUCLEOTIDE

By

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(Received January 22, 1960)

Myofibrils contracted by ATP and myofibrils made to relax by various effects in the presence of ATP were tested for total nucleotide and acid-labile P content. When relaxation was induced at low temperature with EDTA, the acid-labile P/bound nucleotide ratio (P/N ratio) decreased in comparison with the P/N ratio for the contracted myofibril. No decrease in the value of P/N was observed when relaxation was brought about by high ionic strength, urea or concentrations of ATP above the optimal.

Experiments performed with ATP with its P labelled indicated that the difference in the value of P/N is not due to P uptake in the contracted state, but to a dissociation of part of the bound nucleotide, first of all ATP, in the relaxed state.

Muscle converts chemical energy into mechanical work. This process is based on some insufficiently known change in the myofibril which makes to shorten this complex of the two proteins myosin and actin. The energy of contraction is derived from the breakdown of macroergic phosphate compounds, just like in the case of any other cell function. Although there is no direct proof this macroergic compound being ATP, this has been accepted by most investigators of muscle function. The hypothesis is supported by (i) the identity of myosin with the ATP-ase bound to muscle structure [1]; (ii) the fact that actin, the other structural protein of muscle, contains protein-bound ATP, which is broken down on the polymerization of actin [2]; (iii) the obvervation that in certain *in vitro* systems the actin-bound nucleotide takes over phosphate from the creatine phosphoferase-creatine phosphate system and breaks it down [3]; (iv) the fact that the mechanical function released by ATP of the model system composed of actomyosin closely resembles the function of muscle in action [5]; and, finally, (v) the correlation between the function and ATP breakdown of these models [4].

One of the fundamental questions within the framework of this theory of muscle activity is the connection between the energy release due to the rupture of high-energy bonds and the changes in protein structure. According to our hypothesis (based on the idea suggested by Straub [2] when describing the actin-bound nucleotide), the phosphorylated and dephosphorylated ADP (or ATP) bound to actin, would be the very link between the protein structures and free macroergic phosphates. In the course of the contraction cycle the

nucleotide of actin would pass through phosphorylated (ATP) and dephosphorylated (ADP) forms and the difference in energy between the two phases would supply the energy for the structural changes taking place when the protein contracts. The ATP-actin complex may be formed through an exchange of the nucleotide (ADP replaces ATP) or through its phosphorylation (ADP is transformed into ATP by transphosphorylation). To confirm this hypothesis, surviving muscles in the relaxed and contracted state [6] were studied and it was found that it would be advantageous to make experiments with model systems, because in them it could be achieved much easier that the muscles analysed be in the contracted or relaxed state. In the following some experiments of this type will be described.

In the present paper studies on the state of phosphorylation of the bound nucleotide in myofibrils, caused to contract with ATP, and in myofibrils made to relax by various effects (first of all by EDTA), in the presence of ATP are described. The next paper [7] will deal with the metabolism *in vitro* of the bound nucleotide, as analysed by the use of radioactive ^{32}P isotope.

Materials and methods

The *myofibril* suspension was prepared by the method of PERRY [8] with the modification that homogenization and washing were done in 0.02 *M* borate buffer of pH 7.1. Rabbit psoas and latissimus dorsi muscles were minced, then homogenized in a MSE homogenizator with 1 : 10 volume of 0.02 *M* borate buffer (pH 7.1) for 2 minutes. This and every subsequent procedure were carried out at O° C. The homogenate was then centrifuged for 10 minutes at 900 *g*. The precipitate was resuspended in 10 volumes of a 0.02 *M* borate buffer, followed by centrifugation at 900 *g*. This procedure was repeated five times.

After the final resuspension in 1 : 4 volume, water-free glycerol was added to the suspension to a final concentration of 40 per cent. The myofibril suspension thus prepared was indefinitely stable when stored at —10° C.

The *Marsh-factor* was prepared by the method of PORTZEHL [9].

The *ATP* used was prepared by *Kőbányai Gyógyszerárugyár*, Budapest, and contained ADP and AMP as contaminants.

ATP containing ^{32}P was prepared at the Institute of Medical Chemistry, Medical University, Budapest, according to GARZÓ, MILE and MÜHLRAD [10].

Usually, the following mixture was used in the contraction and relaxation experiments:

Myofibril suspension corresponding to 1 g of whole muscle, 2—16 μM/ml ATP, 5 m*M* $MgSO_4$, 0.02 *M* borate buffer pH 7.1, 0.025 *M* KCl, as well as the other reagents specified with the individual experiments. The final volume was 10 ml. The experiments were carried out in a water bath of 20° C with incubation times from 1 to 30 minutes. In some experiments the process was arrested with ethanol, added to reach a final concentration of 20 per cent, while in others, the mixture was centrifuged without adding any other reagent to it. The myofibrils were then repeatedly washed to remove free nucleotide. The washing fluid was borate buffer, or borate buffer containing 20 per cent ethanol; there were six centrifugations at 900 g, in 10-fold volumes (to muscle weight). Subsequently, the bound nucleotide of the myofibril was extracted three times with 2 ml 6 per cent perchloric acid.

The quantity of nucleotide in the perchlorate extract was determined in an *Uvifot* photometer, at 254 mμ. The bound *inorganic P* and the *acid labile P* of the nucleotide were estimated by the slightly-modified method of BERENBLUN and CHAIN [11]. Protein was determined by the quantitative biuret test. In the enzyme activity assays P was determined according to FISKE and SUBBAROW [12].

Results

The experiments now discussed served to compare the bound nucleotide content of the relaxed myofibrils with that of the contracted ones. From the total amount of nucleotide ("N", expressed in μM/g muscle) and from the acid-labile P ("P", μM/g muscle) the P/N ratio was computed and was used to assess the degree of phosphorylation of the bound nucleotide.

Contracted myofibrils were prepared by incubation with ATP in suitable media for from 1 to 30 minutes.

The myofibril preparations used were considered to be relaxed, although in fact they were slightly contracted. The myofibrils incubated with ATP but treated in various ways to inhibit contraction were also considered to be relaxed. Such relaxing treatments were the following.

1. *The relaxing factor* prepared from muscle according to PORTZEHL [9], a suspension of subcellular granule fractions of unknown composition.

There is not sufficient evidence as to the nature of the relaxing factor first described by MARSH [13]. It is a constituent of the muscle cell. Attempts have been made to identify it with various enzymes of muscle, that can phosphorylate ADP by transphosphorylation [14, 15, 16]. Recent data seem to indicate that this view is not tenable [9, 17] and that certain granular components would be responsible for the relaxing action [9, 17], eventually together with some substance of non-protein nature and of unknown composition [18, 19, 20].

2. *EDTA* (ethylene diamine tetraacetate sodium). The relaxant action of EDTA on the glycerol fibre model was first described by BOZLER [21] and WATANABE [22].

3. *Mg-free reaction mixture.* Mg ions must be present when maximum contraction of actomyosin is to be achieved. Therefore, in the absence of Mg the ATP-treated myofibril is considered to be relaxed.

4. *Incubation at 0° C.* It is known that no contraction takes place, or the contracted model relaxes when the medium eliciting maximum contraction at the temperature range of from 20 to 30° C is cooled to 0° C [23, 24].

5. *High ionic strength.* Ionic strength profoundly influences the contraction of actomyosin model systems; the system relaxes above 0.2 μ [24, 25].

The myofibril reacting in a medium whose ionic strength has been increased from 0.025 to 0.20 μ is considered to be relaxed.

6. *Urea* was first described to cause the relaxation of the glycerol fibre (at a concentration of 1.0 M) by BOZLER [26]. The myofibril reacting with ATP in the presence 1 M urea is considered relaxed.

7. *ATP concentration beyond the optimum.* There is an optimum for the contraction of actomyosin model systems in in dependence of ATP concentration [24, 27]. Around an ATP concentration of 10 mM, contraction does

not take place any longer. The myofibrils incubated in media with such high concentrations of ATP are considered relaxed.

The relaxed or contracted state of the myofibrils treated by various methods was checked first of all by assaying the ATPase activity, considering that the contraction of the various actomyosin systems is associated with a high ATPase activity (a few decimal μM P/min/mg actomyosin), while relaxation with a low, inhibited ATPase activity (a few hundredths μM P/min/mg actomyosin). The relaxing treatments employed reduced the ATPase activity to 10 to 35 per cent of that shown by the contracted myofibril. ATPase was not estimated in every experiment, because the settling during incubation offered direct evidence of the contracted or relaxed state of the myofibril.

In Table I and II are presented the results of analysis for the contracted myofibril and for myofibrils caused to relax by treatment with the relaxing

Table I

Incubation in the reaction mixture specified in the text

Final concentrations of relaxants: relaxing factor [9]: 4 ml/10 ml reaction mixture; EDTA: 0.004 *M*. Duration of incubation 2 minutes. Reaction stopped by the addition of ethanol to a final concentration of 20 per cent, followed by washing with borate buffer containing 20 per cent ethanol

	μM nucleotide / g actomyosin "N"	μM hydrolysable P / g actomyosin "P"	P/N
Contracted	7.15	7.60	1.07
Relaxed (Marsh f.)	6.40	4.65	0.73
Relaxed (EDTA)	6.75	5.25	0.78
Relaxed (no Mg^{++})	6.55	5.30	0.81
Relaxed (0° C)	6.63	5.24	0.79

Table II

Experimental conditions as in Table I. At the end of incubation centrifugation, followed by washing with borate buffer

	μM nucleotide / g actomyosin (N)	μM hydrolysable P / g actomyosin (P)	P/N
Contracted	8.1	9.0	1.11
Relaxed (Marsh f.)	7.5	6.3	0.84
Relaxed (EDTA)	7.2	5.8	0.80
Relaxed (0° C)	7.6	6.4	0.84
Relaxed (no Mg^{++})	7.0	5.7	0.82

factor, EDTA, Mg-free medium and incubation at 0° C, respectively. In the experiments shown in Table I, incubation was terminated by the addition of ethanol and the free nucleotide was washed out in an ethanolic medium. In the

experiments contained in Table II, incubation was brought to an end with centrifugation and washing with borate buffer.

Both types of experiments indicated clearly that the relaxed myofibrils contain less nucleotide, which is also less phosphorylated.

Next, we examined in what measure the phosphorylation of the nucleotide is influenced by contraction or by the duration of the reaction. The duration of concentration was 30 minutes, *i. e.* a period during which all of the added free ATP was certainly broken down. According to the enzyme assays, 5 μM/ml ATP was broken down completely within 5 minutes. After 30 minutes the contracted suspension did not contain high-energy P in the free nucleotide, while the relaxed one contained a considerable amount of hydrolysable P. The results of these experiments are presented in Table III.

Table III

Experimental conditions as in Table I, except for duration of incubation

	Duration of incubation, minutes	μM nucleotide / g actomyosin (N)	μM hydrolysable P / g actomyosin (P)	P/N
Contracted	1	6.8	7.55	1.11
Relaxed (EDTA)	1	6.75	5.25	0.78
Contracted	30	8.5	9.2	1.08
Relaxed (EDTA)	30	6.95	6.3	0.91

The results make it clear that the duration of the experiment had no significant influence on the phosphorylation of the bound nucleotide. The fact that after 30 minutes there was no free ATP in the contracted myofibril suspension, while it was still detectable in the relaxed one, and yet the P/N ratio was higher in the contracted suspension, indicate that an imperfect separation of the free nucleotide from the myofibril cannot be held responsible for the phenomenon.

Table IV

Experimental setup as before. After incubation corresponding to the "original state", the process was stopped by centrifugation. Subsequently, the substances specified were added to the single points, as well as $MgSO_4$ to a final concentration of 0,005 *M and the usual buffer. The reaction was then stopped again by centrifugation. Washing with* 0,02 *M borate buffer pH* 7

Original state	After centrifugation	State after 2nd adding	μM nucleotide / g actomyosin	μM hydrolysable P / g actomyosin	P/N
Contracted	+EDTA	contracted	6.6	6.8	1.05
Relaxed	—	relaxed	6.15	4.65	0.75
Contracted	+EDTA+ATP	relaxed	6.05	4.8	0.79
Relaxed	+ATP	contracted	6.95	7.2	1.04

The data in Table IV reveal that the difference in phosphorylation between the contracted and relaxed myofibrils, as well as the change taking place on contraction and relaxation are reversible. When, namely, contracted myofibrils are caused to relax or relaxed ones to contract, the nucleotide of the myofibril will invariably reflect the last condition. It is also clear that EDTA alone, in the absence of ATP, does not cause relaxation (see [21]); the contracted myofibril resuspended in a medium containing EDTA exhibited the P/N ratio apparently characteristic of the contracted myofibril, and it was only after a second resuspension in the presence of EDTA *and* ATP that there appeared a P/N ratio characteristic of the relaxed state.

Other relaxant effects were also analysed. As the data in Table V show, the difference in the measure of phosphorylation between the nucleotide bound

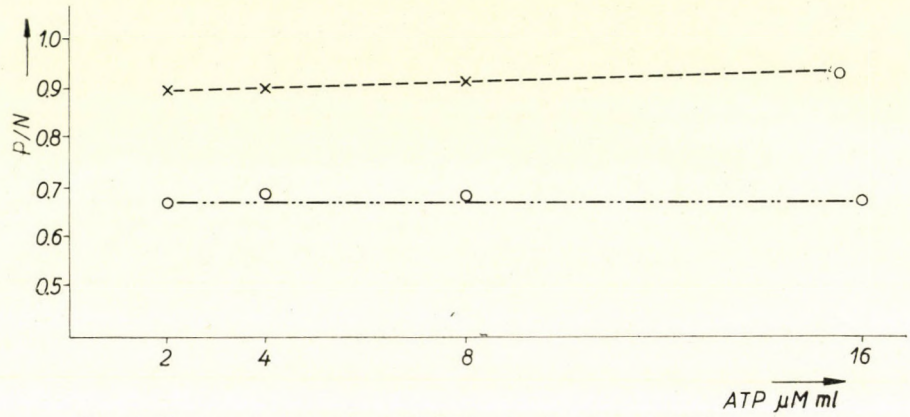

Fig. 1. Changes in the value of P/N in the function of ATP concentration. Experimental conditions as specified in Table I.
— · · — in the presence of EDTA
— — — — no EDTA
× contraction
o relaxation

to the contracted myofibril and that bound to the relaxed myofibril does not characterize any kind of relaxation. Relaxation induced by high KCl concentration and that produced by adding urea increased rather than decreased the value of P/N.

The same applies to the effect of an ATP concentration above the optimum, as it is clear from Fig. 1.

It is noteworthy that in the above cases the P/N ratio decreased after the addition of EDTA (Table V and Fig. 1).

What may be responsible for the decrease of the P/N ratio at relaxation of certain kinds and its increase with contraction? The higher value of the P/N ratio with the contracted state may be due to a transfer of P from the free ATP to the bound ADP on contraction (or the latter is substituted by ATP), but the change may also result from a reduction in the quantity of bound nucleotide

Table V

Incubation and washing as specified in Table I

EDTA, 0.004 *M*; KCl, 0.2 *M*; Urea, 1 *M*

	+EDTA	μM nucleotide / g actomyosin (N)	μM hydrolysable P / g actomyosin (P)	P/N
Contracted	—	6.4	6.8	1.06
Relaxed (EDTA)	—	5.7	5.1	0.89
Relaxed (KCl)	—	7.4	10.3	1.39
Relaxed (KCl)	+	7.4	7.2	0.97
Relaxed (urea)	—	5.46	6.2	1.13
Relaxed (urea)	+	4.95	3.75	0.74

at washing after incubation, due in the relaxed state first of all to an elution of ATP. Radioactive isotope studies seemed to offer the best possibility to decide this issue. If the increase of the P/N ratio in the contracted state is due to an increase of bound ATP, on contraction induced by labelled ATP a considerable proportion (about 30 per cent) of the hydrolysable P of the bound nucleotide might be expected to be substituted, and radioactivity will appear in the bound nucleotide. The experiment was carried out and the results are shown in Table VI.

Table VI

Experimental conditions as specified in Table I

Relative specific activity related to specific activity of the hydrolysable P of the ATP used

	μM nucleotide / g actomyosin (N)	μM hydrolysable P / g actomyosin (P)	P/N	Relative specific activity $\times 100$
Contracted	6.65	6.9	1.06	2.55
Contracted	6.4	6.8	1.04	5.25
Relaxed (EDTA)	6.05	4.05	0.67	3.55
Relaxed (EDTA)	5.6	4.0	0.72	4.2

As seen, no substantial exchange has taken place in the experiment shown. Thus, the difference in the composition of the bound nucleotide between the relaxed and the contracted myofibril was not due to a phosphorylation of the bound nucleotide on contraction or to an exchange of part of the ADP for ATP. The difference must have been due to a release of some of the bound nucleotide by the relaxed myofibril during incubation or during the elution following it, and the nucleotide lost from the relaxed myofibrils was first of all ATP. Relaxation, at least with some of the relaxing effects, influenced the nature of the nucleitide-protein bond and this must have been responsible for the difference observed.

Discussion

The experiments described call attention to certain differences in the phosphorylation of the bound nucleotides between the myofibril suspensions caused to relax by various methods. Evidence has been obtained to suggest that relaxation induced by the Marsh factor, EDTA, 0 °C or Mg^{++} -deficiency is different from that induced by urea, high KCl or high ATP concentrations. This is supported also by BENDALL's statement [28] that there are two types of relaxation: a "Marsh factor-EDTA" type, [20, 31], taking place also when the concentration of Mg^{++} is in excess over, and the "pyrophosphate or high ATP concentration" type, which results when the concentration of Mg^{++} is lower than of the relaxing agent. BÁRÁNY *et al.* [31, 32] have recently found that the inhibition of both ATPase activity and contraction is caused by substances which act on myosin proper or inhibit the interaction between myosin and actin. As we have seen on the basis of the phosphorylation of the bound nucleotide also two types of relaxation can be identified. The connections between this observation and those mentioned above require further investigation.

The experiments with radioactive ATP indicate that the difference in the composition of the bound nucleotide between the relaxed and contracted myofibril is not due to a P-uptake by the bound nucleotide through transphosphorylation. In relaxation, as induced by certain methods, the P/N ratio is, invariably associated with a certain loss of total nucleotide due to a loss of bound nucleotide (mainly ATP) by the relaxed myofibril. That this is actually so and the difference is not due to an imperfect removal of free ATP in consequence to an increased dehydration of the contracted myofibril (Table III), is indicated by the persistence of the difference after the complete breakdown of ATP. In the cases when relaxation is not associated with a decrease of the P/N ratio, there is no reason to assume that the hydratation of the myofibril would be similar to that of the contracted myofibril either. The differences obtained are considered to be real ones, due to differences appearing on relaxation in the protein-nucleotide bond and manifested first of all in a looser linkage with ATP. The radioactive isotope experiments intended to elucidate whether the bound nucleotide would take part in the ATP breakdown associated with contraction, will be described in another report [7].

LITERATURE

1. ENGELHARDT, V. A., LYUBIMOVA, M. N. (ЭНГЕЛЬГАРДТ, В. А., ЛЮБИМОВА, М. Н.) Биохимия **4**, 716 (1939).
2. STRAUB, F. B., FEUER, G.: Biochim. biophys. Acta **4**, 455 (1950).
3. STROHMAN, R. C.: Biochim. biophys. Acta **32**, 436 (1959).
4. WEBER, H. H.: The motility of muscle and cells. Harvard University Press, Boston, 1958.

5. Biró, N. A.: Thesis, Budapest, 1954.
6. Biró, N. A., Nagy, B.: Unpublished data (1956).
7. Biró, N. A., Mühlrad, A.: Acta physiol. hung. **18,** 95 (1960).
8. Perry, S. V.: Biochem. J. **51,** 495 (1952).
9. Portzehl, H.: Biochim. biophys. Acta **24,** 474 (1957).
10. Garzó, T., Mile, I., Mühlrad, A.: in Sugárzó izotópok hazai felhasználása. Az Országos Atomerő Bizottság Izotóp Alkalmazási Szakbizottságának kiadványa, Budapest, 1958, **I,** p. 44.
11. Berenblum, J., Chain, E.: Biochem. J. **32,** 286 (1938).
12. Fiske, C. H., Subbarow, Y.: J. biol. Chem. **66,** 375 (1925).
13. Marsh, B. B.: Biochim. biophys. Acta **9,** 247 (1952).
14. Bendall, J. R.: Proc. Roy, Soc. **B. 142,** 409 (1954).
15. Goodall, M. L., Szent-Györgyi, A. G.: Nature (Lond.) **172,** 84 (1953).
16. Loránd, L.: Nature (Lond.) **172,** 1181 (1953).
17. Ebashi, S.: Arch. Biochem. Biophys. **76,** 410 (1958).
18. Briggs, F. N., Kaldor, G., Gergely, J.: Biochim. biophys. Acta **34,** 211 (1959).
19. Gergely, J., Kaldor, G., Briggs, F. N.: Biochim. biophys. Acta **34,** 218 (1959).
20. Kaldor, G., Gergely, J., Briggs, F. N.: Biochim. biophys. Acta **34,** 224 (1959).
21. Bozler, E.: J. gen. Physiol. **167,** 276 (1954).
22. Watanabe, S.: Arch. Biochem. Biophys. **54,** 559 (1955).
23. Szent-Györgyi, A.: Biol. Bull. **96,** 140 (1949).
24. Biró, N. A., Szent-Györgyi, A. G.: Hung. Acta physiol. **2,** 120 (1949).
25. Bozler, E.: J. gen. Physiol. **33,** 703 (1952).
26. Bozler, E.: Amer. J. Physiol. **167,** 276 (1951).
27. Weber, A., Weber, H. H.: Biochim. biophys. Acta **7,** 339 (1951).
28. Bendall, J. R.: Arch. Biochem. Biophys. **73,** 283 (1958).
29. Perry, S. V., Grey, T. O.: Biochem. J. **64,** 184 (1956).
30. Weber, A.: J. biol. Chem. **234,** 2764 (1959).
31. Bárány, M., Bárány, K.: Biochim. biophys. Acta **41,** 204 (1960).
32. Bárány, M., Jaisle, F.: Biochim. biophys. Acta **41,** 192 (1960

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STUDIES ON THE FUNCTIONAL ROLE OF THE MYOFIBRIL-BOUND NUCLEOTIDE

II. INVESTIGATIONS ON THE METABOLISM OF BOUND PHOSPHATE FRACTIONS BY THE USE OF LABELLED P

By

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The bound acid labile fraction and the bound inorganic phosphate of the myofibril do not become significantly labelled when the myofibril breaks down ATP equally labelled in the β and γ positions. Likewise, there is no decrease in the specific activity of the phosphate fractions of myofibrils labelled *in vivo* with ^{32}P when the isolated myofibril is splitting non-radioactive ATP.

The rate of neogenesis of the myofibril-bound P fractions is several orders of magnitude higher than that of the actin protein (VELICK [5]).

The above results are analysed with a view to a hypothetical intermediary role of the bound nucleotide with special reference to the results published by STROHMAN [9], PERRY [10], ULBRECHT and ULBRECHT [11].

In a previous report [1] it has been concluded that the assumed cyclic change of the bound nucleotide between the phosphorylated and dephosphorylated states cannot be demonstrated by a simple analysis of the bound nucleotide in the various states of the muscle. The cause of this is first of all technical: although there persistently occurred small differences in phosphorylation between relaxed and contracted muscles (P/N ratio, see Tables in [1]), at the same time the total nucleotide content also changed. Therefore it could not be established whether the difference was due actually to a transit from the relaxed to the contracted state or it was an unimportant phenomenon resulting from the washings required for the isolation of the bound nucleotide. It became obvious that the problem could advantageously be investigated by the use of radioactive P, because in this case an eventual exchange of phosphate fractions may yield decisive evidence even if the nucleotide examined would be just a fraction of the bound nucleotide presumably involved in the reaction.

We studied not only the phosphate of the bound nucleotide, but also the myofibril-bound phosphate, *i. e.* that freed by perchloric acid from the thoroughly-washed myofibril. The persistent presence of this phosphate was already noted in our initial studies [2]. As its amount is near to that of the bound nucleotide, and it forms a very firm bond and is linked specifically to actin (as it will be seen later), it is just as justified to attribute a functional significance to it as to the bound nucleotide. Exactly the same conclusions have been drawn recently by HASSELBACH [3] as regards bound inorganic phosphate.

Materials and methods

These have already been described [1], except for some details which will be presented with the experiments.

Results

a) Experiments concerning the nature of the bound inorganic phosphate

In 15 different myofibril suspensions, prepared by the usual method and washed, the inorganic P content averaged 0.18 μM/g fresh muscle, and the adenine nucleotide averaged 0.25 μMg/. Detailed studies showed this inorganic P to be bound to the actin protein of the myofibril. Washed myofibril was treated with an extracting fluid (HANSON and HUXLEY [4]) which extracts exclusively myosin from muscle. To 6.6 ml of myofibril suspension (corresponding to 1.1 g of fresh muscle) 55 ml of the extracting fluid was added. After standing for 1 hour at 0° C and centrifugation for 10 minutes at 900 *g* extraction was repeated. After an interval of 30 minutes followed another centrifugation for 10 minutes at 900 *g*. The volume was then adjusted to 11 ml with 0.02 *M* borate buffer (pH 7), one ml was removed to be tested for non-collagen protein, and the rest of the suspension was tested for inorganic and acid-labile phosphate, as well as for nucleotide, as already described.

The results are presented in Table I.

Table I

	Whole myofibril	After myosin extraction
Protein mg	86	56
P, inorganic, μmole	0.279	0.243
P, hydrolysable, μmole	0.441	0.387
Nucleotide, μmole	0.370	0.332

According to the results, the extraction of myosin was only partial, but while the protein loss amounted to 40 per cent (falling as a whole on myosin), the loss in inorganic P or in adenine nucleotide was only 10 per cent, indicating that the bound inorganic P is bound to actin, like the nucleotide.

Next it was examined to what extent the bound inorganic and the hydrolysable P may be replaced by ^{32}P, to assess the strength of the bond between protein and phosphate. Myofibril was incubated for various lengths of time with orthophosphate containing ^{32}P, washed six times with 0.02 *M* borate

buffer; finally, the bound fractions were extracted with 6 per cent perchloric acid and the measure of exchange was assessed on ground of the specific activity. The results are illustrated in Fig. 1.

The experiment showed the bond between the inorganic phosphate and the protein to be firm; were it merely an adsorptive linkage, an equilibration between the free and orthophosphate would have occurred, corresponding to a 100 per cent exchange. However, only 5 per cent of the bound phosphate was exchanged in 40 hours, a finding definitely pointing to the stability of the bond.

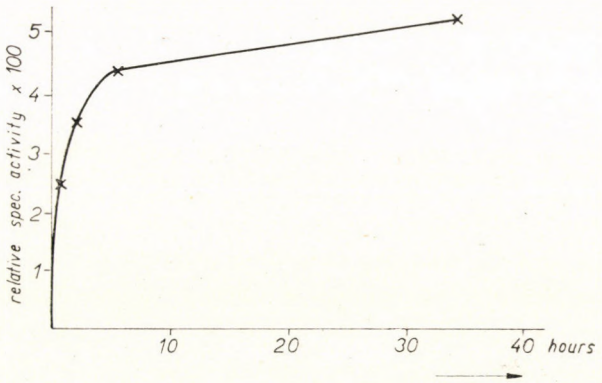
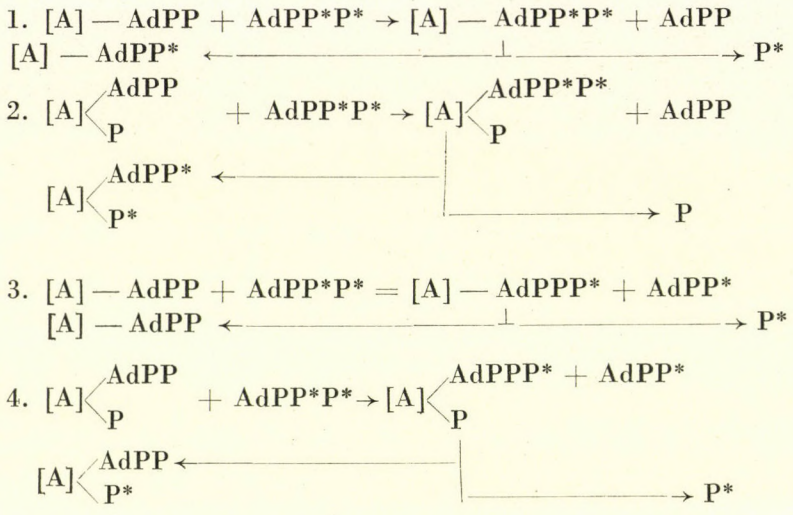


Fig. 1

This confirms the existence of a well-defined inorganic orthophosphate, bound to actin, a fact made probable by HASSELBACH's data [3].

b) Experiments concerning the metabolism of the bound phosphate fraction

On the basis of the assumed intermediary role of bound ADP, or bound ADP and bound inorganic phosphate, four different mechanisms are conceivable. Reactions 1 and 2 would involve an exchange of bound ADP for ATP, while reactions 3 and 4 a phosphorylation of bound ADP by free ATP as the phosphorus donator. In addition, it is assumed that in reactions 2 and 4 in the course of the dephosphorylation of bound ATP the phosphate split off is bound to protein and this would be the protein-bound phosphate mentioned above. ([A] means actin protein, and the adenosine phosphates are represented by AdPPP, etc.). The equations relate to ATP labelled evenly with isotope in the β and γ positions, corresponding to the later-specified experimental conditions, designating the isotope-labelled phosphate groups with P*. As the equations show, in 3 of the hypothetical mechanisms (1, 2 and 4) labelling must take place. In mechanism 1 the bound nucleotide, in mechanism 2 the nucleotide and the bound inorganic phosphate, and in mechanism 4 the bound inorganic phosphate must become labelled.



The experiments corresponding to the above schemes were carried out under various conditions but significant labelling was observed in none of them. One representative experiment is described below.

Radioactive ATP was split with washed myofibril. The end concentration. of ATP was 2 μM/ml. The reaction was stopped after one minute by centrifugation. Borate buffer was used for washing. The borate washing fluid and the perchloric acid extract were tested for phosphorus fractions and their radioactivity. Specific activity was related to the specific activity of the hydrolyzable resp. inorganic P of the post-incubation supernatant (+ washing fluid). The results are shown in Table II.

Table II

	Myofibrils before incubation	Myofibrils incubated with $AT^{32}P$
Bound nucleotide, μM/g	0,224	0.235
P, bound, inorganic μM/g	0,171	0.176
Relative specific activity $\times 100$	—	3.2
P, bound, hydrolyzable, μM/g	0.268	0.272
Relative specific activity $\times 100$	—	0.5

As seen, no significant exchange had taken place either in the nucleotide fraction, or in the inorganic phosphate fraction. The exchange of inorganic phosphate was of the same order of magnitude as that achieved by incubation with labelled inorganic phosphate only. (Fig. 1).

Essentially, the same experimental procedure was used when the phosphate fractions of the myofibril were labelled by the *in vivo* administration of radioactive inorganic phosphate and then non-radioactive ATP was split with labelled myofibril. In accordance with the above hypothesis in this case the labelling of the phosphate fractions of the myofibril (with reaction mechanisms 1, 2 and 4) ought to have decreased. In the experiments with frog muscle neither the labelling of the nucleotide phosphate nor that of the inorganic phosphate decreased significantly.

This experience suggests that if the nucleotide has intermediary role, this may correspond exclusively to mechanism 3, *i. e.* it may be a cyclic phosphorylation-dephosphorylation. However, this mechanism cannot be proved by experiments similar to those described above. Its demonstration would require a fixation in some way of the hypothetical intermediary ATP-state of the bound nucleotide, but the denaturation of the protein makes it impossible to separate free ATP from bound ATP.

All these experiments make it doubtful whether the bound nucleotide has any role directly connected with function. It is namely conceivable that the bound nucleotide is simply a structural element of actin, that is not involved in any specific metabolic process in the course of function. If this is so, the rate of exchange of the bound nucleotide phosphate must be comparable in order of magnitude to the rate of actin neogenesis *in vivo*. However, experimental evidence has been obtained to show that the bound nucleotide was much faster labelled *in vivo* than the neogenesis of actin protein.

5 mC of ^{32}P was injected intraperitoneally into a rabbit. The animal was sacrified 96 hours later, the psoas muscles were prepared out in the usual way. On isolating the myofibril fraction the supernatants were pooled and in this way the specific activity of the free and bound fractions was determined separately. The results are presented in Table IV.

Table IV

	Bound fraction	Free fraction
P, inorganic, μmole	0.435	11.8**
P, inorganic, spec act.*	181	184
P, hydrolyzable, μmole	0.345	1.0
P, hydrolysable, specific activity	157	175

* Specific activity = imp/min/μM P.

** The free inorganic phosphate fraction contains also the P of creatine phosphate. This, being extremely labile, was broken down during the experiment.

The results show no difference in specific activity between the free and bound fractions. VELICK [5] found 67 days for the half-life of actin in rabbit muscle. This would mean a neo-genesis of round 3 per cent of the actin protein in four days. Thus, the possibility that the exchange would be a result of the neogenesis of the whole protein can be ruled out.

Discussion

The above experiments have failed to offer direct proof of the actin-bound nucleotide taking part in that special metabolism of ATP in the course of which the energy of the macroergic phosphate bond is transformed into mechanical work. At the same time, the hypothesis should not be given up completely. The stability of the bond between actin and nucleotide [6, 7], the stoichiometric proportions [6, 8] and the unique nature of the protein—adenosine—phosphate complex characteristic of muscle [2] are by themselves sufficient to be credited with special significance. This view is confirmed by the evidence recently published by STROHMANN [9] that (i) on the reversible depolymerization of actin the bound ADP takes over the terminal phosphate of ATP ensuring reversibility; (ii) under appropriate conditions the actin—H—meromyosin complex can take over P from the creatine phosphate—creatine system and can split this phosphate, which is transphosphorylated to protein bound ADP. These experiments indicate that under suitable conditions the actin-bound nucleotide is in fact capable of being phosphorylated and that the phosphate thus transferred is split off again. This means that STROHMANN has succeeded in demonstrating the existence of the mechanism which on the basis of our above experiments we suggested as the only possible one (reaction 3). Whether this mechanisms actually takes place in the myofibrils when ATP is broken down, it remains an open question, the more so, since PERRY [10] obtained negative results with *myofibrils*, in a system otherwise completely analogous to those prepared by STROHMANN. We therefore feel that this hypothesis, fundamental from the point of view of the mechanism of muscle activity, should be further studied, it being supported also by the labelling experiments *in vivo* which have ruled out the possibility of the bound nucleotide being merely of structural importance.

It is more difficult to form an opinion as to the role of the bound inorganic phosphate. The above experiments make it unlikely that this phosphate would be an intermediary of ATP breakdown. If there is a protein-phosphate intermediary, as it has repeatedly been suggested (more recently by ULBRECHT and ULBRECHT [11]), it is not the firmly bound phosphate found in the washed myofibrils, but possibly some more labile bond.

ULBRECHT and ULBRECHT have suggested the existence of a protein-phosphate intermediary, because they found that in the course of ATP break-

down in the presence of radioactive ADP the ATP became labelled, presumably as a result of a hypothetical protein—phosphate-complex intermediary formation. Their experiments have yielded no evidence as to the nature of this complex, so that if the complex actually exists, it may be identical with the hypothetical ATP in our reaction scheme 3.

LITERATURE

1. Biró, N. A., Mühlrad, A.: Acta physiol. hung. **18,** 85 (1960).
2. Biró, N. A., Nagy, B.: Acta physiol. hung. **8,** 313 (1955).
3. Hasselbach, W.: Biochim. biophys. Acta **25,** 562 (1957).
4. Huxley, H. M., Hanson, J.: Biochim. biophys. Acta **23,** 250 (1957).
5. Velick, S. F.: Biochim. biophys. Acta **20,** 228 (1956).
6. Straub, F. B., Feuer G.: Biochim. biophys. Acta **4,** 455 (1950).
7. Biró, N. A.: Thesis, Budapest, 1954.
8. Mommaerts, W. F. H. M.: J. biol. Chem. **198,** 469 (1952).
9. Strohman, F. C.: Biochim. biophys. Acta **32,** 436 (1959).
10. Perry, S. V.: Biochem. J. **54,** 427 (1954).
11. Ulbrecht, G., Ulbrecht, M.: Biochim. biophys. Acta **25,** 100 (1957).

Note added in proof:

Since this paper was sent to the redaction, Gergely *et al.* [J. Biol Chem. **235,** 1704, 1707 (1960)] questioned the validity of some of the results of Strohman [9], and — in much shorter times as in our experiment — they did not found a labelling of the bound myofibrills *in vivo*. Like our experiments, the experiments of Gergely does not exclude either the cyclical phosphorylation-dephosphorylation mechanism suggested here. A most recent work of Yagi and Noda [Biochim. Biophys. Acta **43,** 249 (1960)] bring clear evidence of the phosphorylation of the bound ADP by creatine-phosphate on the case of myofibrills prepared of glycerol-treated muscle or treated with desoxicholate.

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ON THE MODE OF ACTION OF STREPTOMYCIN

I. EFFECT OF STREPTOMYCIN ON THE TERMINAL OXIDATION OF E. COLI

By

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(Received March 14, 1960)

Bacteriostatic concentrations of streptomycin had no substantial influence on the oxidation of any of the substrates tested with the strains *E. coli* B and *E. coli* 055. Higher concentrations, especially in media of low ionic strength, caused a partial inhibition of oxidation, but this began only after a latency period of from 20 to 40 minutes and proceeded at a slow rate.

Bacteriostatic concentrations of streptomycin inhibited the adaptive development of the terminal oxidation system in *E. coli* cells from semianaerobic cultures. The length of exposure needed for the inhibition varied according to the prevailing conditions and the effect was influenced by the electrolyte concentration and pH of the environment.

There is no theory that would unequivocally explain the mode of action of streptomycin. The action of the antibiotic on the respiration of susceptible bacteria has been discussed in several reports [1—13], but the evidence published is far from being uniform. Inhibition of the oxidation of various substrates in certain microorganisms susceptible to streptomycin has been observed by HENRY *et al.* [1, 2, 3], GEIGER [4], UMBREIT *et al.* [5—8], as opposed to the increased rate of respiration found by BENHAM [9] and WASSERMANN [10]. PAINE and CLARK [11, 12] found no unequivocal correlation between the action on the respiration and the bacteriostatic activity.

According to our preliminary experiments, bacteriostatic concentrations of streptomycin have no substantial inhibitory action on the oxidation of glucose, pyruvic acid, fumaric acid, formic acid, glutamic acid, serine, threonine *etc.* by the strains *E. coli* 055 and *E. coli* B. The oxidation of certain substrates was partially inhibited by higher concentrations of the antibiotic, especially when tested in media poor in phosphate. The inhibition of respiration began after a latency of from 20 to 40 minutes and continued gradually.

It was also found that the *E. coli* cells from semianaerobic cultures slowly oxidized pyruvate. The oxidation of pyruvate could not be increased catalytically with fumarate or oxalacetate; on the other hand, the oxidative activity of such cells greatly increased in time when a suitable oxygen tension was ensured and a complete mixture of amino acids, or an ammonia nitrogen source and pyruvic acid were present. The increase in the activity of the terminal oxidative system is believed to be an enzymatic adaptation, as it has a protracted course, requires the presence of amino acids and is inhibited by chloramphenicol. The effect of streptomycin on this adaptation process was investigated.

Methods

The test organisms were *E. coli* B and *E. coli* 055.

The bacteria were grown in Knight's semisynthetic liquid medium, containing casein hydrolysate completed with tryptophan, and glucose in a salt mixture buffered with phosphate. The pH of the culture medium was 7.5. The microorganisms were cultured for 6 to 20 hours at 37° C, in standing cultures under semianaerobic conditions. The bacteria harvested at different intervals were washed with 0.9 per cent NaCl solution or with distilled water and were suspended in $6.7 \cdot 10^{-2}$ *M*, pH 7.5 phosphate buffer. In some experiments the concentration of the phosphate buffer was changed, in others the pH.

The oxygen uptake by the cell suspensions was measured manometrically in the Warburg apparatus (the CO_2 produced was absorbed by a strip of filtre paper soaked in 20 per cent KOH). The volume of the reaction mixtures in the Warburg vessels was 3 ml. Atmospheric air served as the gas mixture.

The substrates were used in the following concentration: pyruvic acid, 15 μM/ml; fumaric acid, 15 μM/ml; together with pyruvic acid, 1.5 μM/ml); casein hydrolysate (completed with tryptophan), 0.0033—0.066 per cent; dihydrostreptomycin sulphate, 8—75 μg/ml. Each substrate was dissolved in the phosphate buffer and the pH was adjusted to the required value.

The dry solids of the cells were determined nephelometrically, by comparison with an empirically-plotted calibration curve.

The nitrogen content of the cells was estimated by the quantitative Nessler reaction from aliquots taken from the reaction mixtures, after digestion with sulphuric acid in the presence of a selenium catalyst, by the photometric method.

The results are given either as the Q_{0_2} related to cellular nitrogen [Q_{0_2} (N)] or as the amount of oxygen (in microlitre) consumed in 10 minutes by 3 ml of the cell suspension (in this case the designation dO_2 will be used).

Results

As shown in Fig. 1 at the beginning of the experiment the terminal oxidation of the *E. coli* 055 cells was slow. The oxidative activity increased immediately following the addition of casein hydrolysate and pyruvic acid and reached about 3.5 times the initial level in 60 minutes. It is known from other experiments that this increase in the rate of respiration does not take place in the absence of a suitable source of nitrogen. The increased activity of oxidation cannot be explained by an increase in the number of cells, as the increase in the cellular solids did not exceed 25 per cent at the end of the experiment. The *E. coli* 055 cells used in this series were taken from a 18-hour semianaerobic culture.

The adaptive-like development of the oxidation system was inhibited by 50 μg/ml of dihydrostreptomycin. Inhibition was complete after about 40 minutes.

The duration of the latency period was shortened and the inhibition could be made complete, respectively, by altering the permeability of the cells by repeated freezing and thawing prior to the experiment.

To illustrate that under such conditions it was actually a pyruvic acid oxidation system that developed we present Fig. 2. Cells from a 20-hour semianaerobic culture had been frozen and thawed 3 times, then incubated at 37° C with shaking for 90 minutes in a mixture of pH 7.5 containing phosphate buffer (6.7×10^{-2} *M*), pyruvate (15 μM/ml), fumarate (1.5 μM/ml) and

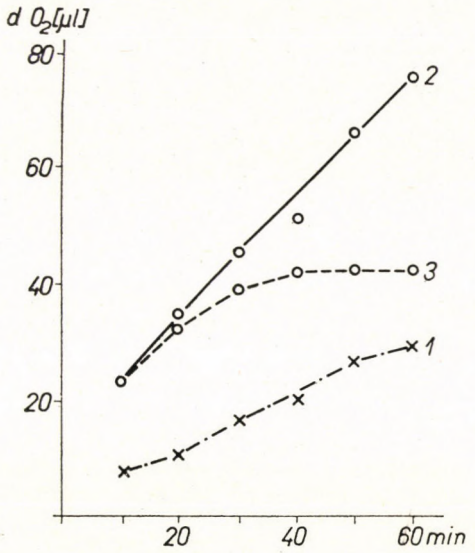

Fig. 1. Effect of dihydrostreptomycin on the terminal oxidation of *E. coli* 055

O_2 uptake measured by the manometric method. Cell density: 1 mg/ml cellular solids
1 : 0.033 per cent casein hydrolysate
2 : 0.033 per cent casein hydrolysate +15 μM/ml pyruvate
3 : 0.033 per cent casein +15 μM/ml pyruvate + 50 μg/ml dihydrostreptomycin

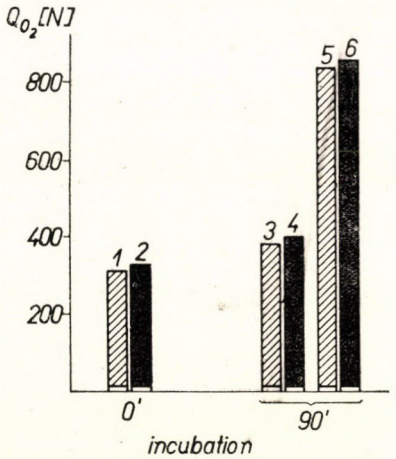

Fig. 2. Effect of dihydrostreptomycin on the terminal oxidation rate of *E. coli* 055.

Pretreatment of cells as specified in text. O_2 uptake measured by the manometric method
Reaction mixture: in $6.7 \cdot 10^{-2}$ *M* pH 7.5 phosphate buffer:
15 μM/ml pyruvic acid, 1.5 μM/ml fumaric acid (columns *2*, *4*, *6*)
Columns *1*, *3*, *5*: the former +50 μg/ml dihydrostreptomycin
Columns *1*, *2 :* non-incubated cells
Columns *3*, *4 :* cells preincubated for 90 minutes in the presence of dihydrostreptomycin (50 μg/ml)
Columns *5*, *6 :* cells preincubated for 90 minutes without dihydrostreptomycin

casein hydrolysate (0.033 per cent), with and without dihydrostreptomycin (50 μg/ml). After centrifugation and washing, the oxygen uptake by the cells was measured in the presence of a mixture of pyruvate and fumarate. After determining the nitrogen content of the cells, the results were expressed in Q_{O_2} (N). During the 90 minutes of preincubation the activity of terminal oxidation in the control cells increased to 2.5 times the initial; this increase was almost completely inhibited by 50 μg/ml of dihydrostreptomycin. The

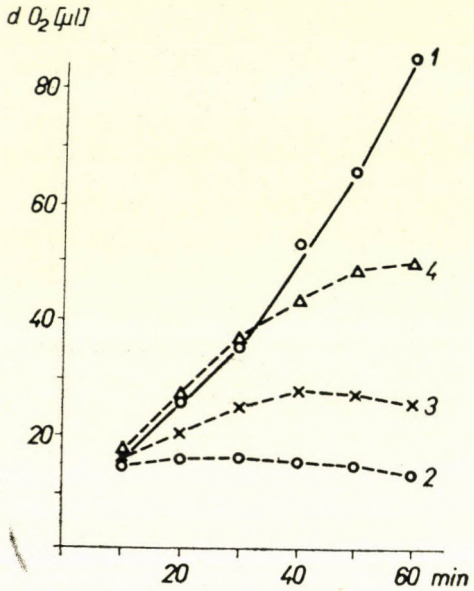

Fig. 3. Effect of phosphate buffer concentration on streptomycin inhibition.

O_2 uptake measured by the manometric method
Cell density: 0.8 mg/ml cellular solids
Reaction mixture: pH 7.5 phosphate buffer, 15 μM/ml pyruvate, 0.033 per cent casein hydrolysate (curve *1*)
Curves *2*, *3*, *4*: the former +33 μg/ml of dihydrostreptomycin
Concentration of phosphate buffer:
1, *2*: $1.1 \cdot 10^{-2} M$
3: $2.7 \cdot 10^{-2} M$
4: $4.3 \cdot 10^{-2} M$

same amount of streptomycin had no significant influence on the oxygen uptake itself.

The inhibition of the enzymatic adaptation by streptomycin was influenced by several factors, including the electrolyte concentration and pH of the medium.

In the experiment shown in Fig. 3 the terminal oxidation of cells from a 6-hour semianaerobic culture was measured in the presence of pyruvic acid and casein hydrolysate, in pH 7.5 phosphate buffers of different concentration.

In the medium containing 1.1×10^{-2} *M* phosphate buffer, 33 μg/ml of dehydrostreptomycin inhibited the enzymatic adaptation almost instantly and completely. With increasing phosphate concentrations complete inhibition took more and more time. At low phosphate concentrations the antibiotic not only interfered with adaptation, but also damaged the already functioning oxidative system.

According to other experiments, it is the phopsphate ions in the first place that antagonize streptomycin. Other electrolytes have a similar action, but at higher concentrations only.

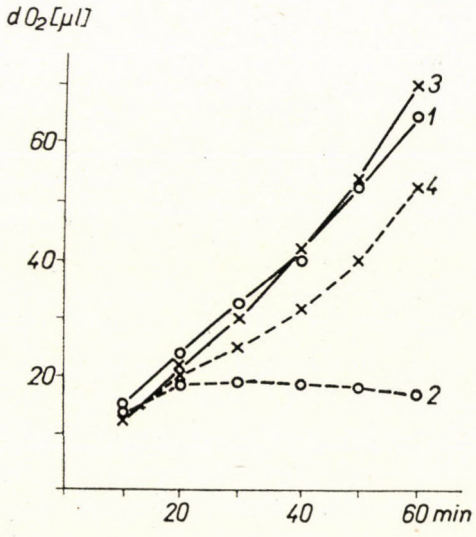

Fig. 4. Action of streptomycin at different pH values. O_2 uptake measured by the manometric method. Cell density: 0.97 mg/ml cellular solids.

Reaction mixture:
1, *3* : $2 \cdot 10^{-2}$ *M* phosphate buffer,8 μM/ml pyruvate,0.606 per cent casein hydrolysate
2, *4* : the former +8 μg/ml dihydrostreptomycin
pH of the medium:
1, *2* : pH 7.5
3, *4* : pH 6.9

In the experiments shown in Fig. 4 the effect of 8 μg/ml of dihydrostreptomycin in a pH 7.5 phosphate buffer (2×10^{-2} *M*) was compared with that in a pH 6.9 phosphate buffer. In the slightly alkaline medium 8 μg/ml of dihydrostreptomycin fully inhibited the adaptive development of the terminal oxidation system after a short latency, while at pH 6.9 inhibition was only partial and did not become complete during the experiment.

The effect of streptomycin was influenced also by the time at which it had come into contact with the cells after the onset of enzymatic adaptation. The significance of this time factor was especially obvious at low (bacterio-

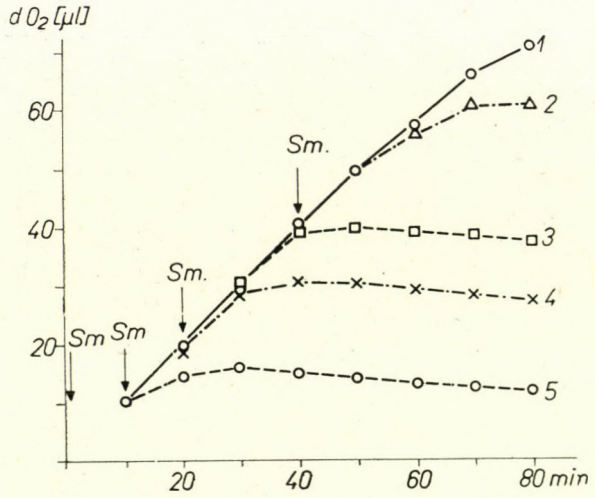

Fig. 5. Inhibitory action of streptomycin added at various points of time.

O_2 uptake measured by the manometric method
Cell density: 0.8 mg/ml cellular solids

Reaction mixture:
1 : 10^{-2} *M* pH 7.5 phosphate buffer, 8 μM/ml pyruvate, 0.0033 per cent casein hydrolysate
2, 3, 4, 5 : the former +8 μg/ml dihydrostreptomycin added at the times indicated by the arrows

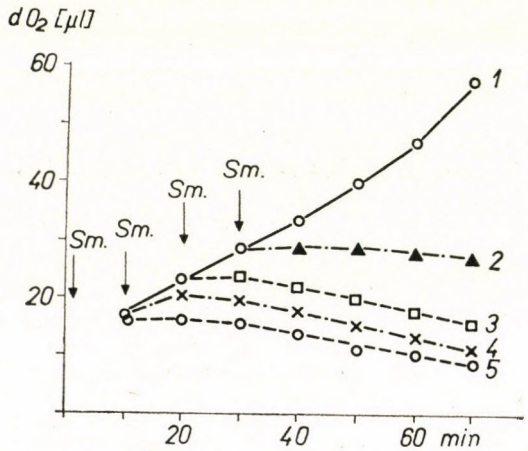

Fig. 6. Inhibitory action of streptomycin added at various points of time.

O_2 uptake measured by the manometric method
Cell density: 0.5 mg/ml cellular solids

Reaction mixture:
1 : 10^{-2} *M* pH 7.5 phosphate buffer, 8 μM/ml pyruvate, 0.066 per cent casein hydrolysate
2, 3, 4, 5 : the former + 33 μg/ml dihydrostreptomycin added as indicated by the arrows

static) concentrations. Let us compare Fig. 5 with Fig. 6 from this point of view. In these experiments the inhibitory action of 8 μg/ml (Fig. 5) and 33 μg/m (Fig. 6) of streptomycin in a medium containing 10^{-2} *M* of a pH 7.5 phosphate buffer was examined on the adaptive synthesis of the terminal oxidation system. The antibiotic was added to the reaction mixture at the points of time indicated by arrows. Eight μg/ml of streptomycin caused complete inhibition only when added to the cells at the beginning of the experiment, together with the substrate. When it was added after adaptation had been progressing for some time, inhibition began after a latency of 30 to 40 minutes. The increase of activity observed until complete inhibition set

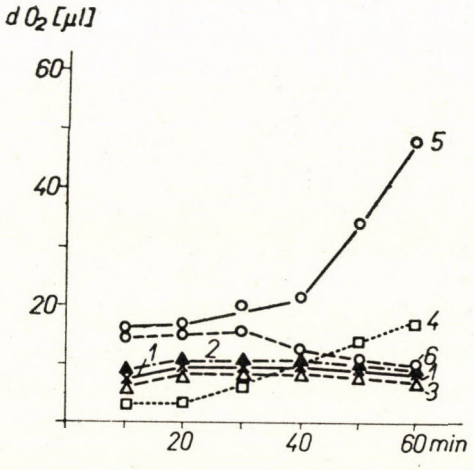

Fig. 7. Effect of streptomycin on the terminal oxidation of *E. coli* B

O_2-uptake measured by the manometric method
Cell density: 0.5 mg/ml cellular solids
Reaction mixture: 10^{-2} *M* pH 7.5 phosphate buffer containing
1 : 10 μM/ml pyruvate
2 : 10 μM/ml pyruvate +3.5 μM/ml fumarate
3 : 10 μM/ml pyruvate +3.5 μM/ml fumarate +17 μg/ml streptomycin
4 : 0.066 per cent casein hydrolysate
5 : 0.066 per cent casein hydrolysate +10 μM/ml pyruvate
6 : as 5. +17 μg/ml streptomycin

in depended on the length of the time between the onset of the experiment and the adding of the antibiotic.

Higher concentrations (Fig. 6) inhibited adaptation completely in a very short time, irrespective of the point of time at which streptomycin had been added to the reaction mixture: after a certain time 33 μg/ml of streptomycin in 10^{-2} *M* phosphate buffer damaged even the terminal oxidation.

Essentially the same results were obtained for the strain *E. coli* B, as illustrated by Fig. 7.

Discussion

Our experiments refute the view that streptomycin or dihydrostreptomycin (the two antibiotics act in the same way) would exert their bacteriostatic action by inhibiting the respiration of susceptible microorganisms, since bacteriostatic concentrations of streptomycin did not inhibit in the susceptible cells the oxidation of any of the substrates tested. Higher concentrations, especially when tested in a medium of low ionic strength, caused a partial inhibition of oxidation, but only after a latency of from 20 to 40 minutes. This indicates that the damage to the oxidative enzyme system is a secondary process, accompanying the dissociation of cell structure or the destruction of cells.

The inhibition of oxidation should be sharply differentiated from the inhibition of the adaptive synthesis of the terminal oxidation system in E. coli cells from semianaerobic cultures. This inhibition is demonstrable even before the oxidative system has been damaged and takes place at low streptomycin concentrations as well.

The experiments did not show clearly which enzyme or enzymes of the extremely complex terminal oxidative system are synthesized during the adaptation to pyruvate. The activity of terminal oxidation in bacterial cells is namely a most complex problem. In the increase of pyruvate oxidation a synthesis of a specific "permease" may also be involved. An adaptive synthesis of such permeases has been described in connection with the metabolism of the Krebs-cycle intermediary products [14, 15]. Although this possibility cannot be ruled out, our experiments tend to indicate that a synthesis of intracellular enzyme or enzymes is more likely in the adaptation process discussed. According to our computations, the oxidation of pyruvate in cells cultured under semianaerobic conditions reaches only the acetate stage, while in the actively oxidizing cells breakdown is more complete, most of the substrate being broken down to water and carbon dioxide.

The described inhibition of adaptation by streptomycin depends in the same way on the electrolyte concentration and pH of the environment, as the bacteriostatic action does. This fact suggests that the two phenomena are closely interrelated.

LITERATURE

1. Henry, J., Henry, R. J., Housewright, R. D., Berkman, S.: J. Bact. **54,** 9 (1947).
2. Henry, J., Henry, R. J., Housewright, R. D., Berkman, S.: J. Bact. **56,** 527 (1948).
3. Henry, R. J., Housewright, R. D., Berkman, S.: J. Bact. **57,** 447 (1949).
4. Geiger, W. B.: Arch. Biochem. **15,** 227 (1947).
5. Umbreit, W. W.: J. biol. Chem. **177,** 703 (1949).
6. Umbreit, W. W.: J. Bact. **66,** 74 (1953).
7. Smith, P. H., Oginsky, E. L., Umbreit, W. W.: J. Bact. **58,** 761 (1949).

8. Wyss, O., Smith, G. N., Hobby, G. L., Oginsky, E. L., Pratt, R.: Bact. Rev. **17,** 37 (1953).
9. Benham, R. S.: Science **105,** 69 (1947).
10. Wassermann, A. E.: Antibiotics & Chemotherapeutics **3,** 997 (1953).
11. Paine, T. F., Clark, L. S.: Science **118,** 73 (1953).
12. Paine, T. F., Clark, L. S.: Antibiotics & Chemotherapeutics **4,** 262 (1954).
13. Di Marco, A.: in Proceedings of the Fourth International Congress of Biochemistry, Vienna 1958. vol. V. Biochemistry of Antibiotics, Pergamon Press, London, 1959, p. 64.
14. Wheat, R. W., Rust, J., Ajl, S. J.: J. Cell comp. Physiol. **47,** 317 (1956).
15. Meadow, P., Clarke, P.: Biochem. J. **69,** 188 (1958).

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ON THE MODE OF ACTION OF STREPTOMYCIN

II. EFFECT OF STREPTOMYCIN ON THE β-GALACTOSIDASE SYNTHESIS IN E. COLI B CELLS

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When added together with the inducer to the reaction mixture, bacteriostatic concentrations of streptomycin completely inhibit the synthesis of β-galactosidase in *E. coli* B cells. When added later, streptomycin blocks the synthesis of the enzyme only after a latency period. The duration of the latency preceding complete inhibition and the amount of enzyme synthesized in the meantime depend on the duration of the interval between the addition of the inducer and the addition of streptomycin. When streptomycin is added 40 to 60 minutes after induction, the inhibition is often preceded by a paradoxical phenomenon, a transient increase in the rate of enzyme synthesis.

In the cells inhibited by streptomycin total protein synthesis continues linearly for a while after the addition of the antibiotic and complete inhibition results as late as 40 to 60 minutes.

Streptomycin inhibits the synthesis of cell proteins and nucleic acids at a practically equal rate. In the period of linear growth preceding complete inhibition the ratio nucleic acid/protein remained constant, at the level measured when adding the streptomycin, as opposed to the exponentially-growing control cells, in which the the ratio of nucleic acid to protein increases during the log phase.

In a previous paper [1] it was reported that bacteriostatic concentrations of streptomycin inhibited the adaptive synthesis of the terminal oxidation system in the cells of certain *E. coli* strains from semianaerobic cultures, without interfering with cellular respiration. A detailed analysis of these phenomena is rendered difficult by the complex structure of the synthesized enzyme system. For this reason we studied the synthesis of an inductive enzyme known for certain to be built up *de novo* of amino acids [2, 3] and the synthesis of which in *E. coli* is well known. Streptomycin has been reported to inhibit the synthesis of certain inductive enzymes [4, 9]. FITZGERALD and BERNHEIM [4—7], ERDŐS and TOMCSÁNYI [8] studied the action of streptomycin on the benzoic acid oxidase system of various apathogenic *Mycobacterium* strains. PERETZ and POLGASE [9] reported that under certain conditions streptomycin inhibits the synthesis of β-galactosidase.

In the present paper an account is given of the inhibition by streptomycin of β-galactosidase synthesis and of the effect of the antibiotic on the total protein and nucleic acid synthesis in the cells.

Methods

E. coli B was used as the test organism. The strains were cultured in Anderson's M9 medium [10], with the modification that glucose was replaced by the same weight of glycerol. Culturing took place in a horizontal shaker at 30° C. Cells from 13 to 16 hour cultures, as well as 3-hour "rejuvenated" cultures were used.

Induction of β-galactosidase was carried out with $5 \cdot 10^{-3}$ *M* lactose in media containing $10^{-3} - 5 \cdot 10^{-3}$ *M* glycerol, 10^{-4} *M* $MgSO_4$, and in some experiments also $5 \cdot 10^{-2}$ *M* NaCl as a supplement. The medium was adjusted to pH 7.5 with $2 \cdot 10^{-2}$ *M* to $6.7 \cdot 10^{-2}$ *M* phosphate buffer. Incubation was carried out in a water bath of 30° C, with shaking.

Streptomycin sulphate *(Pfizer Corp.*, Brussels, Belgium) was used in concentrations of 12 to 30 μg/ml.

The samples taken from the reaction mixture at various intervals were tested turbidimetrically for cellular solids and the cells lysed with toluol were tested for β-galactosidase activity by the method of LEDERBERG [11]. The quantity of the enzyme synthesized is given in units (U). One unit is the quantity of enzyme splitting off 1 mμ*M* o-nitrophenol in one minute at 30° C and pH 7.2.

Cellular total protein was estimated by the method of LOWRY *et al.* [12], after boiling for 15 minutes in 1 *N* NaOH solution.

Cellular nucleic acids were estimated spectrophotometrically, on the basis of absorption at 260 mμ, after suitable extraction with trichloracetic acid at 90° C.

The results were plotted either against time or were represented isometrically.

Results

In the experiments shown in Fig. 1 the β-galactosidase synthesis of 3-hour "rejuvenated" cells was studied, recording the inhibitory action of 20 μg/ml of streptomycin, added at various points of time during the induction. The upper part of Fig. 1, shown the amounts of enzyme synthesized in 1 ml reaction mixture, plotted against time. The lower part of the figure shows the growth curve of the cells. The 20 μg/ml dose of streptomycin caused an almost complete inhibition of enzyme production. At the same time, the cells continued to grow linearly for a while and complete inhibition of growth resulted only after 60 minutes. The same dose of antibiotic, when applied 20 minutes after induction caused complete inhibition only after 40 minutes. This inhibition went parallel with the inhibition of growth. When streptomycin was added 40 minutes after induction, enzyme synthesis, as compared with that of the controls, increased. It is known from other experiments that the initial increase resulting from the addition of the antibiotic is transient and is followed by a gradually-developing inhibition. If streptomycin is applied 40 to 60 minutes after induction, a transient increase occurs but there is no such transient increase in the total protein content or in the growth of the cells.

In the experiment shown in Fig. 2, the inhibitory action of 25 μg/ml streptomycin was examined in media containing different concentrations of phosphate. The inhibitory action of streptomycin added simultaneously with the inducer was not influenced by raising the concentration of the phosphate buffer from $2 \cdot 10^{-2}$ *M* to $6.7 \cdot 10^{-2}$ *M*. The inhibitory action of the antibiotic applied 60 minutes after induction was again preceded by an initial increase. The latency period preceding complete inhibition and the quantity of enzyme

synthesized until inhibition was complete depended on the phosphate ion concentration of the medium.

The experiments presented thus far show that the action of streptomycin on the synthesis of β-galactosidase does not run parallel with the inhibitory

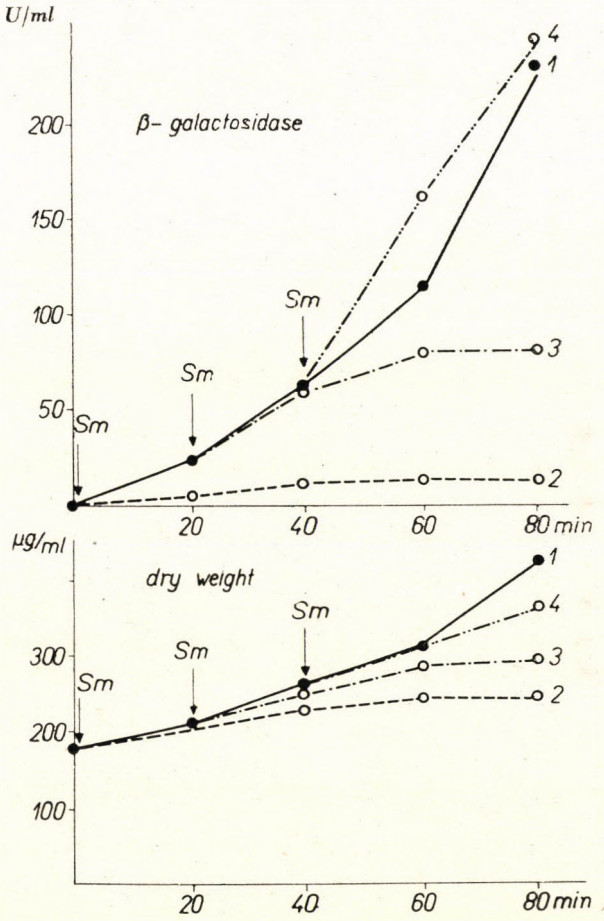

Fig. 1. Inhibition of β-galactosidase synthesis by streptomycin added at various points of time. Reaction mixture:

1: pH 7.5 phosphate buffer $6.7 \cdot 10^{-2}$ *M*, $MgSO_4$ 10^{-4} *M*, NaCl $5 \cdot 10^{-2}$ *M*, lactose $5 \cdot 10^{-3}$ *M*, glycerol $5 \cdot 10^{-3}$ *M*, casein hydrolysate 0.6 per cent
2: the former + streptomycin (20 µg/ml) at 0 min.
3: the same as in 1 + 20 µg/ml streptomycin at 20 minutes
4: the same as 1 + 20 µg/ml streptomycin at 40 min

action on growth. To clarify the conditions and relationships, we have studied side by side the course of enzyme synthesis, total protein and total nucleic

acid formation during induction, as well as the action of streptomycin on these processes. Such an experiment is illustrated in Fig. 3.

In that experiment cells from 13-hour culture were used. Inhibition was effected with 12 μg/ml of streptomycin. In Fig. 3*a* the quantities of β-galactosidase synthesized in 1 ml of the reaction mixture have been plotted against the time elapsed from the onset of induction, The antibiotic added simultaneously with the inductor almost completely-blocked enzyme formation. When streptomycin was added at 60 minutes, the gradually-developing inhibition

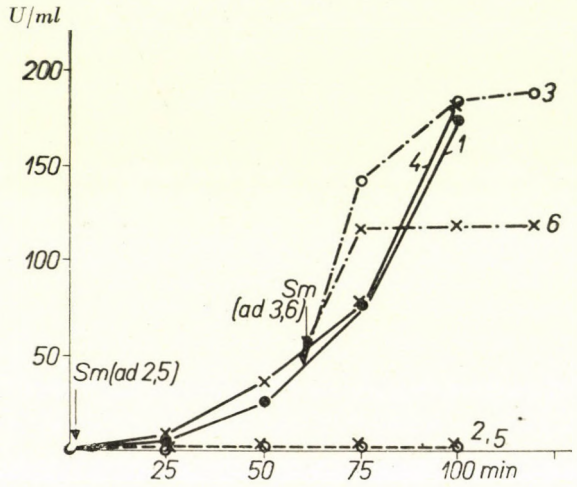

Fig. 2. Effect of streptomycin on the synthesis of β-galactosidase at various phosphate concentrations

Reaction mixture:
pH 7.5 phosphate buffer, $MgSO_4$ 10^{-4} *M*, lactose $5 \cdot 10^{-3}$ *M* glycerol 10^{-3} *M*
Inhibition with 25 μg/ml of streptomycin
Phosphate buffer concentrations:
1, *2*, *3* : $6.7 \cdot 10^{-2}$ *M*
4, *5*, *6* : $2 \cdot 10^{-2}$ *M*
Streptomycin was applied at the points of time indicated by the arrows

was preceded by an initial increase in the rate of synthesis. In Fig. 3*c* the total protein content of 1 ml of reaction mixture (curves 1, 2, 3) and the quantities of nucleic acid in the same volume (curves 1*a*, 2*a*, 3*a*) have been plotted in the same co-ordinate system. In the cells inhibited by streptomycin the total protein content was increasing linearly for a time after the addition of the antibiotic, at the same rate as at the moment of inhibition, independent of the time at which streptomycin had been added. At 40 to 60 minutes following the addition of the antibiotic protein synthesis ceased in the inhibited cells.

Fig 3*b* serves to facilitate comparison of the course of β-galactosidase and total protein synthesis. In this figure the total protein synthesized in 1 ml of the

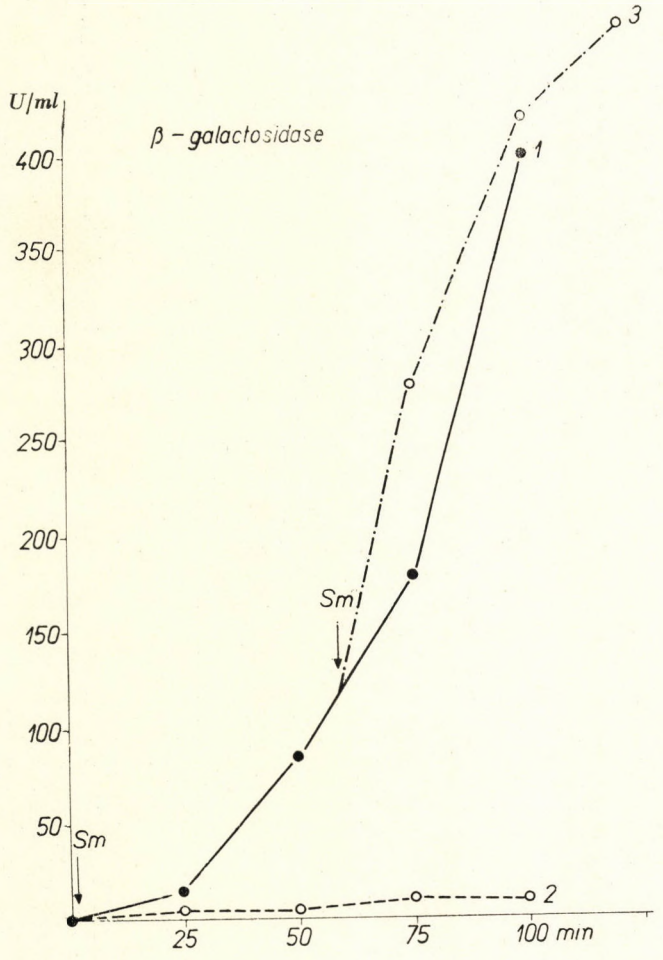

Fig. 3a

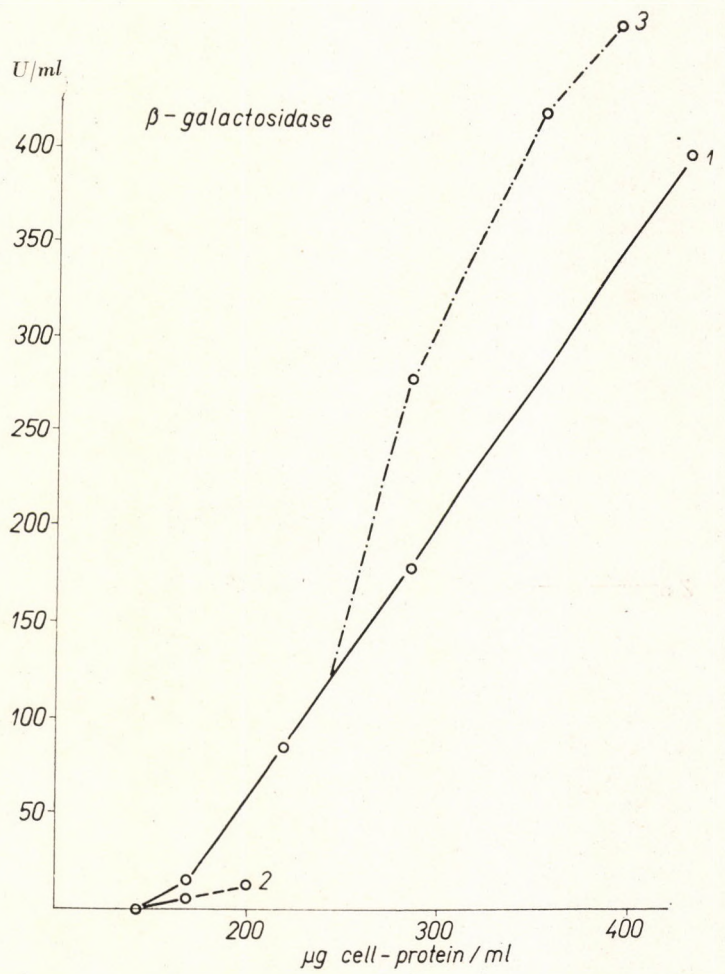

Fig. 3b

Fig. 3a, b, c. Effect of streptomycin the synthetis of β-galactosidase, total protein and nucleic acids

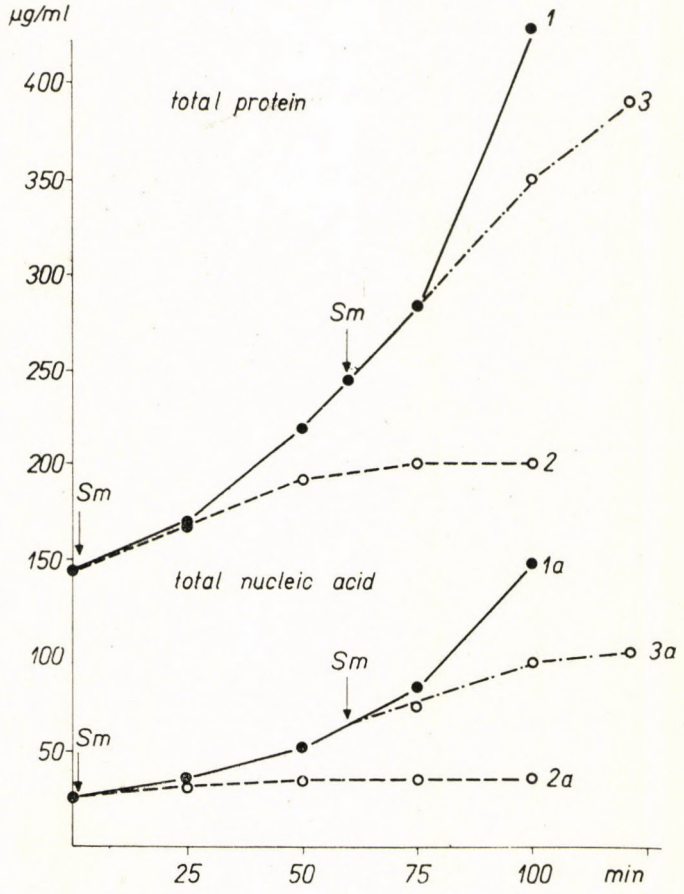

Fig. 3c

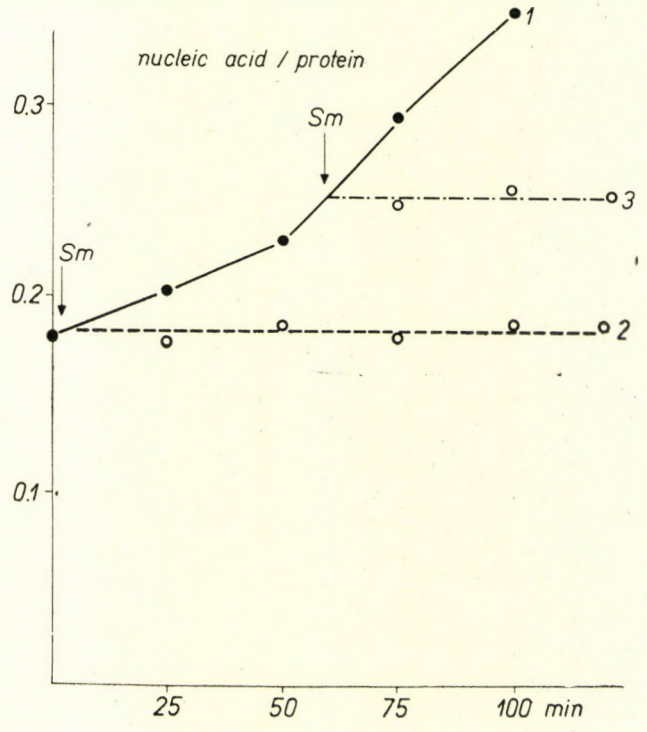

Fig. 3d

Fig. 3. a, b, c. Effect of streptomycin on the synthesis of β-galactosidase, total protein and nucleic acids

Reaction mixture:
pH 7.5 phosphate buffer $3 \cdot 10^{-2}$ *M*, $MgSO_4$ 10^{-4} *M*, lactose $5 \cdot 10^{-3}$ *M*, glycerol 10^{-3} *M*, casein hydrolysate 0.5 per cent
Inhibition with 12 µg/ml of streptomycin
1 : control
2 : control + streptomycin at 0 min.
3 : control + streptomycin at 60 min

reaction mixture has been plotted against the enzyme synthesized in the same volume and during the same time (isometric representation). After a short initial period a linear curve was obtained for the control cells. Streptomycin added together with the inducer brought about such an inhibition that the differential rate of enzyme synthesis became much lower than it was in the controls. Thus, streptomycin added simultaneously with the inducer inhibited the synthesis of β-galactosidase. When the antibiotic was added to the cells 60 minutes after induction, the inhibition of total protein synthesis preceded the inhibition of β-galactosidase synthesis. These results indicate that streptomycin inhibits the synthesis of the various cell proteins to a different extent.

The data in Fig. 3*c* make it also clear that nucleic acid synthesis continues for a while after streptomycin has come into contact with the cells. Fig. 3*d* shows the nucleic acid/protein quotient for the identical points. In the exponentially-growing control cells the value of this quotient increased with time. Under the influence of streptomycin this shift in the value of the quotient did not take place: the nucleic acid/protein quotient was fixed at the level measured when adding the antibiotic. As it has already been mentioned, this is accompanied by the growth turning linear.

Discussion

On the basis of our experiments we do not consider streptomycin to be a drug specifically inhibiting protein synthesis. Following the addition of streptomycin in bacteriostatic concentrations the synthesis of total proteins ceases gradually. The total inhibition of protein synthesis is preceded by a linear phase. Essentially the same applies to the synthesis of β-galactosidase, if the inhibition starts after synthesis has begun.

In the cells exposed to streptomycin the resynthesis of nucleic acids continues for a time and nucleic acid synthesis is inhibited at about the same rate as the synthesis of protein. This observation is at variance with that made by ANAND, DAVIS and ARMITAGE [14] who found that streptomycin blocked nucleic acid synthesis. The divergence might be due to differences in the concentrations of the antibiotic: the authors cited applied streptomycin at a concentration as high as 60 μg/ml.

We therefore consider the inhibition of protein and nucleic acid synthesis to be a secondary effect, because, according to the literature [14], streptomycin is bound to the surface of susceptible cells at an extremely fast rate.

The inhibition of enzyme induction should be separated from that of protein synthesis. The former process is inhibited by a short exposure to low concentrations of streptomycin. Unfortunately, little is known of the process of induction and as long as its mechanism is not clarified, it will not be possible unequivocally to interprete this very early streptomycin effect.

The inhibitory actions discussed suggest a remarkable similarity between the action of streptomycin and the effect of agents interfering with the metabolism of desoxyribonucleic acids.

LITERATURE

1. ZABOS, P.: Acta physiol. hung. In the press. (I).
2. HOGNES, D. S., COHN, M., MONOD, J.: Biochem. biophys. Acta **16**, 99 (1955).
3. ROTMAN, B., SPIEGELMAN, S.: J. Bact. **68**, 419 (1954).
4. FITZGERALD, R. J., BERNHEIM, F.: J. Bact. **54**, 671 (1947).
5. FITZGERALD, R. J., BERNHEIM, F.: J. Bact. **55**, 765 (1948).
6. FITZGERALD, R. J., BERNHEIM, F.: J. biol. Chem. **172**, 845 (1948).
7. FITZGERALD, R. J., BERNHEIM, F., FITZGERALD, D. B.: J. biol. Chem. **175**, 195 (1948).
8. ERDŐS, T., TOMCSÁNYI, A.: Acta physiol. hung. **12**, 310 (1957).
9. PERETZ, S., POLGASE, W. J.: Antibiotics Annual 1956/57. p. 533.
10. ANDERSON, E. H.: Proc. nat. Acad. Sci. (Wash.) **32**, 120 (1946).
11. LEDERBERG, J.: J. Bact. **60**, 381 (1951).
12. LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L., RANDALL, R. J.: J. biol. Chem. **193**, 265 (1951).
13. MONOD, J., PAPPENHEIMER, A. M., JR., COHEN-BAZIRE, G.: Biochem. biophys. Acta **9**, 648 (1952).
14. ANAND, N., DAVIS, B. D., ARMITAGE, A. K.: Nature (Lond.) **185**, 22 (1960).

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A SEMIAUTOMATIC APPARATUS FOR MAKING GLASS MICROELECTRODES

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A semiautomatic apparatus has been constructed for making glass capillaries of 0.25 to 1.5 μ tip diameter from glass capillaries of different initial diameters. The method employed is very simple and may be adopted by any laboratory. The procedures of preparation, filling and storing are described in detail.

Recording the electrical activity of a single cell has become increasingly important in neurophysiological research, with the purpose of gaining insight into the mechanism of nervous functions. Such investigations require the use of microelectrodes. The tip of these glass capillaries varies from 0.5 —1 μ lest they damage the cells. LING and GERARD [1] were the first to use such microelectrodes and since then the use of glass capillaries filled with a 3 *M* KCl solution has become widespread.

At first, the electrodes were prepared by hand, as recommended by LING and GERARD, but this required great skill and patience. To eliminate this difficulty, ALEXANDER and NASTUK [2] constructed an automatic electrode drawer. Their apparatus was rather complicated and uniform electrodes could be prepared exclusively from glass capillaries of identical initial size. We hawe therefore developed a method by which electrodes with almost identical tips can be prepared from glass capillaries of various initial size.

Description of the apparatus

By means of screws, a Bunsen tripod is so adjusted that its shaft be in the vertical position (Fig. 1). Three holders are applied on the shaft that may be brought in any desired position. The upper holder catches the capillary, the one in the middle holds a heatable platinum wire 1 mm in diameter, and the lower one holds an electromagnet (12 000 windings, 2500 ohm, internal diameter 16 mm). This holder system must be so set that the capillary on the upper holder (which may swing freely in any direction) should pass exactly in the centre of the platinum loop and the electromagnet (Fig. 2).

The platinum wire is heated by a transformer. The electromagnet is fed from an anode battery converting AC from the mains to stabilized DC. The wiring diagram is shown in Fig. 3.

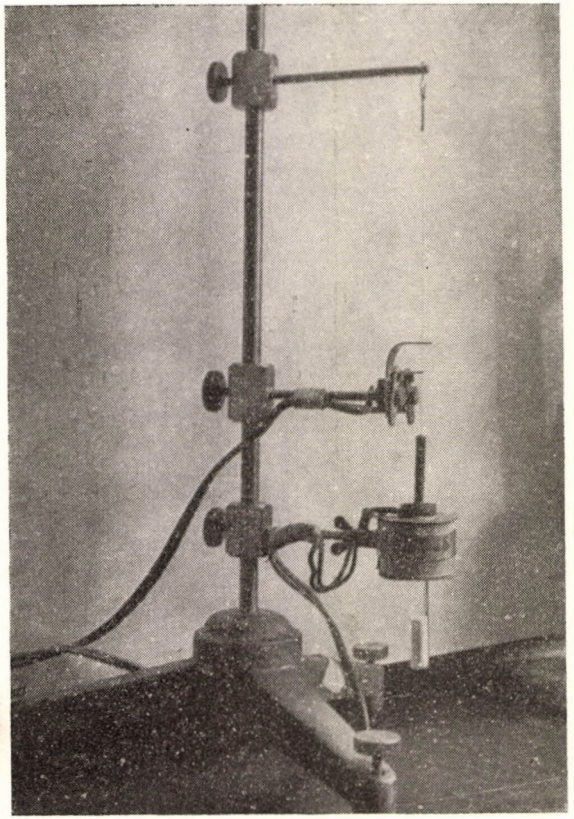

Fig. 1. The apparatus for preparing microelectrodes

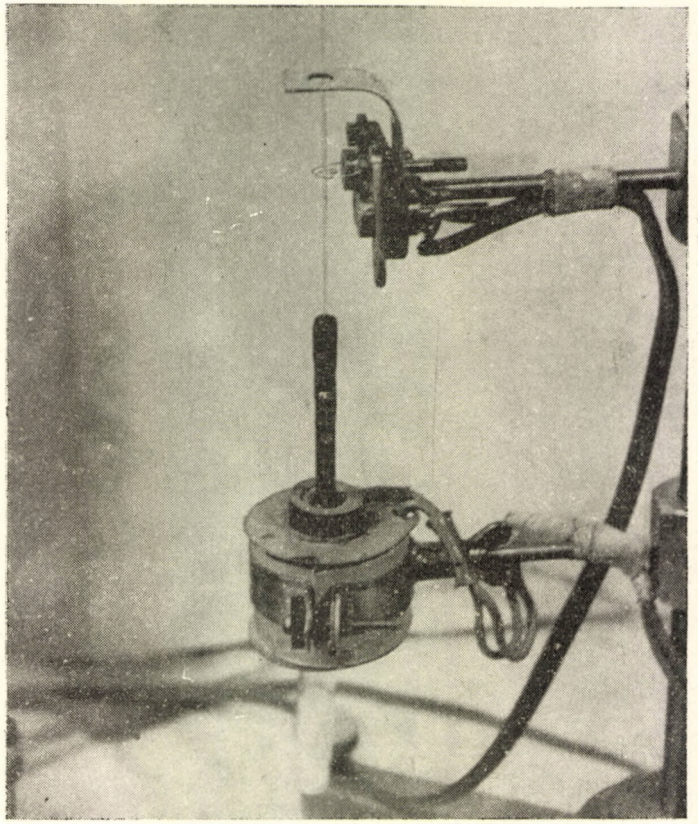

Fig. 2. Position of the glass capillary between platinum wire and electromagnet. The metal plate bent at rectangles above the platinum wire prevents melting of the Mendelejeff paste

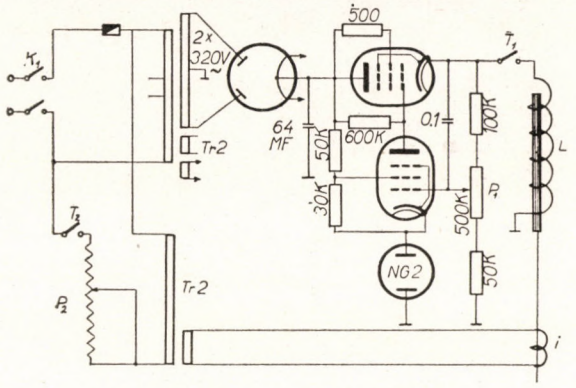

Fig. 3. Wiring diagram of the microelectrode maker apparatus

Preparation of glass capillaries

A pyrex or rasotherm glass capillary, measuring 15 cm in length and 0.8 to 1.5 mm in diameter, with a wall about 0.1 mm thick, is attached to the upper holder by means of sealing-wax, and is directed through the centre of the platinum wire loop, and than an iron cylinder about 6 cm long and weighing 9 g is attached to its end. The bore in the cylinder is filled with sealing-wax to hold the capillary. The iron cylinder hangs about 1.5 cm deep in the hole in the centre of the electromagnet. The glass capillary must not deviate from its vertical position even when the magnet is switched on. This is readily achieved by turning the platinum loop or the magnet. Then the motionless capillary is indirectly heated by the platinum loop. During heating the capillary gradually lengthens by about 5 to 6 mm. Then heating is terminated and the electromagnet is switched on. Subsequently, the platinum loop is heated again, this time with a lower voltage. In about 30 seconds the capillary begins to lengthen faster and faster is response to the pulling force, until it is drawn apart into two parts. The lower half drops through the centre of the magnet into a glass tube which is filled with cotton wool to prevent breaking of the capillary. The capillary is separated from the iron cylinder by melting the sealing wax and is examined under the microscope. It is fit for use and has sufficient strength if the original 1.5 mm diameter is tapering off to 0.5—1 μ in a length of about 1 cm, thinning from 25 μ to 1 μ within a length of about 1 mm. With capillaries of different initial thickness it is advisable to change not the pulling force, but the grade of heating when the electrode is pulled. In our experiments 9.5 to 11 A was required to heat the platinum wire. With little practice 3 or 4 suitable glass electrodes can be prepared from a 15 cm long capillary. There is no difference between rasotherm and pyrex capillaries.

Filling of the capillaries

The capillaries are filled with 3 *M* KCl by the somewhat modified method of LING and GERARD [1]. The 3 to 4 cm long capillaries are placed close to one another on a heat-resistant glass tube measuring 1.5 cm in diameter, and are held in place with their tip downward by means of a rubber ring. This is followed by boiling in a 2.5 *M* KCl solution. Boiling is continued until no bubbles leave the capillaries any longer. Meanwhile (in about 20 to 30 minutes) the concentration of the KCl solution increases to 3 *M*. When the capillaries begin to cool they are filled up with the solution. A China filtre is used to make boiling even. During boiling the dish should be covered. We have found this method suitable for filling capillaries with tips measuring 0.25 to 2 μ.

Capillaries thicker than that will fill spontaneously by capillarity. Filling takes place especially fast when use a tuberculin syringe with a No. 20 cannula. This way the filling by capillarity is restricted to length of about 3 mm, and is complete in 2 or 3 minutes.

Storage of electrodes

The electrodes are stored usually in the dry state; filled electrodes are stored in a closed exsiccator containing 3 *M* KCl solution. We have devised a plexiglass holder for this purpose. Two plexiglass discs 6 cm in diameter and 5 mm thick each are held together by a 6 cm long shaft in the centre, at a distance of about 5 mm. The lower disc is absolutely smooth, in the upper one there are 2 mm bores, 3 to 4 mm from one another, in concentrical order; the capillaries are placed in these bores. The discs are then submerged in 3 *M* KCl, so that the tip of the capillaries should emerge from the solution. Before use, the electrodes are cleaned with distilled water or physiologic saline.

LITERATURE

1. LING, R., GERARD, R. W.: J. Cell. comp. Physiol. **34**, 383 (1949).
2. ALEXANDER, J. T., NASTUK, W. L.: Rev. Sci. Instr. **24**, 528 (1953).

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ADRENOCORTICAL FUNCTION IN THE RAT

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The adrenal venous blood of the rat contains the following corticoids: 6-α-hydrocortisol, aldosterone, a non-identified steroid showing a positive sodium fluorescence but not giving the tetrazolium reaction, corticosterone, 11-dehydrocorticosterone, Δ^4-adrostene-11-ol-3,17-dione and Δ^4-androstene-3,17-dione. By the sensitive tetrasolium reduction method the authors determined the corticoid content of the adrenal venous blood obtained from each animal in 12-minute periods, as well as the aldosterone secretion from the pooled adrenal venous bloods obtained from several animals.

In recent years the rat's adrenocortical function has been studied extensively and it has become clear that in that species the adrenal cortex secretes mainly corticosterone [1, 2, 3]. Some authors mentioned the presence of hydrocortisone, but these claims could not be corroborated [4, 3]. Moreover, 11-dehydrocorticosterone, a polar corticoid of unknown structure, as well as androstenedione have been described to occur in the adrenal venous blood of the rat [2]. The presence of aldosterone was first observed by SINGER and STACK-DUNNE [4].

In the present paper the corticoid pattern of the rat's adrenal venous blood and its quantitative determination are described.

Methods

Eighty albino rats from the same stock, of either sex, weighing approximately 150 to 220 g each, were used. Blood from the left adrenal vein was collected under hexobarbital sodium anaesthesia (10 mg/100 g body weight intraperitoneally), by means of a glass cannula connected with a polyethylene tube. Prior to the insertion of the cannula 5.0 mg/100 g body weight heparin in 1.0 ml physiologic saline had been injected intravenously. During the 60-minute blood collection periods the temperature of the animals was maintained.

Adrenal venous blood was extracted with ether and ethyl acetate, as already described [5]. Haemolysis was brought about with distilled water, then after uniting the 4 : 1 mixture of ether-ethyl acetate of twice the same volume the organic phase was washed with $N/10$ Na_2CO_3 and with $^1/_5$ volume of distilled water, respectively. The residue obtained in vacuum was partitioned between benzene and 70 per cent ethanol, then the residue of the alcoholic phase was dissolved in 1.0 ml ethyl acetate.

Paper chromatographic separation. A complete separation of all of the corticoids required the use of two methods. The steroids less polar than hydrocortisone were separated in a benzene-formamide system [6], while those of higher polarity (including aldosterone) were separated and assayed quantitatively in a modified Bush B_5 system (benzene-methanol-water 14 : 6 : 5). With the formamide-benzene impregnation method the specimens were

run 4 hours with no previous exposure. In the benzene-methanol-water system, after equilibration for 8 hours, the running time changed according to the actual purpose. The quantitative determination of corticosterone requires 4 hours of running, while the separation of aldosterone, or the observation of compounds more polar than hydrocortisone needs 16 hours of running. In this case the solvent dripping off and containing the steroids less polar than cortisone was taken up in china or glass dishes of 10 to 12 ml capacity.

The paper used was *Schleicher-Schüll* 2043 b, previously washed for days in ethylacetate and ethanol. With the impregnation method 2 cm wide, with the benzene-methanolic water system 4 cm wide and 52 cm long strips were used.

The steroids dissolved in a small volume were applied by the usual 0.01 ml micropipette. Considering that for the determination of aldosterone, and for the demonstration of steroids present in traces extracts from several animals had to be pooled, the steroid extract dissolved in 1—2 ml of the solvent (as a reduction of volume without loss is difficult) was applied by prechromatography (BUSH [7]). The procedure, as modified by us, was as follows. Eight cm from one end of the 4 cm wide paper strip the start line is marked and 0.2 or 0.3 of the test material is dropped on that area, taking care lest the solvent reach the end of the paper or the start line. The ethylacetate evaporates rapidly on the large surface; its evaporation may be accelerated by a stream of hot air. Subsequently the start line ends of the strips are dipped into a dish containing a 1 to 2 mm thick layer of ethyl acetate. The corticoids migrate in the front line of the ascending solvent; this is continued until the front line has reached the previously marked start line. Prechromatography experiments with standard corticoids showed that all corticoids were to be found in a few mm wide zone corresponding to the front line. The procedure takes 1 to $1^1/_2$ hours to perform.

Quantitative assay of aldosterone and corticosterone

Aldosterone was determined by running for 16 hours in a benzene-methanol-water system. In this case the aldosterone was found between hydrocortisone and cortisone 0.5 R_f Cpd F—E. The distance between hydrocortisone and cortisone varied from 12 to 16 cm.

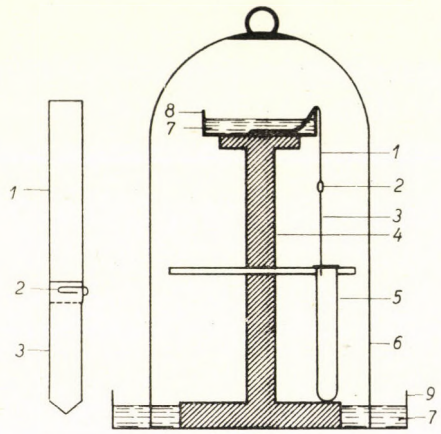

Fig. 1. Diagrammatic representation of the micro-elution apparatus.
1 : guiding paper strip, *2 :* paper-clip, *3 :* paper strip to be eluted, *4 :* rack, *5 :* Wassermann-tube, *6 :* glass cover, *7 :* absolute ethanol, *8 :* glass tube, *9 :* glass dish

The zone 3 cm upward and 3 cm downward from the R_f corresponding to aldosterone was cut out and assayed by the microtetrazolium method after microelution, as described later. A similar procedure was used for the quantitative determination of corticosterone, after 4 hours of running. In both cases the R_f was determined on the basis of free aldosterone and corticosterone, respectively.

Microelution of corticoids. The elution of steroids from the paper chromatograms by the usual methods requires a considerable volume of solvent, is time-consuming and causes a loss of steroids. Moreover, for quantitative assay with this method the small amount of

steroid must be dissolved in a large volume of solvent. On the other hand, the microelution method makes it possible to elute the separated steroids in 0.3 to 0.5 ml of solvent.

Elution is effected in ethanol atmosphere, into reagent tubes measuring 12×0.5 cm. The apparatus used is shown in Fig. 1. The strip cut from the paper chromatogram is attached by means of a paper-clip to a so-called guiding paper 6 to 8 cm long and of the same width, 3 or 4 cm of which is placed into the dish filled with ethanol. Like in descending chromatography, the ethanol reaches the paper strip used for elution, then, concentrating the steroid on the paper in the front line, collects it into the reagent tube. Elution is complete in the first 2 or 3 drops of ethanol already; we usually end the experiment after 0.5 ml of the eluate has collected. The process of elution may be followed up by the use of a paper strip on which one drop of 1 per cent Sudan III solution in alcohol has been placed. With ethanolic elution hydrocortisone, cortisone, corticosterone and aldosterone behaved in the same way and 0.5 ml of the ethanolic eluate contained all of the steroids. (Fig. 1.) *Tetrazolium blue assay of small quantities of corticoid.* For the tetrazolium blue reduction micromethod, the standard steroids are diluted in 1.5 ml of absolute ethanol, in the concentration range of from 0.5 to 5.0 μg. Subsequently, 2.0 ml $CHCl_3$, 0.2 ml of 0.25 per cent tetrazolium blue in ethanol and 0.1 ml NaOH are added to the tubes. The alkali reagent contains 8 g of NaOH dissolved in 100 ml of distilled water and 200 ml of absolute ethanol, added after dissolution. The solution should be freshly prepared every fortnight. Six minutes after adding the alkali photometric determination is done at 520 mμ, in a 5 cm curvette. The calibration values related to hydrocortisone are presented in Table I.

Table I

Hydrocortisone in μg/3.8 ml solution	Optical density at 520 mμ		
1.0	0.150	0.150	0.160
2.0	0.290	0.300	0.320
3.0	0.460	0.450	0.470
4.0	0.610	0.600	0.620
5.0	0.770	0.760	0.750

Results

Corticoids in adrenal venous blood. To demonstrate presence of steroids secreted in traces by the adrenal cortex, extracts of the adrenal venous blood collected of 60 minutes from at least 6 animals had to be pooled. The pooled extracts were chromatographed partly in formamide-benzene, partly in benzene-methanol-water. The corticoids separated in the two systems were demonstrated by tetrazolium blue reduction, sodium fluorescence and the m-dinitrobenzene reaction. Identification, taking into consideration the R_f values of the standard compounds, was tentative. The pooled extracts of adrenal venous blood from at least six animals were shown to contain the following steroids:

1. 6-α-hydroxycortisol
2. aldosterone
3. compound X, not identified
4. corticosterone

5. 11-dehydrocorticosterone
6. Δ^4-androstene-11-ol-3,17-dione
7. Δ^4-androstene-3,17-dione

The chromatographic behaviour of the single compounds is shown in Fig. 2. 6-hydroxicortisol and aldosterone were present in the smallest quantities, while the main compounds were compound X and corticosterone. Both can be assayed quantitatively in adrenal venous blood collected for 12 minutes.

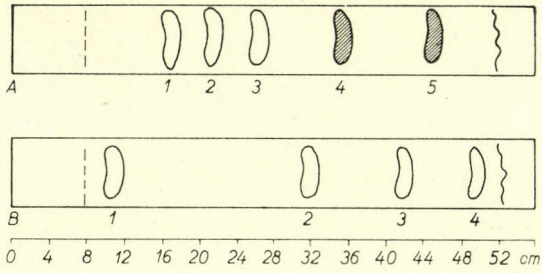

Fig. 2. Diagrammatic representation of the corticoid pattern in the rat's adrenal venous blood after separation by paper chromatography.
A : formamide—benzene system, *1:* Cpd X, *2:* corticosterone, *3:* 11-dehydrocorticosterone *4 :* Δ^4-androstene-11-ol-3,17-dione, *5 :* Δ^4-androstene-3,17-dione
B : benzene—methanol—water system, *1:* 6-α-hydroxy-cortisol, *2:* standard hydrocortisone, *3 :* aldosterone, *4 :* standard cortisone

Tetrazolium blue reduction and sodium fluorescence were observed simultaneously only in the case of 6-hydroxicortisol, aldosterone, corticosterone and 11-dehydrocorticosterone. The unidentified compound X did not reduce tetrazolium, but gave a positive sodium fluorescence test. In the range of from 10 to 20 μg, compound X showed no m-dinitrobenzene reaction. The 17-ketosteroids were positive by both the alkali fluorescence and m-dinitrobenzene tests.

Compound X is always present in adrenal venous blood and can completely be separated from corticosterone only in the formamide—benzene system. In the benzene—methanol—water system compound X moves but slightly slower than corticosterone so that it cannot be perfectly separated from the latter (Fig. 2).

Quantitative distribution of corticoids in adrenal venous blood

As the data in Table II indicate, corticosterone and compound X were present in the largest quantities, as computed for 100 g of body weight and per hour. The 17-ketosteroids and 11-dehydrocorticosterone could also be evaluated quantitatively for single animals. To determine the quantities of aldosterone and 6-hydroxycortisol adrenal venous blood had to be collected for 60 minutes and the bloods from several animals had to be pooled.

Table II

Amount of corticosteroids in adrenal venous blood μg/100 g body weight/hour	
Corticosterone (25)	6.1
Compound X (25)	3.05
11-dehydrocorticosterone (12)	1 to 3 μg
Δ^4-androstene-3,17-dione	on several occasions 1 to 5 μg
Δ^4-androstene-11-ol-3,17-dione	in several tests 1 to 5 μg
6-α-hydroxycortisol	less than 0.5 Δg
Aldosterone (15) μg/kg/hour	0.87

Corticosterone and compound X obtained from the adrenal venous blood of single animals were determined by the sodium fluorescence and tetrazolium reduction tests in the following way. 0.1 ml of the extract dissolved in 1.0 ml ethyl acetate was chromatographed, partly in formamide—benzene and partly in benzene—methanol—water. In the first system the sodium fluorescence method, in the other the tetrazolium blue reaction was used, the latter after micro-elution. Repeated trials showed the results obtained with the two solvent systems and by the two methods to be in satisfactory agreement.

Table III

No.	Corticosterone content of adrenal venous blood μg/100 g body weight/hour	
	Sodium fluorescence test	Tetrazolium blue reaction
1	14.5	12.5
2	13.0	12.5
3	11.8	10.0
4	16.8	15.5
5	13.6	12.5

Considering that aldosterone was present in small quantities, the extracts of adrenal venous blood collected for 60 minutes from 15 rats were pooled. The extract was chromatographed in benzene—methanol—water for 16 hours, then the 6 cm area corresponding to the place of aldosterone was eluted and the aldosterone was determined by the tetrazolium blue reaction. During the 16 hours of chromatography the corticosterone and the solvent had been dripping into the dishes under the strips (2 to 3 ml); after suitable dilution with ethanol, the corticosterone content was determined after repeated running. Table II shows the secretion values for aldosterone, in μg/kg body weight/hour.

Discussion

It has been found that rats under hexobarbital anaesthesia secreted determinable quantities of adrenal cortical hormones even when blood was collected in 10 to 15 minute periods. Unlike in most mammals, the adrenal venous blood of the rat does not contain hydrocortisone, but the animal is capable of secreting 6-*a*-hydroxycortisol in minute amounts. A non-identified steroid is present in considerable quantities; its concentration may attain that of corticosterone.

We found aldosterone secretion to be less than that reported by SINGER and STACK-DUNNE [4] and we were unable to find evidence of hydrocortisone secretion. Differences between the strains of rats may be responsible for this divergence in results.

As it has been observed earlier [5], less corticoid is secreted under general anaesthesia than in the normal state, without surgical intervention. Similarly, the corticoids in adrenal venous blood can be related qualitatively to the resting state. Certain specific stressor effects, the action of other peripheral endocrine organs on the pituitary—adrenocortical system may alter the corticoid pattern and in the adrenal venous blood of the rat steroids may appear which do not occur under normal conditions. For example, hydrocortisone secretion was demonstrable in rats following the administration of calf hypophysis extract, but not after treatment with purified ACTH preparation [8]. Similar observations have been made in other species as well, and the phenomenon has been suggested to involve changes in the enzyme system of the adrenal cortex.

LITERATURE

1. BUSH, I. E.: Ciba Foundation Colloquia on Endocrinology, Churchill, London, 1953.
2. REIF, I. E., LONGWELL, B. B.: Endocrinology **62,** 572 (1958).
3. WEISZ, P., GLÁZ, E., VARENSZKY, SZ.: Orv. Hetil. **94,** 1194 (1953).
4. SINGER, B., STUCK-DUNNE, M. P.: J. Endocrinol. **12,** 131 (1955).
5. ENDRŐCZI, E., LISSÁK, K.: Acta physiol. hung. **15,** 25 (1959).
6. BURTON, R. B., ZAFFARONI, A., KEUTMAN, E. H.: J. biol. Chem. **188,** 763 (1951).
7. BUSH, I. E.: Biochem. J. **50,** 370 (1952).
8. ENDRŐCZI, E.: Unpublished data, 1960.

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THE EFFECT OF WORK PERFORMED IN HYPOTHERMIA AND HYPERTHERMIA ON PITUITARY—ADRENOCORTICAL FUNCTION

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Albino rats were made to swim in water of different temperatures to study the effect of complete exhaustion on the corticoid pattern of the adrenal venous blood and on the hormone synthesis by adrenal tissue incubated *in vitro*. At the optimum water temperature swimming had practically no effect on the corticosterone content, while swimming in water of 18°C caused its reduction. On the other hand, the aldosterone content increased markedly on swimming at 30°C, 18°C and 43°C, and was the highest at the low environmental temperature. Compound X, invariably present in adrenal venous blood, behaved like corticosterone. Corticosterone synthesis *in vitro* was increased even when the adrenal venous blood value did not change or decreased.

Rats in hypo- or hyperthermia have been found to show much poorer performance than at an optimal environmental temperature [1, 2]. These observations revealed that over a certain critical temperature ACTH was no longer capable of influencing performance, presumably in consequence of an insufficiency of adrenocortical synthesis.

In this paper experiments are described, in which rats were made to swim in water at different temperatures, in order to determine the effect of swimming on corticoid secretion.

Methods

A total of 60 albino rats of either sex, weighing 150 to 180 g each, in groups of 15 each, were used. The collection of adrenal venous blood, the estimation of corticoids and aldosterone have been described in detail [3]. The animals were forced to swim in a 45 cm deep vessel holding 50 litres. The temperature of the water was 30°, 18° and 43°C, respectively, with a maximum variation of ± 0.5°C. The animals were subjected to study within 70 minutes after swimming. Hexobarbital sodium (10 mg/100 g body weight) intraperitoneally was used for anaesthesia. Blood clotting was prevented by injecting heparin (5 mg/100 g body weight, 1 mg = 84 U.) into the saphenous vein.

After collecting adrenal venous blood for 60 minutes, the adrenals were removed, weighed and incubated under constant oxygenation for 120 minutes, in a medium containing for every 100 mg adrenal tissue 6 ml of Tyrode's solution, nicotinic acid amide 20 mg, dextrose 6 mg, ATP 2 mg and ACTH (*Organon*, Oss, Holland) 4 I. U. After incubation the solution was centrifuged, then both the supernatant and the tissue were extracted with ether-ethyl acetate, 4 : 1 (using an equal volume with the fluid and 20 volumes with the tissue). The solvents were pooled and prepared for corticoid extraction paper chromatography [3].

The duration of swimming from start until complete exhaustion, and the rectal temperature were recorded.

Results

Adrenocortical activity after swimming at 30°C

The duration of swimming at 30°C was 108 minutes; during this time rectal temperature decreased to 30.9°C. There was no difference from the controles in the corticosterone level of adrenal venous blood, but the corticosterone synthesis of the adrenal tissue increased by 405 per cent. Compound X, a substance always present in the adrenal venous blood of rats and which does not give the tetrazolium blue reaction but can be determined on the basis of sodium fluorescence [3], differed in behaviour from corticosterone. Its level in adrenal venous blood decreased by 40 per cent during swimming, but the adrenal tissue synthesized more of it. It is noteworthy that only traces of compound X occurred in the normal rats which did not swim.

The level of aldosterone in adrenal venous blood was slightly increased after swimming at 30°C. At the same time, aldosterone synthesis in the adrenal tissue increased by 44 per cent.

These results are presented in Table I.

Table I

	Control	Swimming at 30°C	Percentage change
Number of rats	15	15	—
Body weight, g	153	146	−4.5
Adrenal weight, mg/100 g	21.7	23.3	+7.0
Duration of swimming, minutes	—	108	—
Rectal temperature after swimming, °C	37.4	30.9	−17.0
Steroid level of adrenal venous blood, μg/100 g/hour			
Corticosterone	6.1	6.38	+4.5
Compound X	3.05	1.82	−40.3
Aldosterone, μg/kg/hour	0.87	1.05	+20.7
Steroid content of the incubated adrenal tissue, μg/g			
Corticosterone	20.0	101.0	+405
Compound X	—	195.0	—
Aldosterone	3.6	5.2	+44

Adrenocortical activity after swimming at 18° C

The corticosterone content of adrenal venous blood decreased by 34 per cent. At the same time corticosterone synthesis increased by 610 per cent. A significant decrease took place in the secretion of compound X, while the adrenal tissue it was synthesized in considerable quantities. Unlike after swimming at 30°C, aldosterone secretion increased by 360 per cent, and a similarly significant increase was observed in aldosterone synthesis.

Table II

	Controls	Swimming at 18°C	Percentage change
Number of rats	15	15	—
Body weight, g	153	149	−2.6
Adrenal weight, mg/100 g	21.7	26.4	+21.6
Duration of swimming, minutes	—	9	—
Rectal temperature after swimming, °C	37.4	21.5	−42.5
Steroid level of adrenal venous blood, μg/100 g/hour			
Corticosterone	6.1	4.0	−34.4
Compound X	3.05	1.34	−56.0
Aldosterone, μg/kg/hour	0.87	4.0	+360.0
Steroid content of incubated adrenal tissue, μg/g			
Corticosterone	20.0	142.0	+610.0
Compound X	—	44.0	—
Aldosterone	3.6	4.8	+33.0

In agreement with our earlier results [1, 2] the animals could swim at 18°C for much less time than at the optimum temperature. The rectal temperature was 21.5°C.

Adrenocortical activity after swimming at 43°C

After swimming in water of 43°C, corticosterone secretion increased slightly, while there was a significant increase in corticosterone synthesis. The secretion of compound X became less, while its synthesis by adrenal tissue, like in the controls, was not observable. Aldosterone secretion increased significantly, while aldosterone was practically unchanged.

Table III

	Controls	Swimming at 43°C	Percentage change
Number of rats	15	15	—
Body weight, g	153	161	+6
Adrenal weight, mg/100 g	21.7	25.0	+15.2
Duration of swimming, minutes	—	10.0	—
Rectal temperature after swimming, °C	37.4	41.6	+11.0
Steroid level of adrenal venous blood, μg/100 g/hour			
Corticosterone	6.1	7.43	+21.8
Compound X	3.05	1.24	—56.0
Aldosterone, μg/kg/hour	0.87	2.77	+218.0
Steroid content of incubated adrenal tissue, μg/g			
Corticosterone	20.0	99.0	+395.0
Compound X	—	—	—
Aldosterone	3.6	3.14	—13.0

At 43°C swimming lasted 10 minutes as our earlier experiments [1, 2]. Rectal temperature rose to 41.6°C.

The duration of swimming at the different temperatures, the data for adrenocortical activity for the single groups and the values for rectal temperature are presented in Table IV.

Table IV

Group	Rectal temperature after swimming at °C	Duration of swimming, minutes	Adrenal venous blood corticoid level, μg/100 g/hour			Steroid content of incubated adrenal tissue, μg/g		
			Cpd B	Cpd X	Aldosterone*	Cpd B	Cpd X	Aldosterone
Control	37.4	—	6.1	3.05	0.87	20.0	—	3.6
18°C	21.5	9	4.0	1.34	4.0	142.0	44.0	4.8
30°C	30.9	108	6.38	1.82	1.05	101.0	195.0	5.2
43°C	41.6	10	7.43	1.24	2.77	99.0	—	3.14

* In μg/kg/hour.

Discussion

Swimming at extreme temperatures represents a great strain for the organism. This was well reflected by the duration of swimming at 30° C. The

marked reduction in the duration of swimming may be traced back to complex vegetative and metabolic disturbances, as suggested by our earlier finding [2] that swimming at 18° C and at 43° C significantly reduced the rate of oxidative processes. The present experiments have shown that after swimming in cold water corticosterone secretion decreases, although *in vitro* the adrenal tissue synthesized more corticosterone than did the adrenal tissue from the control rats. The same applied to compound X, an unidentified steroid. In contrast with this, aldosterone secretion definitely increased, indicating that the two kinds of corticoid are synthesized by independent mechanisms, or that their synthesis is not controlled by the same regulation. The diminution of hydrocortisone and corticosterone secretion in hypothermia, without work, was demonstrated long ago, although those experiments on dogs supplied no information as regards aldosterone secretion [4]. What may then explain the paradoxical reaction in the adrenocortical secretion? It may be assumed that in a cold environment the ACTH secretion by the anterior pituitary does not become activated, while the factors influencing aldosterone synthesis take effect. In recent years detailed reports have been published claiming that a "glomerulotrophic factor" — and not ACTH — played a role in the maintenance of aldosterone synthesis and secretion [5, 6]. On the other hand, it may be also suggested that at low body temperature the kinetics of adrenal enzymes have been so changed that corticosterone synthesis is inhibited, but the synthesis of aldosterone is not interfered with. The fact confirmed from many sides that corticosterone is the direct precursor of aldosterone, seems, however, to discredit that view [5, 7]. Very probably, the changes in corticosterone and aldosterone secretion are not due to changes in the metabolism of adrenal tissue, but rather to a disturbance in its regulation. This, however, still needs confirmation.

At high environmental temperatures aldosterone secretion increased, while the secretion of corticosterone remained practically unchanged, confirming our earlier observation that hyperthermia enhances aldosterone secretion without affecting corticosterone secretion [8]. Once again, this duplicity of glycocorticoid and mineralocorticoid secretion calls attention to the duplicity of the control mechanism of adrenocortical function. It is questionable, how far the significant differences between corticoid secretion and corticoid synthesis reflect the physiological state. At any rate, the increased synthesis after swimming at low and high temperatures indicates that *in vitro* the activity of the enzyme system was stronger than in the controls. The medium in which synthesis took place provided optimum conditions in both the control and the exhausted animals. The ultimate increase in the synthesis of both aldosterone and corticosterone suggests that factors at present unknown seem to be involved in the control of adrenocortical activity.

LITERATURE

1. YANG, T. L., LISSÁK, K.: Acta physiol. hung. **16,** 47 (1959).
2. YANG, T. L., LISSÁK, K.: Acta physiol. hung. **17,** 63 (1960).
3. ENDRŐCZI, E., YANG, T. L.: Acta physiol. hung. In Press.
4. EGDAHL, R. H., NELSON, D. H., HUME, D. M.: Science **121,** 506 (1955).
5. RAUSCHKOLB, E. W., FARRELL, G. L.: Endocrinology **59,** 526 (1956).
6. FARRELL, G. L.: Endocrinology **65,** 239 (1959).
7. STASHENKO, J., GIROUD, J. P.: Endocrinology **64,** 730 (1959).
8. YANG, T. L.: Sport és Tudomány **5,** 138 (1960).

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CONDITIONED AND UNCONDITIONED CEREBRAL CORTICAL ACTIVATION TO RENAL PELVIC STIMULATION

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In chronic experiments in female dogs with implanted cortical electrodes and unilateral ureteric fistula the weak mechanical stimulation of the renal pelvis caused cerebral electrical activation (orientation reaction). This electrical desynchronization could be extinguished. After lasting extinction a conditioned interoceptive electrical activation could be elicited by combining the renal stimulus with some (auditory or dermal) exteroceptive stimulus.

In a previous paper [1] the changes in cerebral electrical activity in response to intestinal stimulation have been analysed. It has been shown that in chronic experiments the rhythmic, physiologic small intestinal stimulus, as applied for the first time, activates the resting cerebral electrical activity in the dog. This fast electrical activity is extinguished on the repetition of the interoceptive stimulation, or appears in the form of a conditioned intestinal arousal reflex when associated with some auditory or dermal stimulus which by itself elicits arousal.

We now undertook to investigate whether the resting cerebral electrical activity would be desynchronized by a physiological stimulation of the renal pelvis and whether such a pelvic stimulus may be a starting point of a conditioned electrographic change.

Methods

Chronic experiments were performed in female dogs with left ureteric fistula and with stainless steel electrodes inserted through the skull to reach the dura. The experiments were conducted in sound-proof and electrically screened chambers. Prior to applying the stimuli the animals had been allowed time to become accustomed to the environment to extinguish the environmental investigatory reflexes, and were fed before every trial. In this way resting cerebral electrical activity and complete motor inactivity set in soon.

By means of a special device described elsewhere [2], rhythmic pressure was exerted on the wall of the left renal pelvis. The duration of stimulation was 10 sec.

Various sounds and weak electrical stimulation of the skin of the forelimb served as the exteroceptive stimuli eliciting cerebral electrical activation.

An 8-channel BOM EEG apparatus was used and the animals were kept under visual control as well. The size of the responses was measured on the basis of the duration of desynchronization. A total of 34 experiments was made in 5 dogs.

Results

A) Unconditioned renal pelvic arousal reaction

When the renal pelvis was rhythmically dilated for the first time, the high amplitude, slow EEG activity changed into a lower-amplitude, fast one (Fig. 1, *1*). The animal sometimes exhibited a motor reaction. This arousal reaction gradually disappeared on repeating the stimulation (habituation) (Fig. 1, *2*), the orientation reflex had become extinguished.

B) Conditioned renal pelvic arousal reaction

In the next phase the interoceptive stimulus was repeatedly applied until extinction had become permanent. After this had been achieved, no cerebral cortical activation was elicited by the first pelvic stimulation. Then we began to elaborate the conditioned reflex in this state of permanent extinction. The pelvic stimulus was therefore applied in combination with a strong auditory or dermal stimulus which caused by itself a marked cortical desynchronization (Fig. 1, *3*). After 2 or 3 reinforcements the pelvic stimulus alone evoked cerebral electrical activation. Considering that this reaction was extinguished when the interoceptive renal stimulus was applied repeatedly without reinforcement, then reappeared again after repeated trials with reinforcement, it may be accepted as being a regular conditioned arousal reaction (Fig. 1, *4*).

This was proved also by the so-called disinhibition test. If before conditioning, but after the permanent extinction of the interoceptive arousal we apply the sound or the dermal stimulus alone and the renal pelvic stimulus again after the resting activity has set in, there will be two possibilities, *viz.* (i) the extinguished interoceptive arousal has been disinhibited by the sound stimulus and the subsequently applied pelvic stimulus once again elicits desynchronization, or (ii) the auditory stimulus alone does not promote the desynchronization by the subsequently applied renal stimulus, but associated with the renal stimulus makes it possible that later the interoceptive stimulus alone be capable of eliciting the arousal reflex on a conditioned basis. Our experiments have borne out this latter assumption. As seen in Fig. 2, *A*, the renal stimulus applied after the auditory one did not arouse the animal, but when this auditory stimulus was applied a few times in combination with the renal one, the interoceptive arousal took place on a conditioned basis (Fig. 2, *B*).

Discussion

In general, the orientation reflex is considered to be the primary reaction in the conditioned reflex connexion. The nature of this primary response in

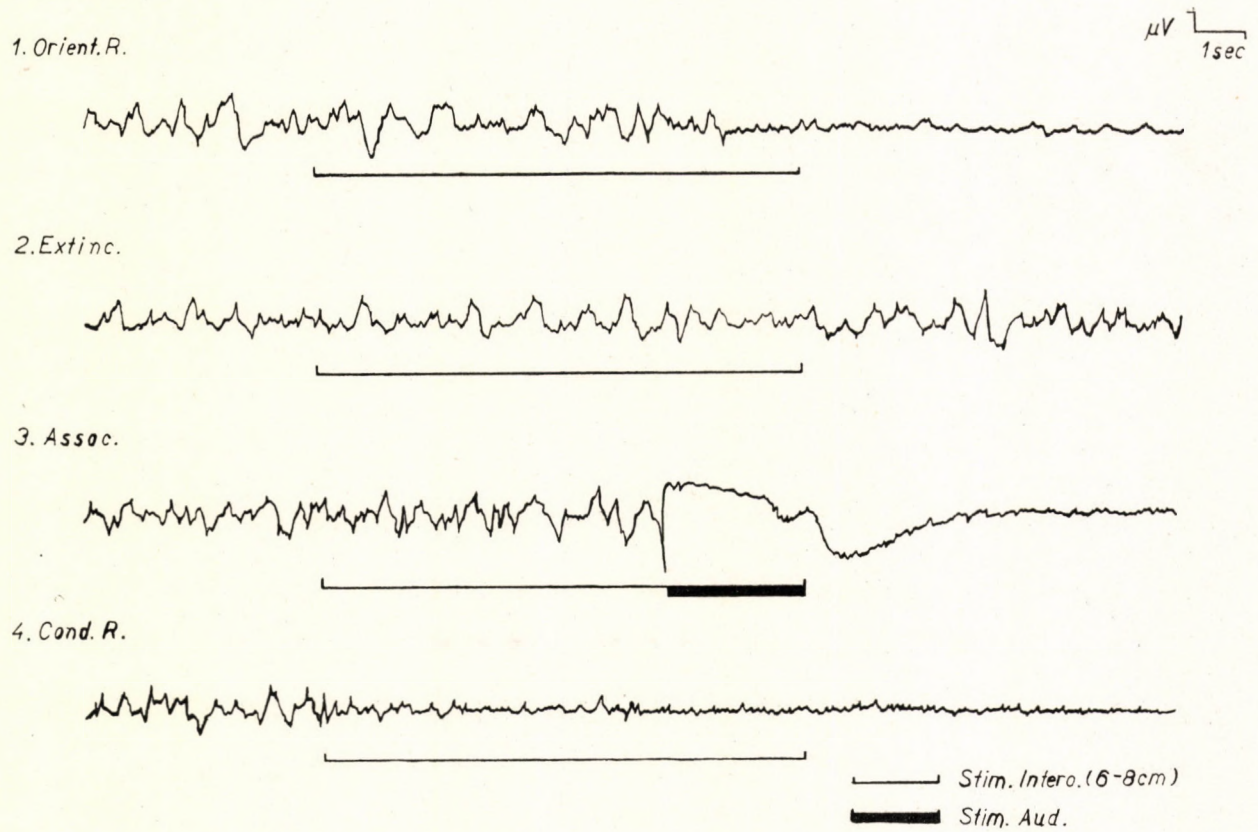

Fig. 1. Dog Morzsa. Procedure to build up a renal pelvic conditioned electrographic reflex. Right temporo-occipital lead. Explanation in the text

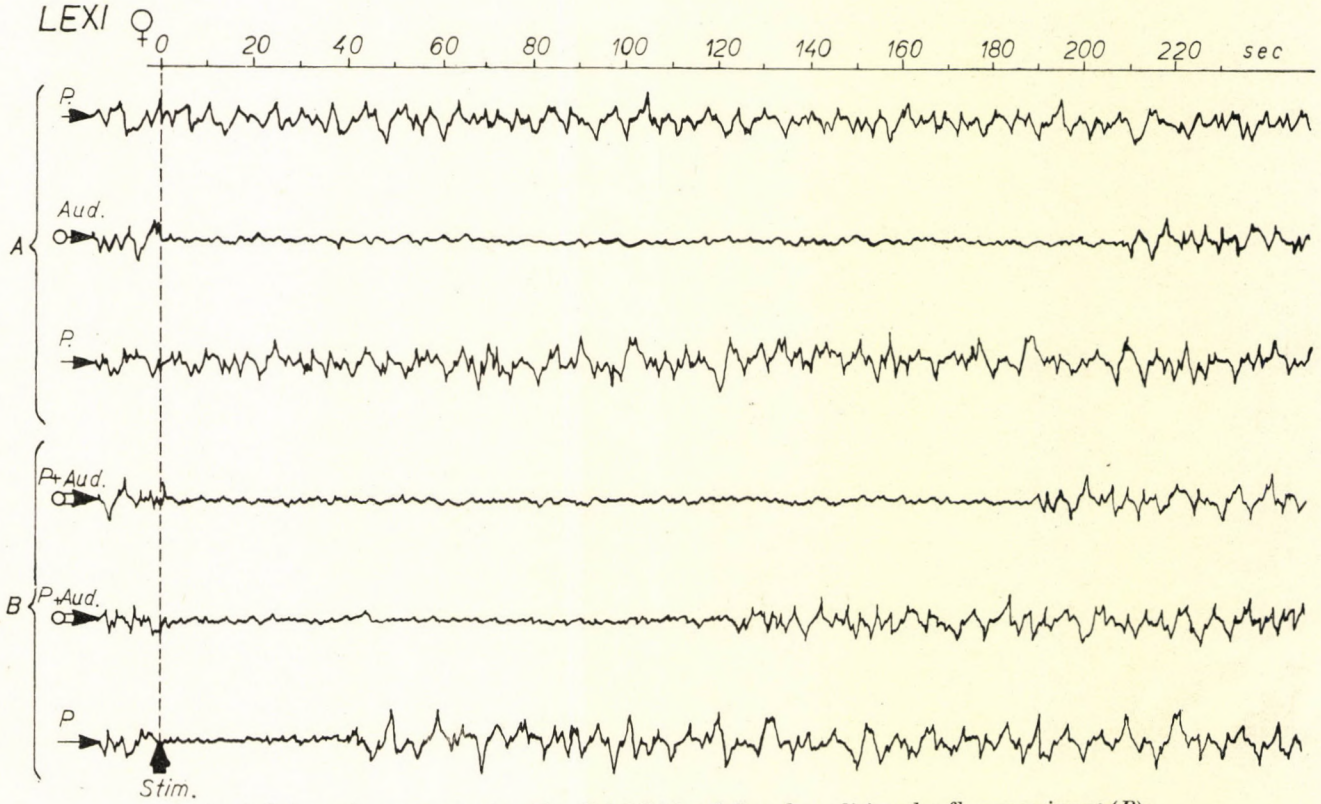

Fig. 2. Schematic representation of a disinhibition (*A*) and conditioned reflex experiment (*B*). Explanation in the text

the conditioned reactions of interoceptive origin is wholly unknown. In the form of cerebral electrical activation we have succeeded in following up this elementary response. According to the evidence obtained, the weak mechanical stimulation of the renal pelvis desynchronizes the resting cerebral activity. This fact is remarkable, it being known that under physiological conditions we do not feel such weak mechanical stimuli in the viscera, they do not become conscious. At the same time it is also known that stimuli similar to those employed by us may elicit conditioned alimentary and defensive reflexes if applied according to the Pavlovian principles [3, 4, 5]. This contradiction merits further elucidation. It is possible on the one hand the "threshold" stimuli applied under our experimental conditions do not occur physiologically. On the other hand, "unconscious" conditioned connexions may also exist. Our studies in man, in which to rectal stimuli an inhibition of the alpha-rhythm was developed independent from consciousness, seem to corroborate this view [6].

The second conclusion that may be drawn is that an exteroceptive orientation reflex may be conditioned to an interoceptive stimulus.

Essentially, we have combined in our experiments two stimuli eliciting orientation reaction. This confirms the view that a conditioned reflex invariably arises from a combination of two unconditioned reactions. In our studies we developed the conditioned response by weakening one of the stimuli (the interoceptive one) so that the orientation reaction to it was extinguished by applying it previously several times. Thus it has become a "neutral" stimulus; therefore this evidence may be interpreted also as proving that if we combine two unconditioned reflexes, the direction of the temporary connexion formed will be from the weaker toward the stronger. In other words, the efferent branch of the reflex will be the biologically stronger effect, *i. e.* that elicited by the reinforcing stimulus (in our case the auditory or dermal stimulus), as it has been proved by the Pavlovian school by the classic alimentary and defensive methods.

LITERATURE

1. Ádám, G., Jouvet, M., Michel, F.: C. R. Acad. Sci. (Paris) **246**, 2042 (1958).
2. Ádám, G.: Acta physiol. hung. **12**, 321 (1957).
3. Bykov, K. M.: (Быков К. М.): Избранные сочинения. Т. изд. медгиз. Москва 1955.
4. Airapetjanz, E. Sh.: Die höhere Nerventätigkeit und die Rezeptoren der inneren Organe. Volk u. Gesundheit, Berlin, 1956.
5. Ádám, G., Mészáros, I.: Acta physiol. hung. **12**, 327 (1957).
6. Tomka, I., Pásztor, E., Ádám, G.: Acta physiol. hung. **16**, Suppl. 35 (1959).

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ON THE ROLE OF THE BRAIN STEM ACTIVATION SYSTEM IN THE CONDITIONING TO VISCERAL STIMULATION

By

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In dogs with implanted cortical and mesencephalic electrodes and intestinal fistula the mechanical stimulation of the small intestine was associated with the direct electrical stimulation of the mesencephalic reticular activation system. This combination of stimuli made it possible to elicit conditioned cerebral electrical activation by the interoceptive stimulus. The evidence obtained indicates that the arousal reaction of mesencephalic character may be conditioned and consequently the conditioned cerebral electrical activation starting from the visceral receptor area is probably travelling through the mesencephalic nonspecific reticular system.

In the conditioned reflex connexion the fundamental mechanism is considered to be the orientation reflex described by PAVLOV [1], in the development of which the activation system of the brain stem, described by MORUZZI and MAGOUN [2], is presumed to play a role.

In previous experiments [3, 4] it has been found that the orientation reaction in response to interoceptive stimulation was objectively demonstrable in the activation of the resting cerebral electrical activity and that such an electrical activation induced by visceral stimulation may be conditioned. Starting out from this observation, in the present experiments we have undertaken to determine whether the unconditioned and conditioned interoceptive cerebral electrical activation would also be connected through the mesencephalic reticular system. We have therefore elaborated such a conditioned arousal reflex by reinforcing visceral stimuli with the direct electrical stimulation of the mesencephalic reticular formation.

Methods

In 11 female dogs a Thiry-Vella type small intestinal fistula was prepared, stainless steel electrodes were implanted into the skull and also bipolar nichrome electrodes into the mesencephalic reticular formation, by means of the stereotaxic technique. The method was previously checked in 30 dogs of about the same weight, to determine by subsequent morphological control the size of variations resulting from differences in the configuration of the head.

The experiments were performed in a sound-proof, electrically screened conditioned reflex chamber. Rhythmic dilatation at a rate of 30/min of the mucosa of the isolated intestinal area was employed as the interoceptive stimulus. The size of the stretching effect was measured by a manometer in the stimulator pneumatic system and the smallest pressure just eliciting the expected cerebral electrical activation (threshold) was used. The mesencephalic

reticular formation was stimulated by rectangular impulses (frequency 300/sec, duration 1 msec, voltage 1.5 to 3 V). The position of the deep electrodes was subsequently controlled by morphological study in every case (Fig. 1).

Before the experiments the animals were allowed time to get accustomed to the chamber and were fed prior to every test. Thereby the environmental orientation reaction was extinguished and thus the resting cerebral electrical activity representing the starting point set in easier, as manifested in motor inactivity. The size of the response was assessed on the

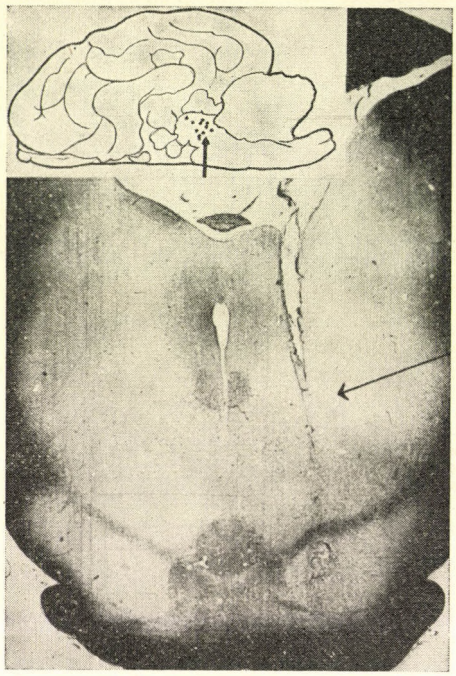

Fig. 1. Histological section from the brain stem of dog Tarka. Position of deep electrodes (arrow) in the mesencephalon. In the left corner the position of the tips of the electrodes as found in 11 dogs is represented diagrammatically

basis of the duration of the desynchronization of the resting activity. A BOM 8-channel EEG apparatus was used. The number of experiments totalled 68.

Results

The mechanical intestinal stimulus, applied in the state of resting, slow cerebral electrical activity elicited a fast, low-amplitude activity, just as in the previous experiments [3, 4]. The duration of this desynchronization decreased gradually, then ceased altogether (habituation) after applying the visceral stimulus 4 to 10 times. The extinction procedure was continued in several experiments until in the course of a given experiment the first intestinal stimulus had no influence on the resting cerebral electrical activity.

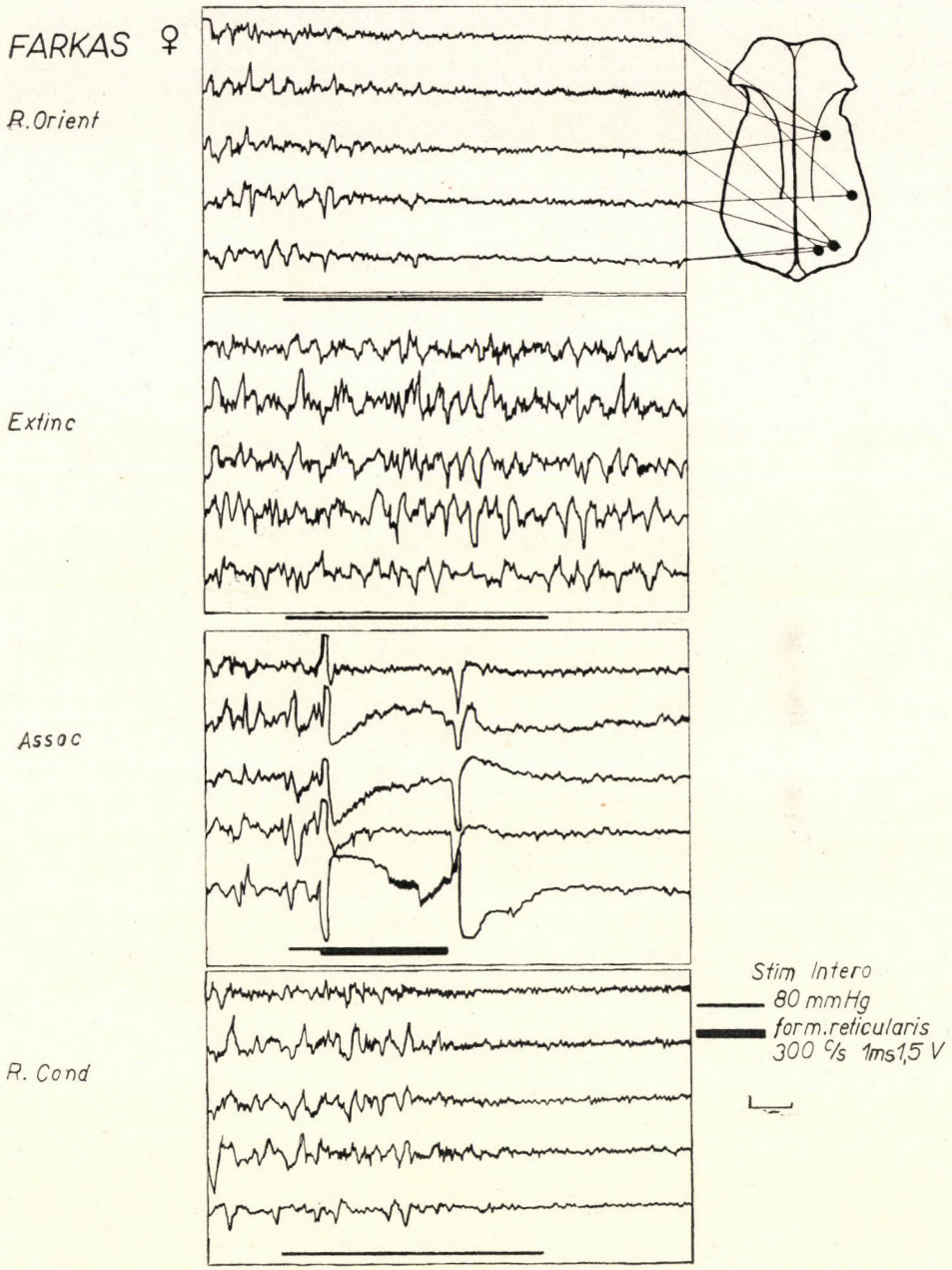

Fig. 2. Dog "Farkas". Elaboration of conditioned intestinal electrographic reflex in four steps. *1.* (R. orient.) arousal reaction to intestinal stimulus. *2.* (Extinc.) extinction of reaction after having been applied a few times. *3.* (Assoc.) association of intestinal with direct electrical reticular stimulation. *4.* (R. cond.) the intestinal stimulus applied by itself elicits a conditioned arousal reflex

In this period of lasting extinction the intestinal stimulus lasting 10 seconds was reinforced during the last 2 or 3 seconds by a direct eletrical stimulation of the reticular formation, known by itself to cause an intense arousal reaction. When applied after such a reinforcement, the visceral stimulus by itself once again became capable to desynchronize the resting electrical activity (Fig. 2), just as it happened in our experiments in which auditory or dermal stimuli were used as the reinforcement [3, 4]. It was remarkable that while in these our previous experiments the reinforcement had to be applied 3 to 4 times to elicit such an interoceptive conditioned arousal reaction, this time a single reinforcement by the direct stimulation of the reticular structures sufficed to enable the intestinal stimulus alone, as a conditioned stimulus, to elicit the typical electrical arousal according to the laws of temporary connexions.

The conditioned reflex nature of the intestinal arousal reaction developed by reinforcing the mechanical stimulation of the intestine with the direct electrical stimulation of the reticular cells was ascertained by means of the extinction and disinhibition tests, as in our previous experiments [4].

Discussion

The results obtained have confirmed our hypothesis that the conditioned arousal reflex is travelling through the mesencephalic afferent activation system. It has namely been shown that if the neutral interoceptive stimulus is reinforced by the direct electrical stimulation of the diffuse nonspecific reticular system described by MORUZZI and MAGOUN [2], the temporary arousal results just as it does in response to strong exteroceptive (auditory, dermal) stimulation that causes arousal by itself. The appearance of the conditioned reaction after a single reinforcement of the intestinal stimulus by reticular stimulation indicates that in the conditioned arousal reaction the adequate "unconditioned" reaction in the Pavlovian sense is invariably an excitation of the reticular activation system. The direct electrical stimulation of this structure is obviously more effective than an exteroceptive stimulus travelling there by way of a distant afferentation. In other words, the cortical electrical activation of mesencephalic origin may be conditioned, even, as our experiments show, to interoceptive stimuli.

LITERATURE

1. Pavlov, I. P. (Павлов, И. П.): Полное собрание сочинений. Т. 3. Изд. Акад. Наук СССР. Москва, 1951.
2. Moruzzi, G., Magoun, H. W.: Electroenceph. clin. Neurophysiol. **1**, 455 (1949).
3. Ádám, G., Jouvet, M., Michel, F.: C. R. Acad. Sci. (Paris) **246**, 2042 (1958).
4. Ádám, G., Mészáros, I., : Acta physiol. hung. **18**, 137 (1960).

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Pharmacologia

THE PHARMACOLOGY OF MYDETON *(MYDOCALM*, 1-PIPERIDINO-2-METHYL-3-p-TOLYL-PROPANONE-3), A NEW INTERNEURONE BLOCKING COMPOUND

By

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Mydeton (1-piperidino-2-methyl-3-p-tolyl-propanone-3), a compound apparently having a central nervous site of action, produces the following characteristic effects: changes in behaviour (which vary from species to species), sedation in the mouse and rat, extreme loss of muscle strength in the rabbit, clonic convulsions followed by loss of muscle tone and total inability to walk in the cat. Only near-toxic doses produce narcosis. Mydeton paralyses multisynaptic spinal reflexes, relieves the hyperreflexia caused by strychnine and reduces the strychnine mortality rate in rats. It is active against the convulsions induced by electric shock. In this respect Mydeton is more potent than Diphedane or Mysoline. Mydeton is active also against nicotine and the tremor caused by harmine.

The drug has no atropine-like, antihistaminic or adrenolytic actions. Its ganglionic blocking potency is 2 or 3 times weaker than that of TEA. It is about twice weaker than papaverine in inhibiting smooth muscle spasm.

Thus, the drug acts first of all on the multisynaptic neurone systems and may therefore be listed among the so called interneurone depressants.

Until recently, atropine and scopolamine were the souveraine drugs in the treatment of extrapyramidal motor diseases, especially parkinsonism. However, they have untoward side effects (dryness of mouth, disturbance of accomodation, tachycardia) and therefore it was desirable to produce drugs with less side effects. The first of such drugs was Parpanit [1]. As far as side effects are concerned, this was only 13 times weaker than atropine and influenced mainly the tremor. Subsequently several new preparations have been recommended for treating the symptoms of parkinsonism, such as Trasentine, Artane, Lysivan, Benadryl, but none of them gained universal acceptance.

Recently, on the basis of the observation that chlorpromazine blocked the bulbar and mesencephalic facilitatory neurone system [2], this compound has been used in the treatment of the widest variety of motor disturbances. However, chronic treatment with chlorpromazine may by itself give rise to changes similar to the symptoms of parkinsonism [3], apparently through interfering with the copper metabolism of the central nervous system, because these symptoms can be ameliorated by the use of BAL (dimercaptopropanol) [4].

We showed in earlier chemical and pharmacological studies that in the rabbit and the cat some aminoketones caused extreme muscle weakness

and complete inability to walk, without making the animals to sleep [5, 6]. Systematic pharmacological investigations showed in particular 1-piperidino-2-methyl-3-tolyl-propane-3 (Mydeton, Mydocalm, of the *Richter Works*, Budapest) to possess such pharmacological properties and to be especially suitable for use the treatment of extrapyramidal motor disorders. The compound is water-soluble, but rapidly loses its activity in the dissolved state; in substance it is stable.

Methods

Acute toxicity was determined in mice, by the method of BEHRENS and KÄRBER [7], using 5 animals each for the doses increasing in steps of 100 mg/kg. The effects on the somatic nervous system were studied in the mouse, rat, rabbit and cat; eventual psychic effects were also recorded.

The studies concerned with the inhibition of the tremor produced by harmine were made as follows. The mice were placed into a closed wire cage 15 cm high and 8 cm in diameter. Exactly in the centre of the bottom of the cage was an axle allowing the cage to swing right and left The cage was suspended by a rubber band which served to prevent extreme swinging. The motions of the cage were recorded kymographically. A few trials in the cage sufficed to adapt the animal to its new environment and to make it behave normally. The compound was injected subcutaneously 15 minutes before the subcutaneous injection of 20 mg/kg of harmine. In other experiments the inhibition of the tremor was observed visually. In this series 5 animals were used to test each dose of the drug. The activity was expressed by a quotient, with the number of animals showing tremor in the numerator and with that of the animals treated with Mydeton in the denominator.

The anti-nicotine effect was studied in mice against 3 and 6 mg/kg doses of nicotine tartarate administered intravenously, by a method already described [8]. The doses corresponded to the seasonal LD_{100} of nicotine [6].

Inhibition of electroshock was studied in the following way. Previous studies in 10 Wistar rats of our own breed showed the optimum parameter of stimulation producing strong tonic convulsions in all four limbs in every animal to be 100 Hz, 5 msec, 27 V. Electroshock treatment with these parameters applied at intervals of 30 minutes elicited typical convulsions of identical intensity for hours. In evaluating the anticonvulsive effect of the decrease in the intensity and the duration of convulsions were recorded. The intensity of convulsions is given in points, in the average for 10 animals each. The action on the intensity of metrazol convulsions was evaluated in the same way. A more detailed description will be given in the appropriate sections.

The action on striated muscle was studied in the masseter muscle of the rat, by the method of HOTOVY and ERDNISS [9]. The compounds were injected into a tail vein. Muscle contractions were elicited 18 to 20 times a minute.

Of the spinal reflexes only the ipsilateral flexor and the contralateral extensor ones were elicited, by stimulating the central stump of the tibialis posterior nerve. The contractions of the corresponding muscles were recorded by a Marey capsule transmission. The reflexes were elicited 18 to 20 times a minute, by rectangular impulses 5 msec in duration.

The actions on circulation, respiration and autonomic ganglia were studied in cats under urethane (300 mg/kg) + chloralose (35 mg/kg) anaesthesia. Blood pressure was measured in the left femoral artery by means of a mercury manometer, respiration was traced kymographically by means of a Marey capsule attached to a tracheal cannula. The cervical preganglionic fibre was stimulated with rectangular impulses and the contractions of the nictitating membrane were recorded by an isotonic lever (transmission 1 : 20).

Cardiac action was studied in isolated frog hearts beating in 1 ml Ringer's solution. The activities of the mammalian heart were recorded by means of a cardiometer, and ECG tracings were made to detect eventual disorders in the formation and conduction of impulses.

The action of the compound on the cerebral bioelectric potentials was studied in unanaesthetized cats, using an 8-channel Kaiser EEG apparatus. The electrodes were fixed into the surface of the frontal and occipital lobes and the activation area of the reticular formation of the brain stem. The Horsley-Clarke co-ordinates of the latter were: anterior 2 mm, lateral 3 to 4 mm, and vertical 3 to 4 mm. The animals were experimented on 2 weeks after operation.

Results

Toxicity and general effects

Mice. LD_{100} 800 mg/kg, LD_{50} 620 mg/kg. The 100 mg/kg dose produced a very mild ataxia, the 200 mg/kg dose extreme muscle weakness with marked ataxia. The rectal temperature dropped to 34 to 35 °C. After administration of a 400 mg/kg dose the animal could hardly walk and its body temperature decreased to 30 °C. 600 mg/kg produced narcosis and some animals died of respiratory paralysis.

Rats. Smaller doses produced a remarkably strong sedative effect. This was obvious even after a dose as small as 50 mg/kg subcutaneously. The

Fig. 1. Rabbit. Response to 100 mg/kg of Mydeton subcutaneously. Total loss of muscle power. The animal cannot stand up, drops its head, the limbs give way

formerly irritable, aggressive animals became tame, rough interventions were tolerated passively. Larger doses affected motor functions, first causing inability to walk. Narcosis was observed after near-toxic doses only.

Rabbits. In response to the subcutaneous injection of 100 mg/kg the changes appeared in the following order. After five minutes mild ataxia, after 15 minutes mild muscle weakness with maintained posture correction and slipping of limbs, after 30 minutes inability to walk, extreme muscle weakness were observed; the animal tried to escape from strong painful stimulation, but could move just a few steps. This characteristic posture is shown in Fig. 1. The ability to correct posture was maintained throughout, no symptom of narcosis was noted.

Cats. Muscle weakness was observable in response to the 50 mg/kg dose. The 100 mg/kg dose was followed in 10 to 15 minutes one or two clonic convulsions, after which the animal became inable to walk. In this phase the animals were responding to external stimuli, felt no pain and showed no evidence of narcosis. The cats were mewing desperately, breathed rapidly and salivated. The duration of the action of this dose was 3 to 4 hours.

Antinicotine and antiharmine actions

According to BOVET and LONGO [10] the compounds improving the symptoms of human parkinsonism inhibit the nicotine-induced convulsions in the rabbit. CAHEN [11] has suggested the inhibition of the nicotine tremor to be a valuable method in the search for drugs active on paralysis agitans. The method has been widely accepted so that now the nicotinolytic compounds have separate chapters in the text-books [12].

It seemed therefore interesting to examine whether Mydeton possessed an antinicotinic action and how its potency compared with that of Parpanit.

Table I

Mydeton subcutaneously mg/kg	Nicotine tartarate intravenously		Parpanit subcutaneously mg/kg	Nicotine tartarate intravenously	
	6 mg/kg	3 mg/kg		6 mg/kg	3 mg/kg
100	3/10	0/5	100	0/5	
75		0/5			
50	5/10	1/5	50	1/5	0/5
25		2/5			0/5
25			25	1/5	2/5
12.5			12.5	1/10	3/5
0		5/5			

Mice in groups of 5 were given the test drug subcutaneously 30 minutes before the lethal dose of nicotine tartarate, and the potency of antinicotine action was judged on the basis of the number of survivors. This was expressed in the form of fraction, with the number of survivors in the numerator and the animals treated with the test drug in the denominator. The results are presented in Table I. It can be seen that the antinicotinic potency of Mydeton is about $\frac{1}{2}-\frac{1}{3}$ of that of Parpanit; 25 mg/kg of Mydeton saved about 50 per cent of the animals, while of Parpanit 12.5 mg/kg subcutaneously afforded the same protection. 100 mg/kg of Mydeton afforded 75 per cent protection against twice the lethal dose of nicotine, while the same dose of Parpanit 100 per cent protection.

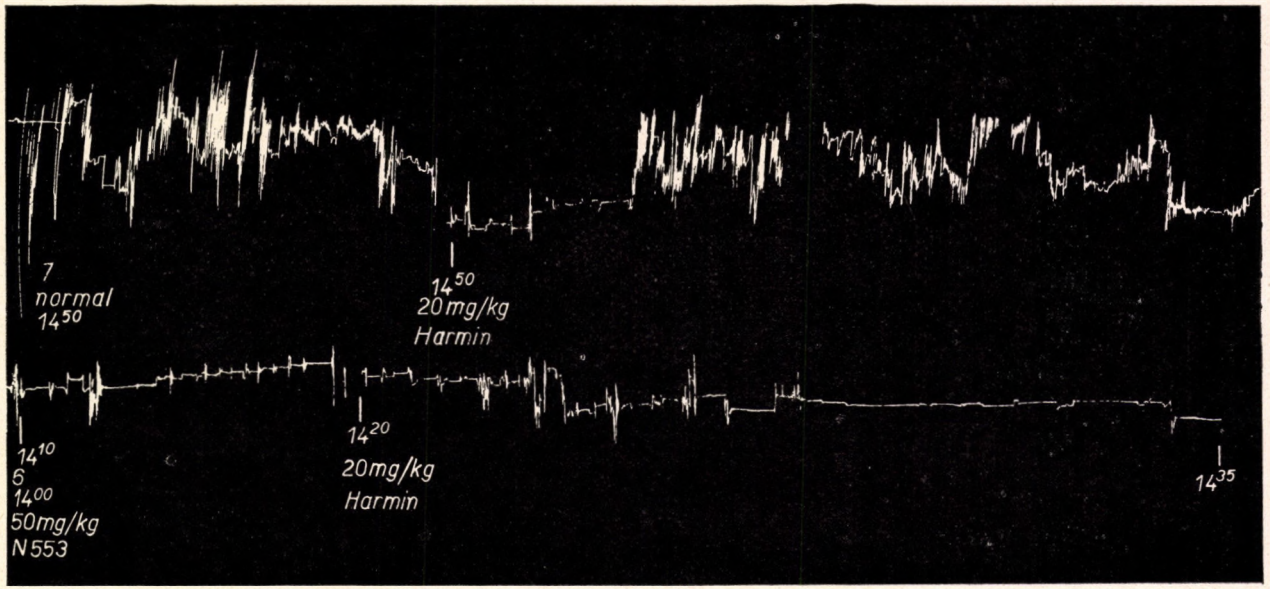


Fig. 2. Mouse. Inhibition of tremor produced by harmine. Curve above: Motor activity of normal animal, before and after harmine 20 mg/kg subcutaneously. Curve below: Effect of Mydeton 50 mg/kg, subcutaneously, before and after the subcutaneous injection of 20 mg/kg of harmine

According to ZETTLER [13], the inhibition of the harmine tremor may also be used in the search for drugs against parkinsonism. This action of Mydeton was also studied. An experiment of this type is shown in Fig. 2. The curve above shows the motor activity of an untreated control mouse, the one below that for a mouse pretreated with Mydeton. The control animal responded to placing into the cage by an intense orientation reaction, causing the cage to swing wildly. Then following the subcutaneous injection of 20 mg/kg of harmine the animal became quiet and developed tremor and motor agitation for about 30 to 40 minutes. The curve below shows the motor activity of a mouse which had been given a subcutaneous injection of 50 mg/kg of Mydeton 10 minutes before it was placed in the cage. The animal was absolutely calm. The two curves make it clear that just like in the rat, Mydeton has a sedative action in the mouse, too. The animal was indifferent to the new environment and exhibited no orientation reaction. A 20 mg/kg subcutaneous dose of harmine given 20 minutes after the injection of Mydeton caused motor agitation for a short time, but no tremor. Parpanit differs from Mydeton also in that it may by itself cause tremor. Harmine makes the Parpanit-induced motor agitation more marked and therefore an eventual inhibition of tremor could not be assessed. Mydeton in a dose of 25 mg/kg did not prevent the development of tremor, but considerably reduced its duration.

Action on striated muscle

It is characteristic of Parpanit that it antagonizes not only the nicotine convulsions, but normalizes the muscle acitivity increased by prostigmine. At the same time it has no influence on the muscle response to indirect neural stimulation. According to HOTOVY and ERDNISS [9], the inhibition of the prostigmine-increased muscle activity may be suitable for determining whether a compound is capable of influencing spastic hypertension in man; they succeeded by this method in confirming the therapeutic action of Belladonine a compound which has no antinicotinic activity. A typical experiment of this

Table II

Drug	Dose mg/kg, intravenously	Percentage decrease of contraction amplitude	Number of experiments
Mydeton	5	67.9	4
Parpanit	5	57.0	2
Mydeton	2	63.5	2
Parpanit	2	27.4	3

type is shown in Fig. 3. The intravenous injection of 2 mg/kg of Parpanit diminished the muscle activity increased by prostigmine, and this action was further enhanced by Mydeton. In the experiment shown in Fig. 3, 5 mg/kg of Mydeton proved to be more potent than the same dose of Parpanit. The results have been tabulated (Table II).

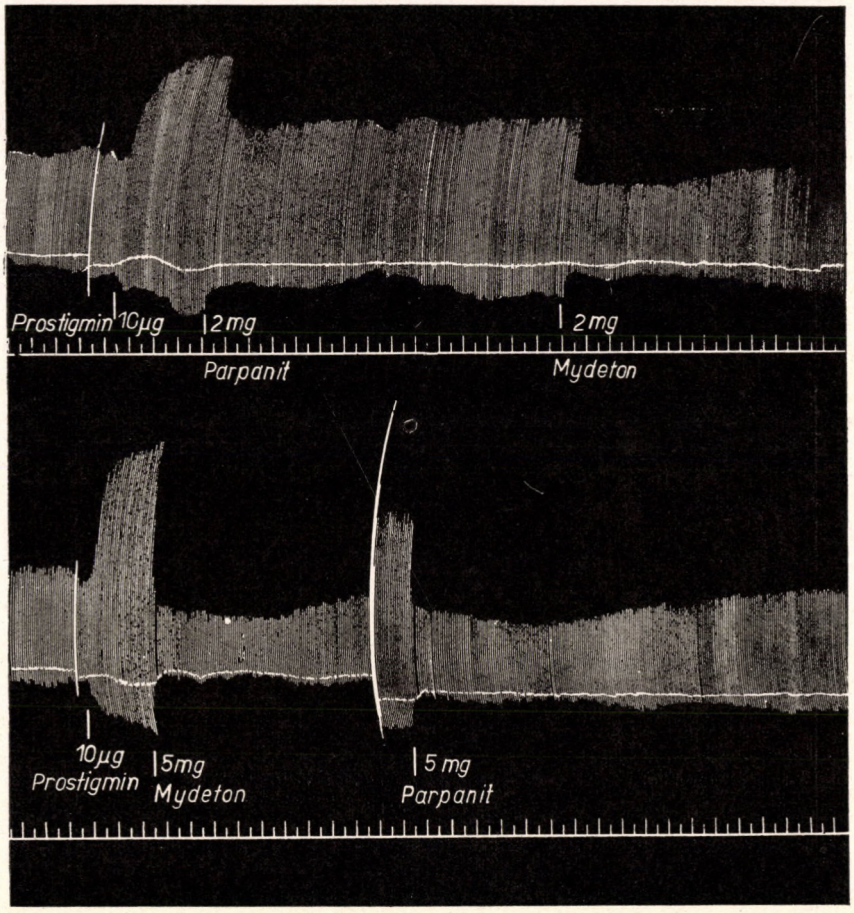


Fig. 3. Rat, 160 g. Masseter muscle hyperactivity, induced by the intravenous injection of prostigmine, 10 µg/animal. Diminution of muscle tone in response to Parpanit (2 to 5 mg/kg) and Mydeton (2 to 5 mg/kg, intravenously).
Parameters of stimulation: 100 Hz, 15 msec., 5 to 8 V

Action on spinal reflexes

Parpanit is known to block the multisynaptic spinal reflexes, while it leaves the monosynaptic knee jerk unaffected [14]. In our earlier studies on the pharmacology of aminoketones it was found that the aminoketones with an

antinicotinic activity possessed such an action, similar in potency to that of Parpanit and Myanesin [6]. Studies on the action of Mydeton on spinal reflexes have been particularly justified by BERGER's observation [15] that no connection existed between the intraneurone-depressant and flaccid paralysis-inducing effect of the compound. This applies in particular to Parpanit and Myanesin, both of which inhibit the flexor and especially the extensor

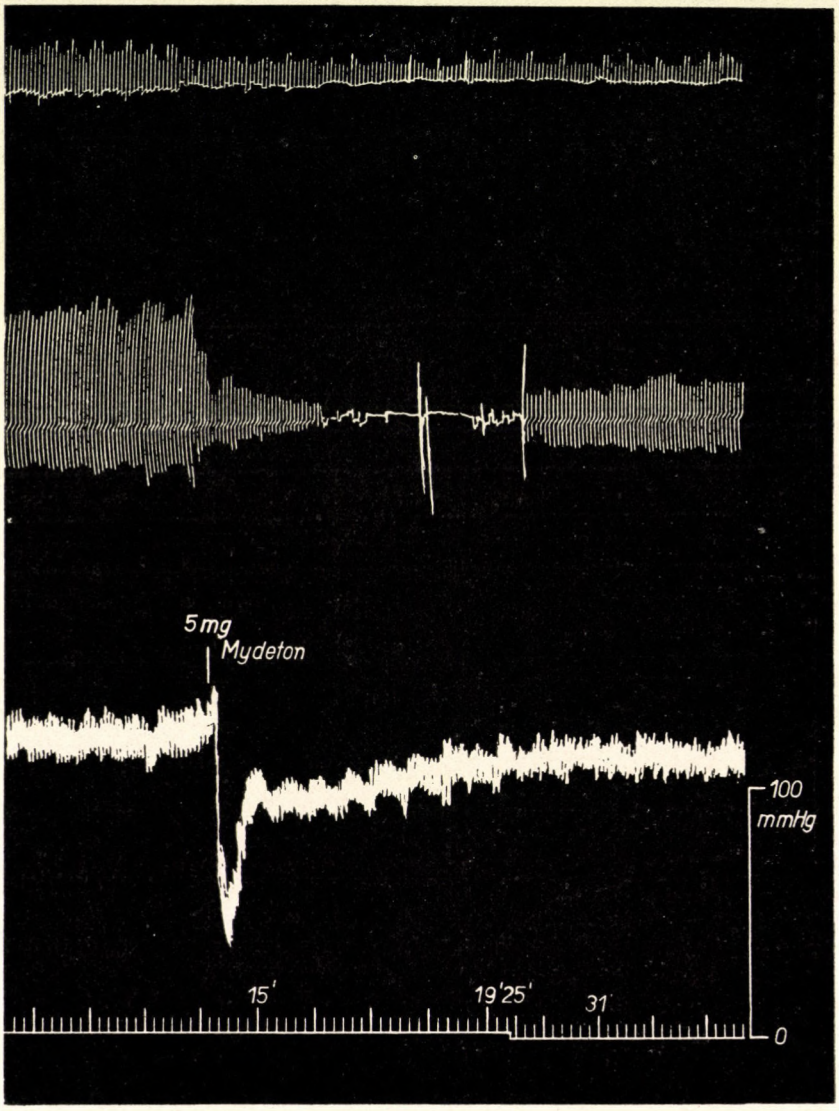


Fig. 4. Cat, 3500 g. Effect of an intravenous dose of 5 mg/kg of Mydeton on spinal reflexes. Top: ipsilateral flexor reflex. Centre: crossed extensor reflex. Bottom: blood pressure. Time signal: 10 sec

reflex, but only Myanesin causes a flaccid paralysis similar to that produced by Mydeton.

Fig. 4 shows that Mydeton depressed especially the crossed extensor reflex, while it had no influence on the amplitude of the ipsilateral flexor reflex. The reflex depression was not correlated with the vasodepressor action, because it persisted after the blood pressure had become normal. The reflex depression caused by the intravenous injection of 5 mg/kg Mydeton was lasting; the initial amplitude was resumed only after 20 to 30 minutes.

To elucidate the site of action of this reflex depression, the effect of the compound on the strychnin-induced reflex facilitation was studied. GUALTIEROTTI [16] showed that strychnine blocked the so-called collateral neurones of RENSHAW and the reflex irradiation was a result of this. As seen in Fig. 5, Mydeton was capable of normalizing the hyperreflexia caused by the intravenous injection of 20 μg/kg of strychnine. As our investigations showed Mydeton to have no or very little influence on the ipsilateral flexor reflex and on the amplitude of the neurodirect muscle contraction, this reflex depression could have been ascribed to a diminished activity of the motoneurones in the anterior horn. If this were so, the increased ipsilateral flexor reflex should have also been diminished. Considering the absence of this effect we assumed that the site of action of Mydeton was in some higher cerebral facilitatory systems.

Mydeton is a more potent reflex depressant than Parpanit, especially as regards the inhibition of strychnine hyperreflexia. In many instances Parpanit in a dose of 5 mg/kg failed to influence the reflex irradiation caused by strychnine, while the same dose of Mydeton diminished the amplitude of the reflexes.

The antagonism of strychnine and metrazol convulsions in the rat

The above investigations have made it clear that Mydeton is a specific inhibitor of the central nervous multineuronic synapses. As it was also capable of blocking the strychnine-hyperreflexia, we investigated whether it lessened the toxicity of strychnine. We found the LD_{100} of strychnine to be 3 mg/kg (s. c.) in rats. This dose killed the animals in 22.5 $\pm$ 15.7 minutes. The duration of this period increased to 119.5 $\pm$ 69 minutes and 3 of 10 animals survived when 100 mg/kg of Mydeton had been injected subcutaneously 20 minutes prior to the injection of strychnine. Thus, Mydeton has a marked antistrychnine action and the reason why it can prevent death in but 30 per cent of the cases is the protracted action of strychnine, and the faster elimination of Mydeton.

The inhibition of metrazol convulsions was also studied in rats. The dose was 70 mg/kg of metrazol while the dose of Mydeton was 50 mg/kg subcutaneously, 20 minutes before administering metrazol. The results have been summarized in Table III, where the time of appearance and the intensity of convulsions are also shown. The intensity of convulsions is given in points,

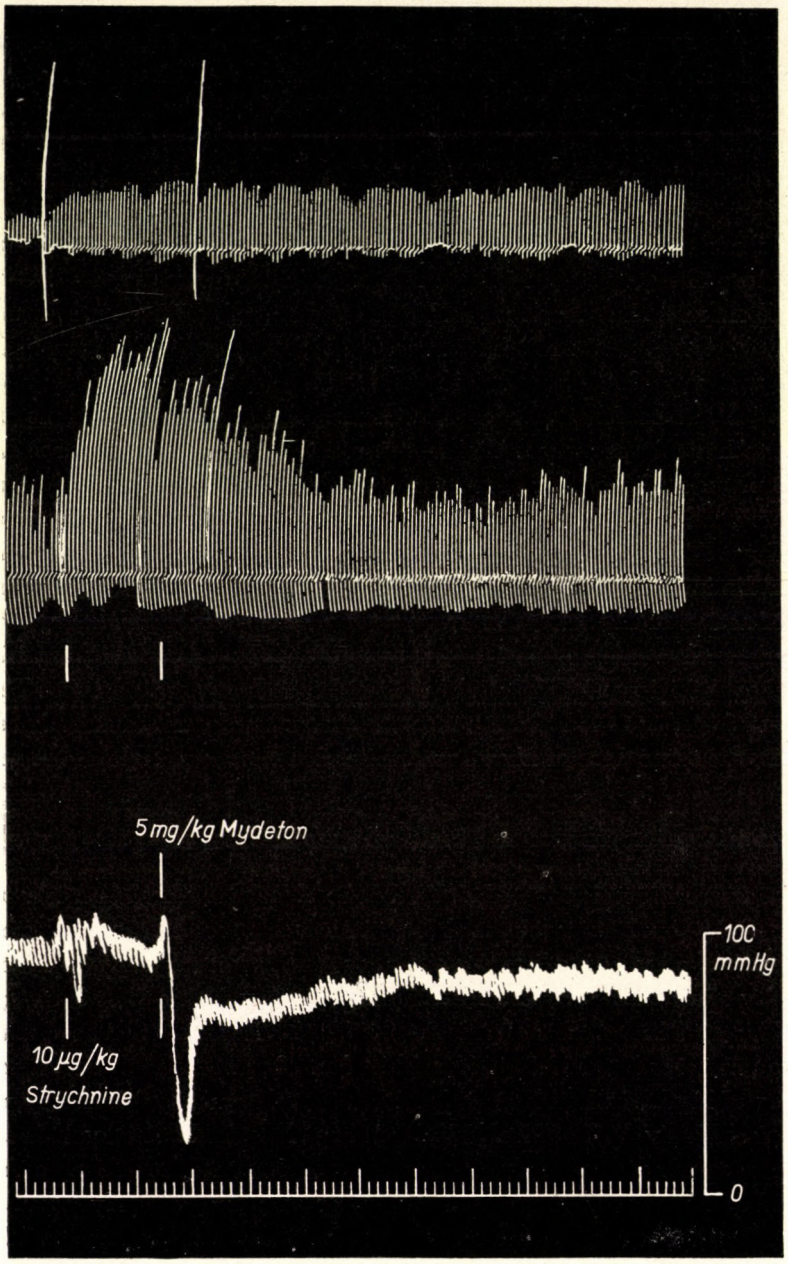


Fig. 5. Cat, 3800 g. Hyperreflexia (flexor and crossed extensor) induced by strychnine (10 µg/kg intravenously), relieved by the intravenous injection of 5 mg/kg of Mydeton. Top: flexor reflex. Centre: extensor reflex. Bottom: blood pressure. Time signal: 10 sec

and because there were several seizures the scores have been added up. One hundred points mean a maximum increase of tone in all four limbs, 75 points an increased tone in the fore limbs and a clonus in the hind ones, 50 points stand for clonus in all four limbs and 25 points indicate clonic motions in the fore limbs only. Mydeton significantly diminished the intensity of convulsions,

Table III

Inhibition of metrazol convulsions in the rat

Metrazol 70 mg/kg subcutaneously

Animal No.	Weight g	Time of injection	Note	Points
1	160	14^{45}	14^{52}++++	100
2	180	14^{46}	15^{02}++	50
3	155	14^{47}	15^{00}++	50
4	120	14^{48}	14^{50}++ 15^{05}++++ 15^{17}++++	250
5	160	14^{50}	14^{58}++ 15^{20}++	100
			Total	500

After treatment with 50 mg/kg of Mydeton subcutaneously

Animal No.	Weight g	Time of injection	Note	Points
1	175	15^{55}	16^{50}+ succumbed	25
2	130	15^{56}	17^{25}+	25
3	125	15^{57}	16^{35}+ succumbed	25
4	130	15^{58}	16^{35}+ 16^{40}++ 16^{50}++ 16^{55}++	125
5	175	15^{59}	16^{45}+ 16^{47}++ succumbed	75
			Total	275

the total score decreased from 500 to 275, but at the same time the toxicity of metrazol was significantly increased. This was shown by the fact that 3 of the 5 animals pretreated with Mydeton died, while all of the controls survived.

Inhibition of electroshock in the rat

It is known [17] that certain compounds not influencing the metrazol convulsions considerably inhibit the appearance of the convulsions produced by electroshock. Considering its strong central actions, Mydeton was examined also for this effect in comparison with Parpanit, Diphedane and Mysoline, to

determine to what extent and for how long the test drugs influenced the response to electroshock. The results obtained in groups of 10 animals each are presented in Fig. 6, showing the time elapsed from the subcutaneous injection of the test compounds in 30 minute intervals on the abscissa and the intensity of the convulsions in "points" on the ordinate. The scores should be interpreted as specified above with metrazol. This method was thought more reliable than that in which the convulsive threshold is determined by increasing

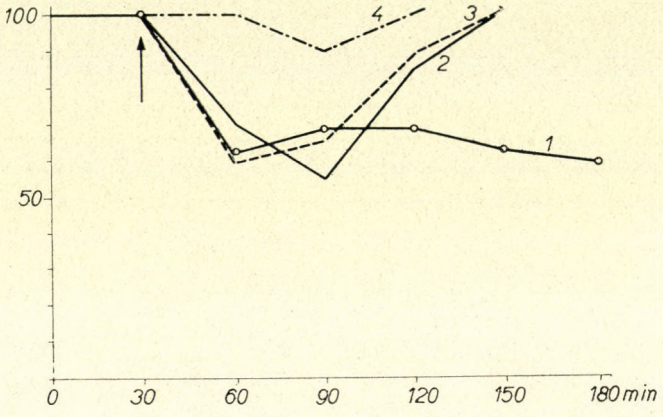


Fig. 6. Rat. Electroconvulsion. Abscissa: time. Ordinate: intensity of convulsion, in points Each curve shows the mean for 10 animals. The arrow marks the subcutaneous injection of the test substance

1: Mydeton, 50 mg/kg
2: Parpanit, 50 mg/kg
3: Diphedan, 50 mg/kg
4: Mysolin, 50 mg/kg

the voltage or intensity. Mysolin injected subcutaneously had the weakest and Mydeton the most lasting action; it was still demonstrable after 150 minutes, while the effect of Parpanit and Diphedan was over in 90 to 120 minutes. Parpanit showed a remarkable anti-electroshock action, concerning which we have been unable to find data in the literature.

The effect of Mydeton on the cortical and subcortical bioelectric activity in the cat

In these studies large doses (100 mg/kg) of Mydeton were administered, to produce marked changes in behaviour and posture. A typical electro- and subcortigram of this type is reproduced in Fig. 7. In part *a*, showing the basic activity faster and slower activities were alternating. This was particularly conspicuous in the occipital cortex. A sound stimulus of 200 Hz applied at this point elicited an intense arousal reaction. The activity of the frontal cortex increased sharply and the sleep spindles, appearing at about 4 to 5 sec intervals, disappeared in the occipital cortex. Part *b* shows the bioelectric activity 8

minutes after the administration of 100 mg/kg of Mydeton. The sleep spindles are absent, frequency is increased, the curve is similar to that of the arousal reaction. A sound stimulus applied at that time had no effect. Shortly afterwards, in part *c*, the picture of a typical attack of clonic convulsions is visible, followed by isoelectric silence. Part *d* shows the electric activity 30 minutes after Mydeton administration. There is no trace of narcosis, instead of it a high-frequency, low-amplitude activity was recorded. A sound stimulus

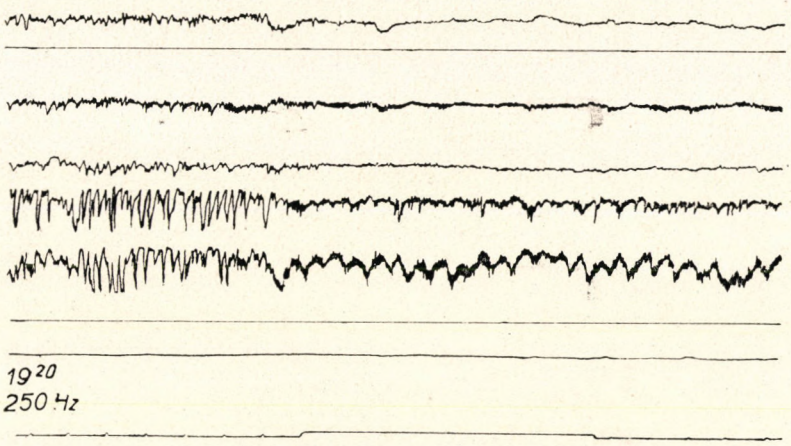
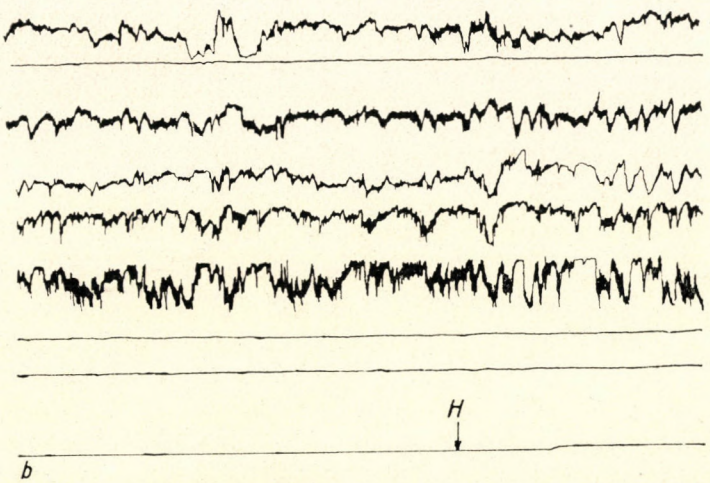
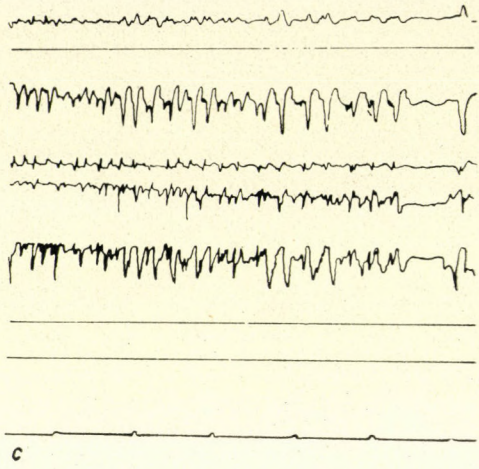


Fig. 7. Effect of 100 mg/kg of Mydeton on the corticogram of the cat
Leads, downward: bifrontal, right longitudinal, left longitudinal, bioccipital, right reticular formation.
Part *a*: Before Mydeton, desynchronization caused by sound (250 Hz)

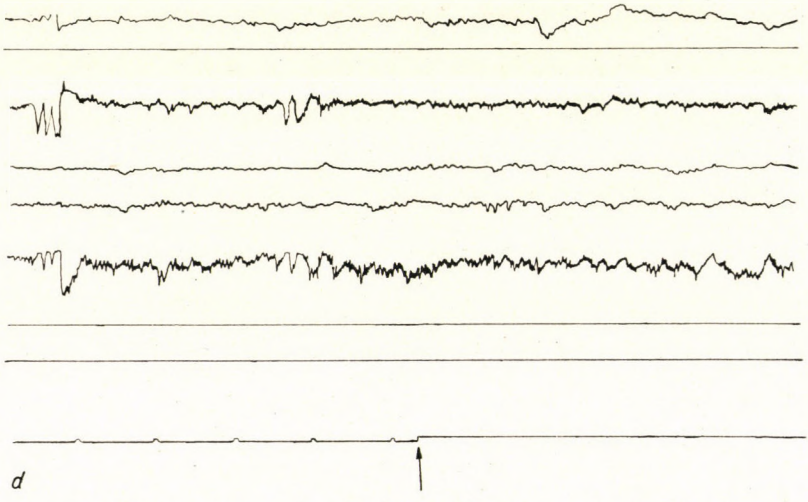


Part *b*: Seven minutes after Mydeton. Fast activity, occasional slow waves

(200 Hz) applied at that point had no effect. At the same time, the symptoms noted were lying on the side, tachypnoea, hypersalivation, motor activity resembling running, loss of tone in the limb muscles, absolute inability to walk.



Part *c*: Clonic convulsions at the end of the convulsive period. Note onset of bioelectric silence



Part *d*: Thirty minutes after Mydeton. No response to 200 Hz sound

These findings make it clear that Mydeton has a very potent action on the central nervous system and, presumably through stimulating the rostal reticular formation, gives rise to a typical arousal reaction in the cat. Further investigations are needed to clarify the exact site of action. Such convulsions

occurred exclusively in cats and therefore the results cannot be considered valid for other species. The action of morphine, too, is known to vary from species to species, for example it causes motor excitation in the mouse, a cataleptic state in the rat, tonic-clonic convulsions in the cat, and inertia, sleep in the dog.

Action on the circulation, respiration and autonomic ganglia in the cat

An intraveneous dose of 0.5 mg/kg Mydeton caused transient fall in blood pressure. The intravenous injection of 5 mg/kg caused a vasodepression of 80 to 90 mmHg, which was normalized in about 1 minute at the beginning of the experiment. The vasodepression in response to the fourth injection was more protracted, associated with apnoea lasting 10 to 20 sec. This did not occur after bilateral vagotomy, while the drop in blood pressure was unaffected. Thus, the Mydeton-induced apnoea is apparently a pulmonary chemoreflex. Apnoea was often followed by hyperpneoa. This was independent of the hypotension, because it persisted after blood pressure had become normalized. All these effects are illustrated in Fig. 9. Atropine had no inhibitory effect either on the vasodepressor response or on the apnoea.

Mydeton did not affect the hypotensive response to acetylcholine and histamine (Fig. 9), *i. e.* it has no atropine-like and anti-histamine actions. It greatly reduced the hypertensive response to choline phenylether (Fig. 9) and decreased the vasopressor action of adrenaline.

The protracted falls in blood pressure in some cases and the inhibition of the vasopressor activity of choline phenylether have justified investigations into an eventual action on ganglia, although in our earlier studies the aminoketones were found to possess no or very little such activity [5, 6]. It is seen in Fig. 9, that in response to 5 mg/kg of Mydeton the amplitudes of the nictitating membrane contractions elicited by electrical stimulation of the cervical preganglionic sympathetic fibre at 30 sec intervals were decreased by about 20 to 30 per cent. More detailed studies showed the ganglionic blocking action to be about 2 or 3 times weaker but more lasting than that of TEAB.

Action on the heart

Cardiac activity in the cat was recorded *in situ* by a cardiometer. Fig. 9 shows that after opening the chest and applying the cardiometer blood pressure set at around 50 mm Hg. An infusion of 5 μg/kg/min adrenaline increased diastolic filling and decreased systolic volume, in other words, it increased the output. Mydeton 5 mg/kg intravenously caused a very short decrease, then an increase and finally a gradual decrease of the stroke volume, which reached its maximum 3 to 4 minutes after the injection. To decide whether the diminution

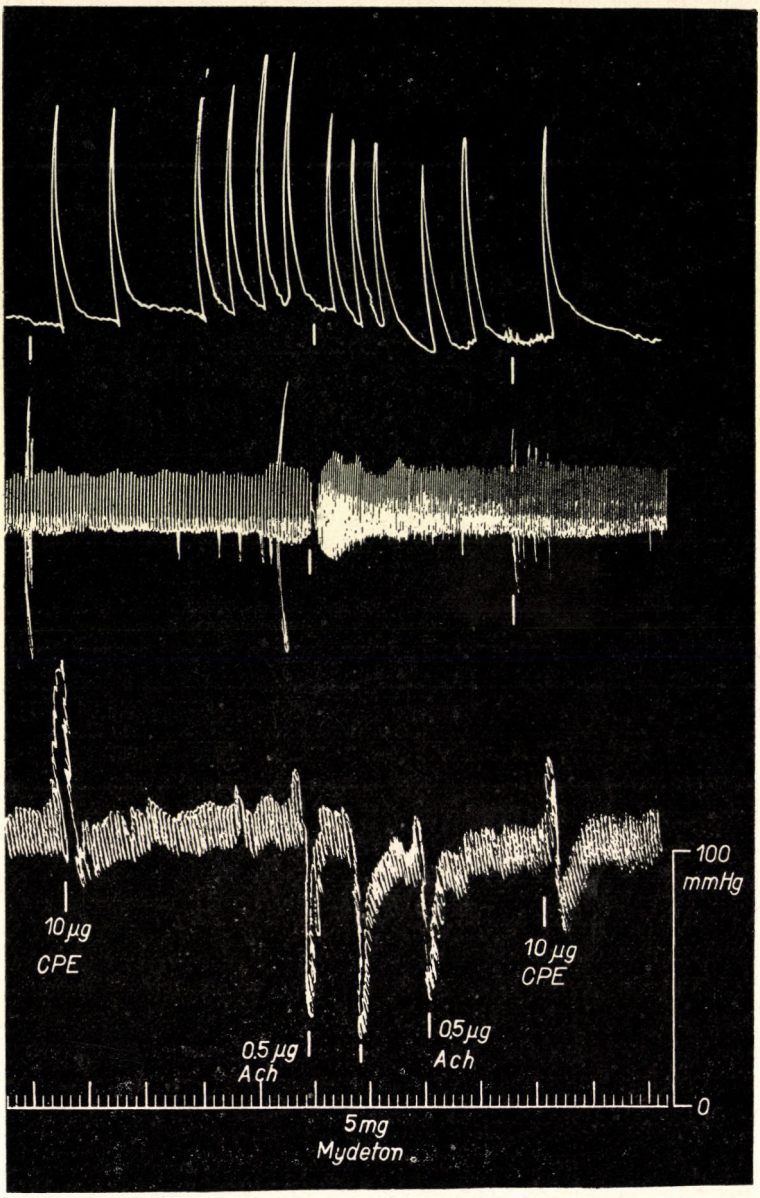


Fig. 8. Cat, 3000 g, Chloralose-urethane anaesthesia. Effect of Mydeton on the vascular and respiratory actions of choline phenylether and acetylcholine and on the nictitating membrane response to the electrical stimulation of the cervical preganglionic fibre.
In downward order: nictitating membrane, respiration, blood pressure.
Time signal on 0 line: 10 sec

of cardiac output was due to peripheral vasodilatation, or to a weakness of heart action as indicated by the cardiometer tracings, the bioelectric ativity of the heart was studied by ECG. The results are presented in Fig. 10. About 3 seconds after the administration of Mydeton the height of the R waves decreased markedly. This lasted about 3 seconds, then the QRS complex gradually increased in height, reaching the initial size in about 2 to 3 minutes, *i. e.* at a

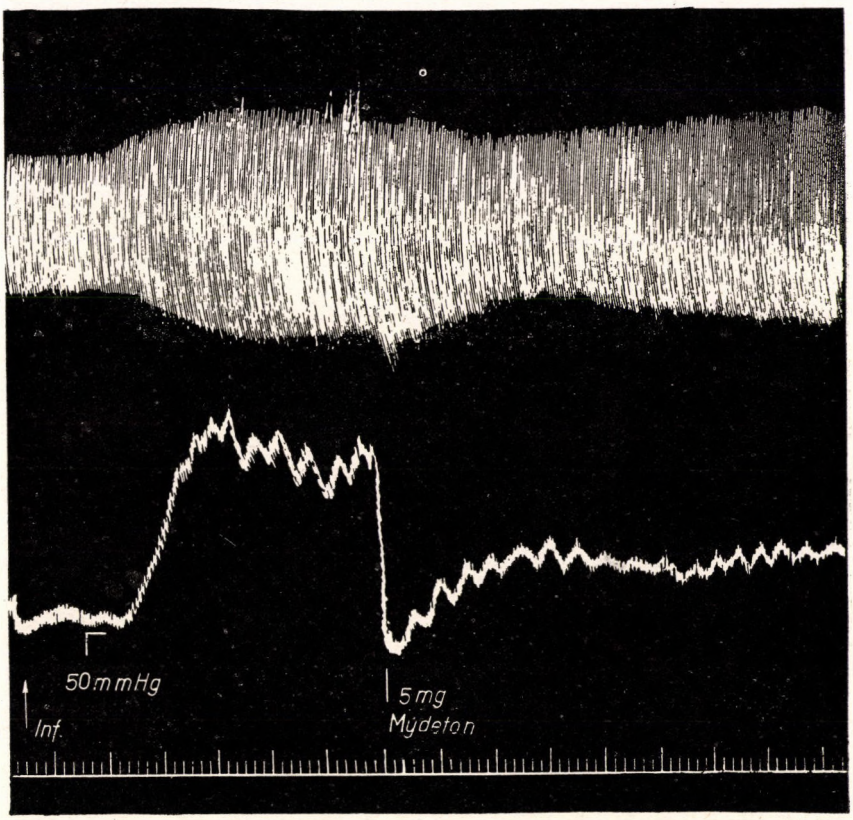


Fig. 9. Cat, 3600 g. Chloralose-urethane anaesthesia. Effect of Mydeton on stroke volume. In downward order: cardiometer, blood pressure, time signal (10 sec). In cardiometer curve: top: diastole, bottom: systole. Inf.: onset of adrenaline infusion, 5 μg/kg/min

ime when the decrease of cardiac output was the greatest. Thus, the fall in blood pressure seems to be due to a decrease of peripheral resistance and the diminution of the stroke volume must have been a result of this. The decrease of peripheral resistance may be traced back to a mild ganglionic blocking action, on the one hand, and, as it will be seen later, to a spasmolytic action, on the other.

Parpanit, too, is known to cause a fall in blood pressure and for this reason we have examined its effect on the ECG of the cat. Fig. 11 shows that 5 mg/kg of Parpanit injected intravenously caused a decrease in the height and even splitting of the R waves, but for a short while only.

The cardiac actions of Mydeton and Parpanit were studied in frog hearts, too. In concentrations of from 25 to 100 μg/ml (depending on the hearts),

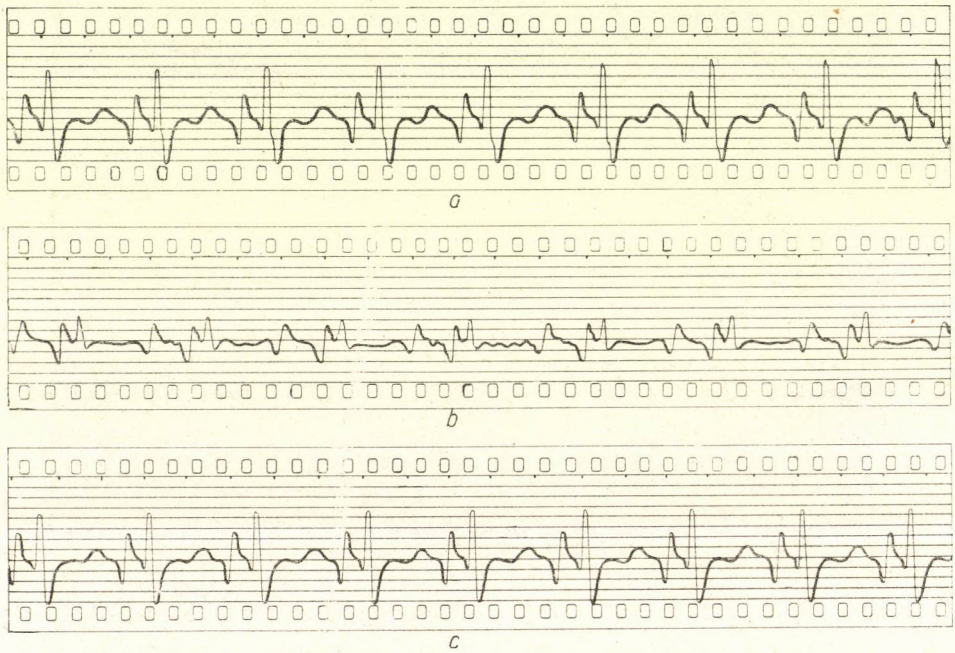


Fig. 10. Cat. Effect of Mydeton on the ECG. Lead II. 5 mg/kg Mydeton intravenously. Split R, negative T, prolonged PQ interval.
a) Before Mydeton. *b)* During Mydeton. *c)* 3 Minutes after Mydeton

Mydeton had a negative inotropic action on the isolated frog heart and did not interfere with the formation and conduction of impulses. The heart kept on beating in the original rhythm, and no diastolic arrest resulted even at a concentration of 100 μg/ml. In contrast with this, similar concentrations of Parpanit markedly weaken the heart and produce not only a negative inotropic action but also arrhythmia and bradycardia.

Mydeton is easy to wash out from the heart muscle, thus its effect is reversible, as opposed to Parpanit, which is difficult to remove even by repeated washing and which alters the amplitude for as long as $\frac{1}{2}$ to 1 hour.

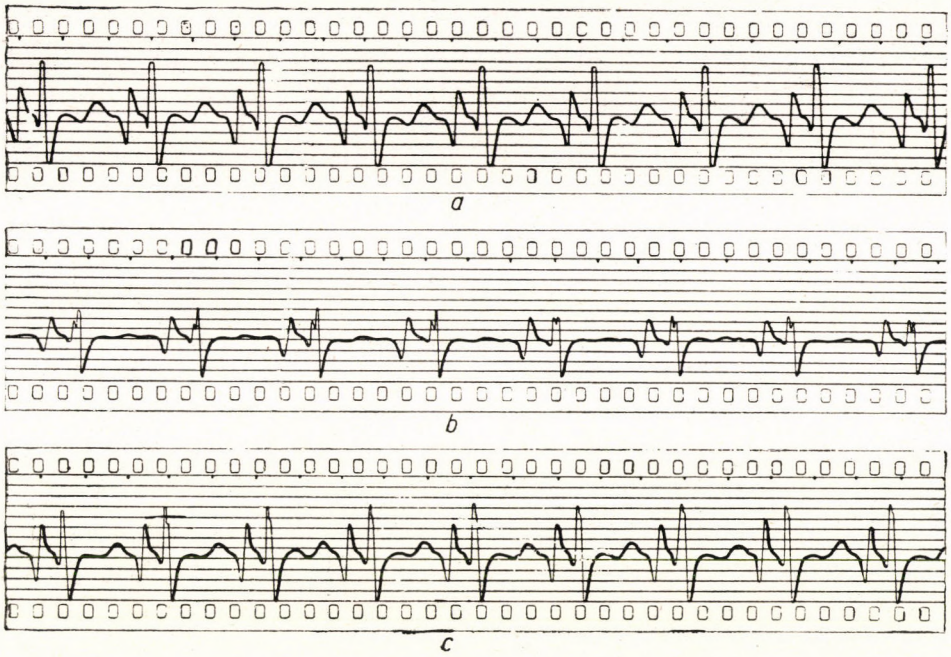


Fig. 11. Cat. Effect of Parpanit on the ECG. Lead II. 5 mg/kg Parpanit intravenously. Split R, isoelectric T, prolonged PG.
a) Before Parpanit. *b)* During Parpanit. *c)* 3 minutes after Parpanit

Actions on smooth muscle

The aminoketones have been found to possess a weak spasmolytic effect [18]. It has therefore been thought necessary to study Mydeton from this respect. Parpanit and papaverine were used for comparison. These studies were made on the isolated guinea pig ileum, in a Magnus dish containing 18 ml of Tyrode's solution, under constant oxygenation, at 38°C. The results are presented in Table IV. Mydeton was about 25 to 50 times less potent than Parpanit in the inhibition of the contraction induced by acetylcholine, nicotine and histamine.

The difference between Mydeton and Parpanit was not so marked in the inhibition of $BaCl_2$ spasm. The spasm caused by 0.28 mg/ml of $BaCl_2$ was completely inhibited by 0.05 mg/ml of Mydeton; 0,042 mg/ml of Parpanit and 0,03 mg/ml of papaverine were needed to produce the same effect. Since ten times smaller doses of Mydeton were required to inhibit the acetylcholine spasm, it appeared as if Mydeton had also an atropine-like activity. This, however, is not true, because identical doses of Mydeton were required to relieve the spasm caused by acetylcholine, nicotine and histamine, respectively. Parpanit is most potent against acetylcholine spasm, *i. e.* it has chiefly an atropine-like action.

Table IV

Action on smooth muscle in the guinea pig intestine

Compound	Dose µg/18 ml	Compound, µg/18 ml					Spasmolytic action, %				
		Ach	Hist	5HT	Ni	$BaCl_2$	Ach	Hist	5HT	Ni	$BaCl_2$
Mydeton	5	1					60				
	10		0.1					70			
	20	1					75				
	25		0.1	25	30			100	60	80	
	50	1		25	30		100		100	100	
	1500					5000					100
	4000					5000					100
Papaverine	50					5000					100
Parpanit	1	1			30		95			95	
	2.5	1					100				
	5		0.1		30			100		100	
	10			25					100		
	300					5000					15
	750					5000					72
	1000					5000					83

Abbreviations: Ach: acetylcholine,
Hist: histamine,
5HT: 5-hydroxytryptamine,
Ni: nicotine.

Discussion

Mydeton is active on the central nervous system, in the first place. This conclusion was drawn from the following findings:

a) its action varies with the species of animal tested;
b) it inhibits multisynaptic spinal reflexes;
c) it lessens the toxicity of strychnine;
d) it inhibits the convulsions in electric shock;
e) it has a marked anti-nitocine action; and
f) it inhibits the tremor caused by harmine.

Mydeton has a very weak effect on the autonomic nervous system; it possesses neither atropine-like, nor antihistamine, nor adrenolytic activities. Its ganglionic blocking action is about 2 or 3 times weaker than that of TEA. It is a less potent spasmolytic agent than papaverine.

On the basis of these pharmacological data Mydeton is recommended for use in the treatment of such nervous diseases in which extrapyramidal motor

disturbances predominate. LEHOCZKY [19] has shown Mydeton to be superior to chlorpromazine in relieving the symptoms and in side effects.

The central nervous site of action needs further investigation. Informative experiments proved Mydeton to be capable of relieving the decerebration rigidity in the cat. A dose of 5 mg/kg intravenously caused immediate relaxation of the muscles maintaining extensor tone. One of its most important sites of action seems therefore to be in the bulbar facilitatory system. We believe this action to be of decisive importance in the inhibition of the reflex irradiation caused by strichnine. At the same time, an inhibitory action on the spinal synapses cannot be ruled out.

The inhibition of electroshock convulsions is believed to be an important property of Mydeton, which make its use beneficial in the treatment of grand mal and eventually of petit mal seizures [20]. Further research into the mechanism of the inhibition of electro-convulsions may bring to light new evidence helping to elucidate the mode of action of anticonvulsant drugs. Electrophysiological studies have namely shown such drugs to have different sites of action. Phenobarbital lessens the excitability not only of the diencephalon, but also of the Ammon's horn [21]. Phenuron, used in temporal epilepsy, has no influence on the threshold of rhinencephalic after-discharges, but prolongs the slow components of the multispike after-discharges. Tridione does not act on the hippocampus, but depresses the cortex and the diencephalon. Diphenylhydantoine has no effect on the after-discharges of the cortex and rhinencephalon, but considerably increases the diencephalic after-discharge threshold [22].

It has been mentioned and illustrated by EEG curves that Mydeton causes convulsions in the cat. The convulsions seem to be subcortical in origin. Although such an effect was not observed in species other than the cat, and the results obtained in cats cannot be applied without restrictions to other species, it may be worth while to point out that for example according to PRESTON [23], chlorpromazine produces convulsive activity in the hippocampus, and clinical evidence is available to show that chlorpromazine can produce epileptic spikes in the EEG [24].

Mydeton causes narcosis in extremely large, near-toxic doses only. However, even very small doses cause muscle weakness, as well as a normalization of the hyperactivity produced by prostigmine, so its therapeutic range is very wide. Pharmacological studies show Mydeton to be closely similar to Myanesin, which is a selective paralyser of the interneurones [25], and for this reason Mydeton may be listed among the so-called interneurone blocking agents.

Mydeton causes a relaxation of the nictitating membrane contracted as a result of prolonged preganglionic stimulation. It may therefore possess a ganglionic blocking action. As, however, the blood pressure changes in response to Mydeton are different from those seen in the characteristic responses to

ganglionic blocking agents, Mydeton may cause the relaxation of the nictitating membrane directly, *i. e.* postganglionarly, through a spasmolytic action similar to that of papaverine.

This may explain also its heart-weakening action. Intravenously injected, papaverine is namely known to cause heart failure and a weakening of the function of the isolated papillary muscle [26]. This action of Mydeton is clearly visible in the cardiographic and ECG tracing. However, it is also possible that the severe ECG changes are due to the fall in blood pressure and the consequential marked reduction of the O_2-supply to the heart.

Experiments on frog hearts suggest that Mydeton may act directly on the heart. However, it weakens the heart much less than papaverine or Parpanit do and its effect is definitely reversible.

These pharmacological properties may make Mydeton a valuable drug for the treatment of extrapyramidal motor disturbances of various origin, as it is indicated also by pertaining clinical observation (LEHOCZKY, BLAZSÓ).

LITERATURE

1. DOMENJOZ, R.: Schweiz. med. Wschr. **76**, 1282 (1946).
2. LONGO, V. G., BERGER, G. P., BOVET, D.: J. Pharmacol. **111**, 349 (1954).
3. LEHMANN, H. E., HANRAHAN, G. E.: Arch. Neurol. Psychiat. (Chicago) **71**, 227 (1954).
4. SOMOGYI, I, TASS, GY.: Orv. Hetil. **98**, 937 (1957).
5. NÁDOR, K., PÓRSZÁSZ, J.: Arzneimitt. Forsch. **8**, 313 (1958).
6. PÓRSZÁSZ, J.: Thesis, Budapest, 1955, p. 186.
7. BEHRENS, B.: Arch. exp. Path. Pharmak. **167**, 365 (1932).
8. ISSEKUTZ, B. SEN., PÓRSZÁSZ, J., ISSEKUTZ, L., NÁDOR, K.: Acta physiol. hung. **6**, 95 (1954).
9. HOTOVY, R., ERDNISS, F.: Arch. exp. Path. Pharmak. **209**, 204 (1950).
10. BOVET, D., LONGO, F.: J. Pharmacol. **102**, 22 (1951).
11. CAHEN, R. K., LYNES, TH.: J. Pharmacol. **103**, 44 (1951).
12. HAUSCHILD, F.: Pharmakologie und Grundlagen der Toxikologie. Thieme, Leipzig, 1956, p. 638.
13. ZETLER, G.: Arch. exp. Path. Pharmak. **231**, 34 (1957).
14. TAUGNER, R., CULP, W.: Arch. exp. Path. Pharmak. **220**, 423 (1953).
15. BERGER, F. M.: Pharmacol. Rev. **1**, 243 (1949).
16. GUALTIEROTTI, T.: J. Physiol. **128**, 320, 326 (1955).
17. HAUSCHIELD, F: Pharmakologie. p. 803. Thieme, Leipzig, 1956.
18. PÓRSZÁSZ, J., NÁDOR, K., GIBISZER-PÓRSZÁSZ, K., WIESZT, T., PADÁNYI, R.: Acta physiol. hung. **7**, 139 (1955).
19. LEHOCZKY, T.: Gyógyszereink, 1959, 41. sz.
20. BLAZSÓ, S.: Personal communication.
21. GANGLOFF, H., MONNIER, M.: Pflüger's Arch. ges. Physiol. **261**, 421 (1955).
22. GANGLOFF, H., MONNIER, M.: Ther. Umsch. **13**, 257 (1956).
23. PRESTON, J. B.: J. Pharmacol. **118**, 100 (1956).
24. BETHE, D., ITIL, T.: Arzneimitt. Forsch. **8**, 418 (1958).
25. KING, E. E., UNNA, KL. R.: J. Pharmacol. **111**, 293 (1954).
26. TARDOS, L.: Acta physiol. hung. **7**, 319 (1955).

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Orvostudományi Egyetem Élettani Intézete, Szeged, és

Magyar Tudományos Akadémia Gyógyszerkutatási Osztálya, Budapest (VIII. Üllői út 26.).

Vth INTERNATIONAL CONGRESS OF BIOCHEMISTRY

Moscow (USSR), August 10—16, 1961

General Information

According to the resolution of the General Assembly of the International Union of Biochemistry, the *Vth International Congress of Biochemistry* will be held ind Moscow, August 10—16, 1961.

The USSR Biochemical Society cordially invites everyone interested in the broadest aspects of biochemistry to participate as an active member. Each active member is allowed to introduce any member of his family as an associate member.

Those who would like to participate in the work of the Vth International Congress of Biochemistry are requested to fill in the enclosed Registration Forms (in duplicate) and to send them to the Executive Committee of the Congress (USSR, Moscov, B—71, Leninsky Prospekt, 33). The latest date at which Registration Forms will be accepted is March 31, 1961 and December 31, 1960 for the forms I and II, respectively.

Members who intend to contribute to one of the sectional meetings either a communication or a demonstration should send in three copies of the summary of their communication (up to 200 words) by December 31, 1960.

The registration fee has been fixed at $ 13 for the active members and at $ 2 for the associate members. Payment of the registration fee is requested to the account of the Congress No. 380 083 in the USSR Vneshtorgbank by March 31, 1961. This registration fee is not returnable.

Active members who register before the deadline will receive, before the Congress opens, a second circular detailed information on the organization of the Congress, the final programme, a copy of the abstracts of sectional communications, and the preprints of 2 of the symposia at each member's choice and, after the Congress, a copy of the texts of the two lectures given at the plenary sessions. The preprints of the other symposia can be obtained for extra payment. Registered members who will not be able to attend the Congress will receive this documentation by mail.

Scientific Programme of the Congress

In accordance with the recommendation of the General Assembly of the International Union of Biochemistry, the organizers of the Congress intend to draw attention to a restricted number of the most burning problems of contemporary biochemistry, With this purpose in view there will be organized:

I. Lectures given at plenary sessions

II. Symposia

III. Sectional sessions for members' communications and demonstrations.

I. Two general lectures will be given at the plenary sessions of the Congress :

a) D. Green (USA) — "*Subcellular Structure and Enzymatic Function*".

b) F. Šorm (Czechoslovakia) — «*Microstructure of Protein in Connection with its Biological Function*».

II. It is planned to organize the following symposia :

1. Biopolymers: Molecular Structure and Biochemical Activity.
2. Functional Biochemistry of Cell Structures.
3. Evolutionary Biochemistry.
4. General Mechanisms of Action and Specific Inhibition of Enzymes.
5. Phosphorylating and Non-phosphorylating Pathways of Oxidation.
6. Mechanism of Photosynthesis.
7. Principal Mechanisms and Pathways of Biosynthesis.
8. Biochemical Principles of Food Industry.

III. The following sections will be organized :

1. Chemistry of Compounds of Biological Interest
2. Biochemistry of Proteins and Amino Acids
3. Biochemistry of Nucleic Acids and Nucleoproteins
4. Biochemistry of Viruses
5. Enzymology
6. Comparative Biochemistry
7. Biochemistry of Antibiotics
8. Industrial Biochemistry
9. Cyto- and Histochemistry
10. Muscle Biochemistry
11. Biochemistry of the Nevous System
12. Vitamins and Nutrition
13. Biochemistry of Hormones
14. Biochemistry of Microorganisms
15. Plant Biochemistry
16. Clinical Biochemistry
17. Biochemistry of Carbohydrates
18. Biochemical Pharmacology
19. Biochemical Genetics
20. Immunochemistry
21. Biochemistry of Malignant Tumors
22. Biochemistry of Photosynthesis
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Die von der Medizinischen Kommission der FIR (Internationale Föderation der Widerstandskämpfer) einberufene

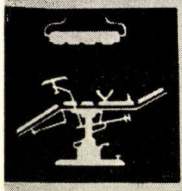
III. INTERNATIONALE MEDIZINISCHE KONFERENZ

wird vom 17. bis 19. März, 1961 in Lüttich (Belgien) zusammentreten. Dabei sollen insbesondere die Behandlungsmethoden der progressiven Asthenie und der vorzeitigen Vergreisung besprochen werden.

Anfragen und Anmeldungen zur Teilnahme an der Tagung sind an die Medizinische Kommission der FIR, Wien II, Castellezg. 35, zu richten.

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Farkas Sándor
A kézirat nyomdába érkezett: 1960. VIII. 17 — Terjedelem: 8 (A/5) ív, 38 ábra

1960.51901 — Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György



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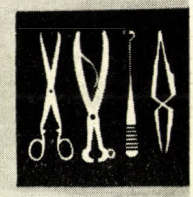

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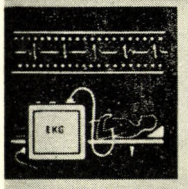
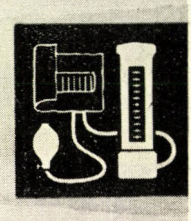


Electro-medical apparatus



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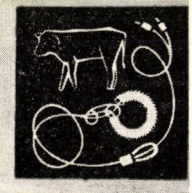
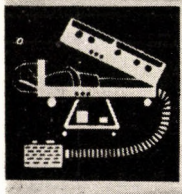
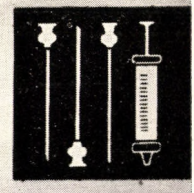
Dentistry chairs, dental apparatus type "Unit", dental material



Veterinary instruments

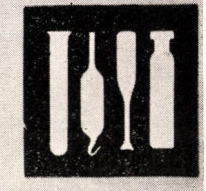


Various types of iron lungs



Injection needles and syringes

Rocking respirator and other apparatus for paralysis treatments



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РЕЗЮМЕ

ИССЛЕДОВАНИЕ ФУНКЦИОНАЛЬНОГО ЗНАЧЕНИЯ СВЯЗАННЫХ К МИОФИБРИЛЛАМ НУКЛЕОТИДОВ

I. Исследование состояния фосфориляции связанных к миофибриллам нуклеотидов *in vitro*

Э. БИРО и А. МЮЛРАД

Авторы исследовали общее количество и содержание гидролизируемого фосфора нуклеотидов, связанных к миофибриллам, сокращенным аденозинтрифосфатом, и находящимся вследствие различных воздействий в присутствии АТФ в расслабленном состоянии. В случае релаксации, вызванной расслабляющими факторами, этилендиаминтетраацетатом, при низкой температуре, количество гидролизируемого фосфора (соотношение «Р.И» связанных нуклеотидов) уменьшается по сравнению с фосфором в нерасслабленных миофибриллах, — в случае релаксации, вызванной большой ионной силой, мочевиной, или же концентрацией АТФ больше оптимальной, не наблюдалось данного уменьшения содержания фосфора.

Согласно опытам с меченным в фосфоре АТФ разница имеет место не вследствие приема фосфора в сокращенном состоянии, а путем диссоциирования в расслабленном состоянии одной части связанных нуклеотидов, прежде всего АТФ.

ИССЛЕДОВАНИЕ С ФУНКЦИОНАЛЬНОГО ЗНАЧЕНИЯ СВЯЗАННЫХ К МИОФИБРИЛЛАМ НУКЛЕОТИДОВ

II. Исследование обмена веществ связанных фосфорных фракций с помощью меченного фосфора

Э. БИРО и А. МЮЛРАД

Существенного мечения поддающейся гидролизу связанной фракции как и связанного неорганического фосфата миофибрилл не состоится, если миофибриллы в позициях γ и В расщепляют равномерно меченный АТФ. Подобным образом не снижается радиоактивность фосфорных фракций миофибрилл, меченных введенным *in vivo* P^{32}, если изолированная миофибрилла расщепляет не радиоактивный АТФ.

Скорость новообразования связанных к миофибриллам фосфатных фракций многократно больше, чем, согласно результатам Велика (5), скорость новообразования актиновых белков.

Вышеизложенные результаты обсуждаются с точки зрения предположенной промежуточной роли связанных нуклеотидов, в частности сопоставляя их с результатами экспериментов Штромана (9), Перри (10) или же Ульбрехта и Ульбрехта (11).

К МЕХАНИЗМУ ДЕЙСТВИЯ СТРЕПТОМИЦИНА

I. Действие стрептомицина на терминальное окисление *E. coli*

П. ЗАБОШ

Стрептомицин в бактериостатической концентрации, при исследованиях на *E. coli B* и *E. coli* 055, не оказывает значительного действия на окисление применяемых автором субстратов. При более высоких концентрациях, особенно, в среде с небольшой ионной силой, можно было обнаружить частичное торможение окисления, но оно наступает только после 20—40 минутного скрытого периода и постепенно прогрессирует.

Стрептомицин в бактериостатической концентрации в зависимости от условий, после более или менее продолжительного воздействия, прекращает адаптивного характера синтез терминальной окислительной системы в клетках *E. coli*, происходящих из полуанаэробных культур. На указанное тормозящее действие стрептомицина влияет концентрация электролитов и pH окружающей среды.

К МЕХАНИЗМУ ДЕЙСТВИЯ СТРЕПТОМИЦИНА

II. Действие стрептомицина на синтез β-галактозидазы в клетках штаммов *E. coli B*

П. ЗАБОШ

В бактериостатической концентрации стрептомицин совершенно прекращает синтез β-галактозидазы в клетках *E. coli B* в том случае, если его добавляют к реакционной смеси одновременно с добавлением индуктора. Если же стрептомицин добавляется позднее, то он прекращает синтез энзима только по истечении определенного скрытого периода. Продолжительность предшествующего полному торможению, скрытого периода, и количество синтезированного между тем энзима, зависит от того, сколько времени прошло между добавлением индуктора и добавлением стрептомицина. Если добавить стрептомицин на 40—60-ой минуте, то всегда наблюдается парадоксальное явление: развитию торможения предшествует временное ускорение синтеза энзима.

Синтез общих белков в заторможенных стрептомицином клетках некоторое время продолжается дальше, линейно с измеренной при добавлении антибиотика скоростью, и лишь по истечении 40—60 минут наступает полное торможение.

При действии стрептомицина синтез клеточных белков и нуклеиновых кислот тормозится приблизительно одинаковым образом. В предшествующей полному торможению линейной фазе роста, отношение нуклеиновая кислота/белок устанавливается на величине, измеренной при введении стрептомицина, в то время, как рост контрольных клеток идет экспоненциально и относительное количество нуклеиновых кислот в них, повышается в логарифмической фазе.

ПОЛУАВТОМАТИЧЕСКИЙ АППАРАТ ДЛЯ ВЫТЯГИВАНИЯ СТЕКЛЯННОГО МИКРОЭЛЕКТРОДА

Я. ПОРСАС и Ф. САБО

Авторами был разработан полуавтоматический аппарат для вытягивания стеклянного электрода, при помощи которого можно из стеклянных капилляров с различным исходным диаметром изготовить стеклянные капилляры с верхним диаметром от 0,25 до 1,5 микронов. Метод весьма простой, осуществляемый в любой лаборатории. Дается подробное описание метода изготовления стеклянных капилляров, их заполнения и хранения.

ИССЛЕДОВАНИЯ НА КРЫСАХ ФУНКЦИИ КОРЫ НАДПОЧЕЧНИКОВ

Э. ЭНДРЁЦИ и ЯНГ ТЕН-ЛА

Кровь надпочечной вены крыс содержит следующие кортикоиды: 6—α-гидроксикортизол, альдостерон, не идентифицированный стероид с положительной флюоресценцией натрием, но не дающий восстановления тетразолем, кортикостерон, 11-дегидрокортикостерон, Δ^4-андростерон-11-ol-3,17-дион и Δ^4-андростерон-3,17-дион. Авторы с помощью чувствительного метода восстановления тетразолем определили содержание кортикоидов в собранной в течение 12 минут крови надпочечников отдельных животных, или же в вытяжке соединенной от нескольких животных крови надпочечной вены секрецию альдостерона.

ДЕЙСТВИЕ РАБОТЫ, СОВЕРШЕННОЙ ПРИ ГИПОТЕРМИИ И ГИПЕРТЕРМИИ НА ФУНКЦИЮ ГИПОФИЗ-КОРА НАДПОЧЕЧНИКОВ

ЯНГ ТЕН-ЛА и Э. ЭНДРЁЦИ

Авторы исследовали в опытах по плаванию белых крыс в воде различной температуры действие полного утомления на состав кортикоидов в крови надпочечной вены и на синтез гормонов инкубированной в условиях *in vitro* надпочечной тканью. Было установлено, что содержание кортикостерона в случае плавания при оптимальной температуре

практически не изменяется, в то время как оно при 18 °С начинает снижаться. В противоположность этому содержание альдостерона выраженно повышается в случае плавания как при температуре 30°, так и 18° и 43 °С, причем повышение было больше всего в случае низкой температуры среды. Изменение соединения X, наблюденного в венозной крови надпочечников в качестве постоянного компонента, было подобно изменениям величины кортикостерона. Надпочечная ткань показала *in vitro* повышенный синтез кортикостерона, даже в тех случаях, когда его величина в крови надпочечной вены не изменялась, или же снижалась.

УСЛОВНАЯ И БЕЗУСЛОВНАЯ ЭЛЕКТРИЧЕСКАЯ АКТИВАЦИЯ КОРЫ ГОЛОВНОГО МОЗГА ПРИ РАЗДРАЖЕНИИ ПОЧЕЧНОЙ ЛОХАНКИ

Г. АДАМ и И. МЕСАРОШ

В хронических опытах на собаках-самках, имеющих короковые электроды и свищ одного мочеточника, было выявлено, что слабое механическое раздражение почечной лоханки обусловливает электрическую активацию мозга (ориентировочную реакцию). Эта электрическая десинхронизация может быть погашена. После длительного угашения этой реакции, при сочетании раздражения почек с какими либо экстероцептивными (звуковыми или кожными) раздражителями, возникает условная интероцептивная электрическая активация.

О РОЛИ АКТИВИРУЮЩЕЙ СИСТЕМЫ СТВОЛА МОЗГА В ОБРАЗОВАНИИ УСЛОВНОГО РЕФЛЕКСА НА ВИСЦЕРАЛЬНОЕ РАЗДРАЖЕНИЕ

Г. АДАМ, И. МЕСАРОШ, К. ЛЕХОЦКИ, А. НАДЬ и А. РАЙК

У собак с хронически введенными в кору головного мозга и в средний мозг электродами и со свищем тонкой кишки, авторы сочетали механическое раздражение тонкой кишки с прямым электрическим раздражением восходящей активирующей системы среднего мозга. После такого сочетания раздражений применением интероцептивного раздражения можно вызвать условную электрическую активацию мозга. Из результатов авторов следует, что на основе реакции пробуждения мезенцефалического типа можно выработать условный рефлекс, и следовательно является вероятным, что исходящая из висцеральной рецепторной области условная мозговая электрическая активация включается через неспецифическую ретикулярную систему среднего мозга.

ФАРМАКОЛОГИЯ МИДЕТОНА (МИДОКАЛМ, 1-ПИПЕРИДИНО-2-МЕТИЛ-3-*p*-ТОЛИЛ-ПРОПАНОН-3), НОВОГО СОЕДИНЕНИЯ, БЛОКИРУЮЩЕГО ИНТЕРНЕЙРОНЫ

Я. ПОРСАС, К. НАДОР, К ГИБИСЕР-ПОРСАС и Т. БАРАНКАИ

Наиболее характерное фармакологическое действие Мидетона, 1-пиперидино-2-метил-3-*p*-толил-пропанона-3 указывает на то, что его точкой воздействия является центральная нервная система. Это действие заключается в следующем: влияние Мидетона на поведение животных различных видов различное: на мыши и крысы это соединение оказывает успокаивающее действие, на кроликах наблюдается значительная мышечная слабость, на кошках мышечная слабость, полное расстройство ходьбы наступает лишь после появления клинических судорог. Наркоз вызывается только почти токсическими дозами. Мидетон тормозит многосинаптические спинальные рефлексы, снижает у крыс повышенные стрихнином рефлексы и смертность от стрихнина. Мидетон препятствует наступлению электрического шока. В этом отношении его действие сильнее чем действие Дифенала или Мизолина. Он обладает выраженным антиникотиновым действием и препятствует вызванному гармином дрожанию.

Мидетон не имеет атропиноподобного, антигистаминового, адреналитического действий. Парализирующее ганглии действие в 2—3 раза меньше действия ТЕА. Его тормозящее спазм гладкой мышцы свойство проявляется прибл. в 2 раза слабее действия папаверина.

Следовательно, данное соединение оказывает выраженное действие, главным образом, на многосинаптические нейронные системы и поэтому его можно причислить к так наз. ослабляющим интернейроны препаратам.

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Biochemia

PURIFICATION OF THE RIBONUCLEIC ACID INDUCING PENICILLINASE FORMATION IN B. CEREUS CELLS

By

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(Received July 15, 1960)

Two methods for the purification of RNA extracted from constitutively penicillinase-forming *B. cereus* cells have been applied and compared. These purified RNA preparations displayed essentially the same effect in giving rise to penicillinase formation in inducible *B. cereus* cells, as a crude extract described earlier.

The RNA preparations obtained by the two methods as well as the crude extract have been compared quantitatively as to their effect concerning penicillinase synthesis.

It has been shown that the saturating level of the RNA still having full activity must be in the order of 10^2—10^3 molecules RNA per cell.

The supposed role of the specific RNA and that of the RNAse treatment have been discussed.

As described earlier [1, 2], a *M* NaCl-extract from *B. cereus*-NRRL-B-569/H, a penicillinase-constitutive strain [3], if added to ribonuclease-pretreated *B. cereus*-NRRL-B-569 (penicillinase-inducible) cells is capable of giving rise to a transient production of penicillinase in the recipient cells under suitable conditions. The effect seems in many aspects to be different from the natural induction, *i. e.* from induction with penicillin. However, the increased activity of the enzyme under the effect of the added RNA is due to a real synthesis of enzyme protein *de novo* [4].

We worked under the assumption that the active principle in our crude extract was a specific RNA and in order to prove this we tried to purify the extract and collect qualitative as well as quantitative data about its nature.

Materials and methods

Organisms. Throughout the whole series of experiments we were using the *B. cereus* NRRL-B-569/H cells, as sources of the active principle, and *B. cereus* NRRL-B-569 cells, as receptors. The first one is a one-step mutant of the second, isolated by Sneath [3] and has the ability of forming considerable amounts of exopenicillinase constitutively. The 569 strain is inducible with penicillin. The induced synthesis of penicillinase in the 569 cells was thoroughly studied by Pollock [5, 6] and the proof of the penicillinase formed by the inductive and constitutive cells being identical was also shown by Pollock *et al.* [7, 8]. We obtained both strains from Dr. Pollock and are very indebted to him both for that and for his interest in our work.

Medium. The nutrient medium as well as the reaction mixture for studying penicillinase synthesis was essentially the same as used by Pollock and Kramer [9], the only modification being the replacement of the amino acid mixture by 1 per cent (w/v) casein hydrolysate.

Enzymes. Crystalline bovine ribonuclease and deoxyribonuclease were made in our Institute, according to Kunitz [10]. Both enzymes were free of proteolytic activity.

For assaying nucleic acids we used a Type DU Beckman Spectrophotometer at 260 mμ wavelength. The amount of nucleic acids is always expressed as adenine-equivalent, *i. e.* μmole adenine/ml.

For rapid determination of penicillinase activity we used the iodine-method, described by PERRET [11] and modified by CITRI [12]. We calibrated the values on the basis of the manometric technique and found a good agreement between the two different methods (in the order of ±5 per cent). As we accepted only considerable amounts and differences and employed every conceivable control, — the iodine-method proved convenient and reliable in our experimental circumstances. — The values of penicillinase activity are expressed in penicillinase units (μmoles penicillin hydrolysed/hour, at 30°C, pH 7.1).

Bacterial dry weight was measured as optical density by a Biró—Fedorcsák type photometer, based on a calibration of direct gravimetric assay of dried bacterial cells.

Preparation of the receptor (569) cells

569 cells were grown in Erlenmeyer flasks containing fluid to $^1/_5$ of their volume. The flasks were shaken on a horizontal shaker at 37°C. At the logarithmic phase (0.3—0.6 mg b. d. w. /ml) the cells were harvested, resuspended in 0.9 per cent NaCl and shaken in the presence of 25 μg/ml ribonuclease for 60 min. at 37°C. The cells were then collected, washed twice with saline and resuspended in fresh medium to a concentration of approximately 1 mg b. d. w./ml.

Preparation of the active principle (RNA) from 569/H cells

569/H cells were grown in 200 ml of medium with shaking at 37°C until the late stationary phase (appr. 15—16 hours). The cells were harvested, frozen at —10°C and crushed as quickly as possible in a mortar with added starch *(amylum solani)*, using about 15 times as much starch as the weight of the wet, packed cells originally present. Every piece of equipment and material used during this procedure had been precooled at —10°C. After about 4—5 minutes of hard crushing with a pestle there resulted a homogeneous yellowish, slightly wet mass which was rapidly taken up in a small volume of saline and centrifuged at 1000 *g* for a few minutes. The yellowish opalescent supernatant contained a considerable amount of nucleic acids, as well as protein and other soluble cell-components, but not intact cells. The nucleic acids were extracted from this cell-free supernatant by either of the following methods.

a) *Purifications of RNA by the "chloroform-method"*

This procedure was adopted from the method described by HOTCHKISS [13] for the purification of the transforming principle, and slightly modified for our purposes.

To the supernatant obtained after centrifuging the crushed cells an equal volume of chloroform and 2.5% of the total volume of *iso*-amylalcohol was added at 0°C. After 10 min. vigorous shaking the mixture was centrifuged to 3 distinct layers. The bottom layer consisted of chloroform, the top layer of the water-phase, while the surface-denaturated, precipitated protein took place in the middle. The nucleic acids were precipitated from the separated water-phase with 3 volumes of ice-cold 96% ethanol. After centrifugation the precipitate was redissolved in the original volume of saline and incubated with 10 μg/ml deoxyribonuclease at 30° C for 15 min. in order to depolymerise and so remove DNA. The solution was cooled and the shaking in chloroform and the precipitation by ethanol repeated at least twice more, to remove proteins (including the added DNA-ase) as completely as possible. Lastly the precipitated RNA was dissolved in a small volume of 0.9 per cent NaCl. This solution had no penicillinase activity whatever and contained but traces of protein or DNA, the bulk of it being RNA (C-RNA).

b) *Purification of RNA by the "phenol-method"*

The method had been described by KIRBY [14] and applied later by GIERER and SCHRAMM [15] for the preparation of pure TMV-RNA.

The supernatant of the crushed cells was shaken vigorously with an equal volume of freshly distilled and water-saturated ice-cold phenol, then centrifuged. There were 4 phases distinguishable. At the bottom of the tube some precipitated material which proved to be DNA and part of the proteins; these were precipitated by the phenol, on the top of which could be seen the water-phase. Between the phenol and the water-phase there was another precipitate consisting of denatured protein. The phenol-phase together with the precipitate in it was discarded and the water-phase shaken out at least 3 times with fresh samples of phenol, taking care of keeping the temperature always below 3°C. After discarding the last

phenol-phase, the water-phase was shaken out 6 times with an equal volume of cold peroxide-free ether, to remove the phenol dissolved in water. Finally, the ether was removed by bubbling N_2 gas. The DNA contamination of this RNA (P-RNA) was always below 0.5% as measured by the indole-method [16].

Experiments and results

C-RNA was prepared from stationary-phase cells of 569/H. The concentration of the RNA was 0.0153 μmole adenine/ml. The 569 cells were grown to an optical density corresponding to 0.6 mg/ml b. d. w., taken up in saline and treated with ribonuclease. After thorough washing the cells were resuspended in 10 ml of medium, giving a concentration of 1.4 mg/ml b. d. w.

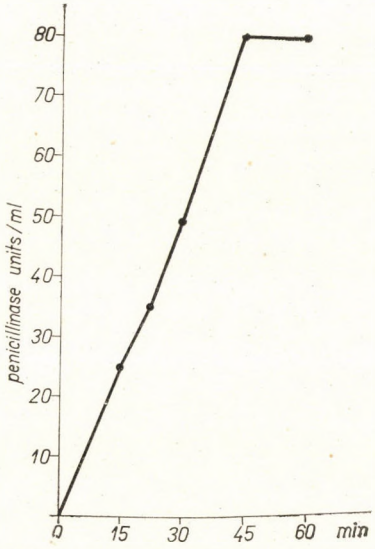

Fig. 1. Effect of C-RNA on penicillinase synthesis of B. cereus 569 cells

At zero time 1 ml of RNA was added and the first 2 ml sample was immediately pipetted into 2 ml of ice-cold chloramphenicol solution (50 μg/ml) to stop protein synthesis. The remaining cell-suspension was shaken at 37°C and samples were taken every 15 min for assaying penicillinase activity. As can be seen (Fig. 1) we could detect a considerable amount of newly formed enzyme after 15 minutes — a phenomenon never occurring after induction with penicillin. This means that the initial lag so characteristic of induced penicillinase synthesis disappears or at least becomes too short to be detectable.

To establish whether only the RNA possessed the ability of producing the enzyme-forming effect, after the first chloroform treatment we halved the water phase (at that time still containing both RNA and DNA) and incubated one half with DNAse (Fraction R), the other with RNAse (Fraction D),

using in both cases 10 μg/ml of the corresponding nuclease at 30°C, for 15 min. Subsequently both fractions were again halved. Half of both fractions was deproteinized as usual, the other half of Fraction R treated with ribonuclease (Fraction R_{RNAse}), and that of Fraction D with deoxyribonuclease (Fraction D_{DNAse}), then both were deproteinized. The four fractions were simultaneously added to 4 samples of RNAse-pretreated cells and penicillinase formation was followed. Table I shows the results of such an experiment.

Table I

Fractions

Minutes	D	R	RDNase	DRNase
	Penicillinase units/ml			
0	0	0	0	0
30	0	80	0	0
45	0	160	0	0
60	0	160	0	0

* 0 represents an enzymic activity lower than 10 units/ml.

As can be seen, only the highly polymerized RNA was capable of "inducing" penicillinase formation, DNA and depolymerized RNA had no effect.

Up to that point the C-RNA had served quite satisfactorily our purposes, except that it had to be prepared fresh almost every day, since a considerable loss of activity occurred on storage at —10°C after 24 hours and practically no activity was left after 2 days. Primarily for that reason we tried to prepare the RNA by a completely different method. We must say at once that from this point of view we have not had the least success, the P-RNA being even more unstable than the C-RNA. On the other hand, however, the specific "inducing" activity of the P-RNA was usually very high as compared with C-RNA.

In the experiments represented in Fig. 2 we used 3 different kinds of the active principle prepared from the same source. In every case the nucleic acid content was diluted to the same concentration. In the first series of experiments (Fig. 2a) the final concentration of the added nucleic acids in the reaction mixture was $3 \cdot 10^{-2}$ μmoles/ml adenine eq., in the second $3 \cdot 10^{-4}$ μmoles/ml (Fig. 2b).

Among the 3 preparations P-RNA showed by far the highest specific activity. Another remarkable finding was the lack of a significant difference due to the concentrations in the range tested. Addition of 20 μg/ml chloramphenicol naturally resulted in complete inhibition of enzyme synthesis.

As the RNA concentration used before was always well above the saturating level, it seemed absolutely necessary to determine the lowest RNA concentration still effective in producing penicillinase synthesis. These experiments were carried out with a P-RNA (Fig. 3).

In this particular experiment, the saturating concentration of RNA must have been approximately between $3.2 \cdot 10^{-4}$ and $8 \cdot 10^{-5}$ μmoles/ml adenine

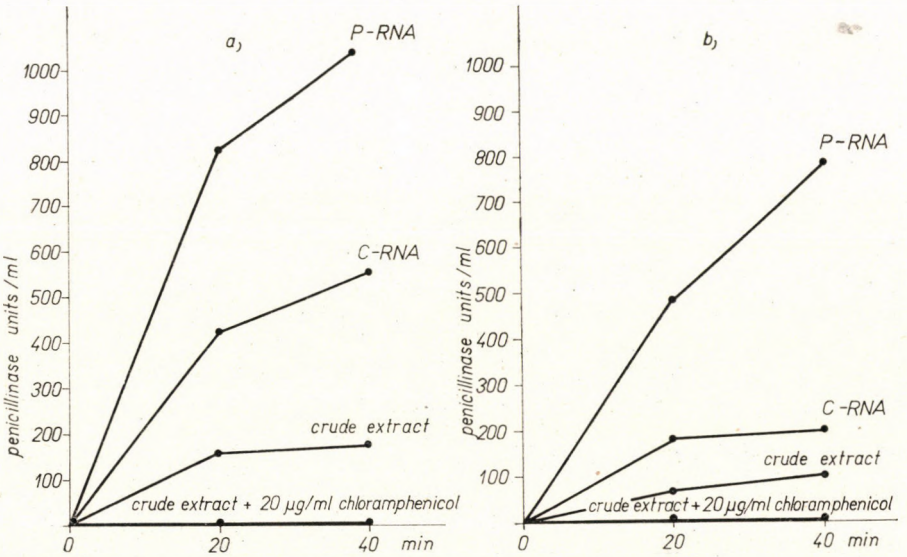

Fig. 2. Effect of different RNA-s on penicillinase synthesis
a) Concentration of RNA-s = $3 \cdot 10^{-2}$ μM adenine equ/ml
b) Concentration of RNA-s = $3 \cdot 10^{-4}$ μM adenine equ/ml

eq. Below that concentration the activity of the RNA seemed to run more or less parallel with the dilution.

Though in this paper our main purpose was to present the proofs of the active principle being in fact an RNA, and that its specific activity depends to a great extent upon the method used for obtaining it, we think it necessary to mention that the effect seems to be an extremely complex one. There are many known, and even more as yet unknown, factors influencing the success or failure of the given experiment. We cannot discuss the details of these aspects here. The only point we wish to call attention to is the composition of the medium in which penicillinase synthesis takes place. A substantial difference may be observed both in kinetics of the formation of the enzyme, and of the amount formed depending upon the source of protein building blocks (*i. e.* peptone, casein hydrolysate or broth), and upon the concentration of certain ingredients, for instance citrate. It needs further study to analyse these environmental effects, from the very beginning of the

cultivation of both the donor and the receptor cells until the composition of the reaction mixture. We know very little about these, but have observed that seemingly unimportant environmental changes can cause remarkable effects.

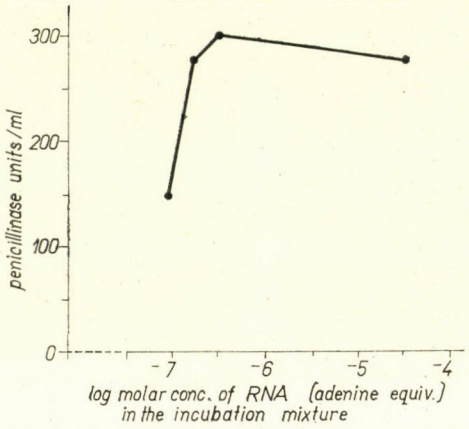

Fig. 3. Effect of P-RNA in different concentrations. The penicillinase values were estimated after 40 min. incubation with the amount of RNA/ml represented on the abscissa. — 569 cell concentration: 0.4 mg/ml b. d. w.

Discussion

The role of RNA in the synthesis of proteins (and particularly of enzymes) has been one of the main subjects of biochemical investigation and discussions since the classical discoveries of BRACHET [17] and CASPERSSON [18]. But in spite of many efforts, of the wide variety of the different systems and techniques used by a great number of authors, there is so far no unequivocal concept about the relationship between the synthesis of the two biologically most important polymers and the specific part one of them plays in the production of the other. Our experiments do not tell us anything about the mechanism involved in the formation of enzymes in general, or even that of penicillinase in particular. However, they tell us at least one thing which earlier has not been established, that specific RNA can play an important, indeed indispensable, role in the course of building up specific inducible enzymes. It has been known for long that specific RNA-s can act as genetic factors and so determine a number of specific proteins — as they do in the case of TMV—RNA [19]. Still, in that case one may rightly assume many intermediary substances and steps between the genetic factors itself and its phenotypical expression, *i. e.* the specific set of virus proteins formed under the influence of TMV—RNA. In our experiments the case was clearly different. The added specific RNA had no genetic influence, the receptor 569 cells recovered after the experiments were just as inducible as they had been before

and even their basal enzyme level did not seem any higher than that of normal, untreated, inducible cells. The RNA here must act somehow directly on the process of synthesis of at least one particular protein, namely penicillinase. How it works, we do not know as yet. The most plausible hypothesis is that it takes some part in the process as a specific template by arranging the amino acids to their proper place in the polypeptide chain. Only one fact is quite certain: that this RNA acts completely specifically, it cannot be substituted either with DNA or degraded RNA extracted from the same organism, or by native RNA obtained from the organisms so far tested (uninduced 569, yeast, *E. coli)*. The other remarkable thing about the phenomenon is that the added RNA is capable of exerting its full effect at an extremely low concentration. Calculating on the basis of the experiment represented in Fig. 3, and on an assumed molecular weight of one million for the RNA, the amount still giving rise to a considerable penicillinase formation must be as low as approximately 10^{-3} μg/ml RNA $= 10^{-10}$ $M = 10^{13}$ molecules of RNA. We know furthermore from other experiments aimed at following the fate of added RNA in the receptor cells (the detailed results will be published later) that the cells take up but a small fraction of the added RNA, approximately 10^{-12} M, *i. e.* 10^{11} molecules when the number of viable cells/ml is about 10^8—10^9. So the number of RNA molecules per cell might be in the order of 10^2—10^3. Taking in account the amount of penicillinase formed under such conditions we have to assume that the specific RNA must act catalytically.

As far as the RNAse treatment is concerned, we still do not know anything about its function — except, of course, that it is indispensable. In the light of the "repressor model" hypothesis of inductive enzyme synthesis [20] according to which the repressor present in inducible cells only is supposed to be an RNA [21] we might perhaps assume that the RNAse acts by destroying the repressor on the one hand, and a number of other RNA-s necessary for penicillinase synthesis on the other, so that the RNAse effect might be much more important and specific than we had first believed. Experiments to prove this possibility are in progress in our laboratory.

LITERATURE

1. Kramer, M., Straub, F. B.: Biochim. biophys. Acta **21,** 401 (1956).
2. Kramer, M., Straub, F. B.: Acta physiol. hung. **11,** 139 (1957).
3. Sneath, P. H. A.: J. gen. Microbiol. **13,** 561 (1955).
4. Kramer, M.: MTA Biol. Csoport Közl. (Budapest) **2,** 223 (1958).
5. Pollock, M. R.: in Gale, E. F., Davies, R. (Ed.): Adaptation in Micro-organisms. Cambridge University Press, 1953, p. 150.
6. Pollock, M. R.: in Boyer, P. D., Lardy, H., Myrbäck, K. (Ed.): The Enzymes. Academic Press, New York, 1959. Vol. I., p. 619.
7. Pollock, M. R.: J. gen Microbiol. **14,** 90 (1956).
8. Kogut, M., Pollock, M. R., Tridgell, E. J.: Biochem. J. **62,** 391 (1956).
9. Pollock, M. R., Kramer, M.: Biochem. J. **70,** 665 (1958).

10. KUNITZ, M.: in NORTHROP, J. H.: Crystalline Enzymes. Columbia University Press, New York, 1948, p. 280.
11. PERRET, C. J.: Nature (Lond.) **174,** 1012 (1954).
12. CITRI, N.: Biochim. biophys. Acta **27,** 277 (1958).
13. HOTCHKISS, R. D.: in COLOWICK, S. P., KAPLAN, N. O. (Ed.): Methods in Enzymology. Academic Press, New York, 1957. Vol. III. p. 692.
14. KIRBY, K. S.: Biochem. J. **64,** 405 (1956).
15. GIERER, A., SCHRAMM, G.: Nature (Lond.) **177,** 702 (1956).
16. BONTING, S. L., JONES, M.: Arch. Biochem. Biophys. **66,** 340 (1957).
17. BRACHET, J.: Biochemical Cytology. Academic Press, New York, 1957, p. 510.
18. CASPERSSON, T. O.: Cell Growth and Cell Function. Morton & Co., New York, 1950.
19. GIERER, A.: in HAYES, W. and CLOWES, R. C. (Ed.): Microbial Genetics, Cambridge University Press, 1960, p. 248.
20. PARDEE, A. B., JACOB, F., MONOD, J.: J. molec. Biol. **1,** 165 (1959).
21. PARDEE, A. B., PRESTIDGE, L. S.: Biochim. biophys. Acta **36,** 546 (1959).

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METABOLISM IN VITRO OF HYDROCORTISONE IN DOG, CAT, GUINEA PIG AND RAT LIVER

By

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Metabolism *in vitro* of hydrocortisone in dog, cat, rat and guinea pig liver tissue has been investigated. It has been found that the liver is capable of transforming hydrocortisone into less polar free Δ^4-3-keto corticosteroids.

The transformations observed are as follows.

(i) Production of corticosterone through 17-α-dehydroxylation.

(ii) Appearance of REICHSTEIN's S compound through 11-β-dehydroxylation.

(iii) Conversion of corticosterone into 11-dehydrocorticosterone. This reaction is catalyzed by 11-β-dehydrogenase and is lacking in guinea pig and cat livers.

(iv) On the effect of 11-β-dehydrogenase, hydrocortisone is metabolized into cortisone.

Numerous studies in the past years have revealed the liver to be the main centre of peripheral corticosterone metabolism. It has been stated that, with the concomitant disappearance of its biological activity, hydrocortisone is metabolized in rat [1, 2], guinea pig [3] and dog [4, 5] liver into (i) di- and tetrahydrocortisol by reduction of the ring A; (ii) 17-keto-derivatives by splitting of the side chain attached to carbon atom 17; and (iii) to a lesser extent, into Δ^4-pregnene-11,17,20,21-tetrole-3-one (REICHSTEIN's compound E) by reduction of the keto group C-20. At the same time, it has been demonstrated by MARTIN *et al.* [6] in this laboratory that hydrocortisone is rapidly transformed by the liver into less polar corticosterone, but with the Δ^4-3-keto structure, unchanged, thus with the maintenance of the biological activity.

The present paper deals with experiments showing liver tissue *in vitro* to be capable of transforming hydrocortisone into less polar free Δ^4-3-keto-corticoids.

Methods

Liver tissue freshly removed from rats, guinea pigs, cats and dogs of both sexes was homogenized or cut into 3 to 5 mm slices. Ten g of such tissue was incubated at pH 7.4 in 40 ml Krebs—Ringer-bicarbonate buffer containing 100 mg glucose per 100 ml, $9 \cdot 10^{-4}$ *M* ATP and $1 \cdot 10^{-2}$ *M* nicotinamide. Hydrocortisone acetate *(Richter)* was added in an amount of 25 mg per 10 gm tissue and the oxygenated mixture was incubated at 37°C for 180 minutes. After the incubation period the proteins were removed by precipitation with 2 volumes of acetone. This was then evaporated under reduced pressure, followed by extraction of the free corticoids with a 2 : 1 mixture of ether and ethyl acetate. The solvent phase was washed with 0.1 *N* sodium carbonate and distilled water. Partition was made in a 1 : 1 mixture of 70 per cent ethanol and benzene-petrolether [7]. Separation and identification of free corti-

coids were performed by paper chromatography, both according to the procedure of BURTON, ZAFFARONI and KEUTMANN [8] using benzene-formamide, and employing the system B_5 of BUSH [9]. Identification and quantitative determination were made as follows.

(i) Presence of the Δ^4-3-keto group was demonstrated by the alkaline fluorescence method of BUSH [9]. The same method, with its accuracy of 15 per cent and sensitivity of 0.5 μg served for semi-quantitative determination.

(ii) The α-ketol side chain of carbon atom 17 was demonstrated by the tetrazolium blue reduction test.

(iii) Compounds reducing tetrazolium were microeluted and the degree of reduction was quantitatively determined [10].

(iv) The keto group 17 was demonstrated by the m-dinitrobenzene colour reaction.

(v) Finally, the R_f values of individual components were compared to those of the crystalline standard preparations.

Results

In the native state, dog and cat livers contain very little hydrocortisone and corticosterone. On the contrary, corticosterone was demonstrated to be

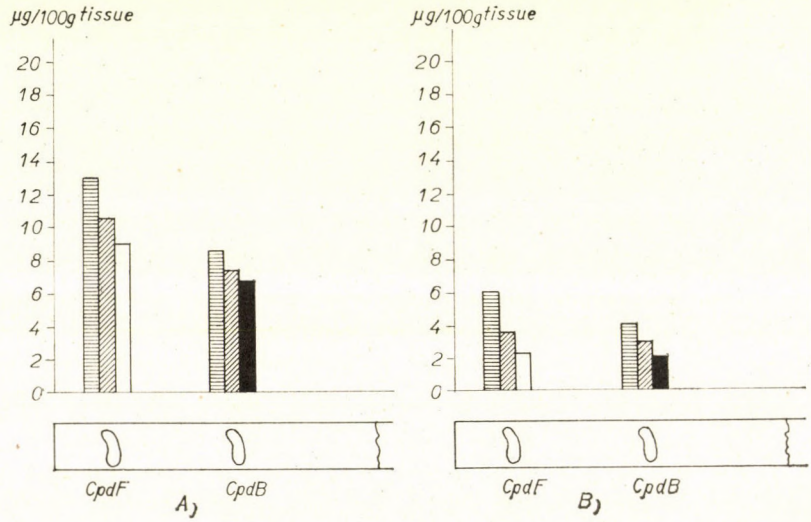

Fig. 1. Chromatographic pattern of native (A) and incubated (B) liver in benzene-formamide system
Column with horizontal lines = dog
Column with oblique lines = cat
White column = guinea pig
Dark column = rat

present in the liver of the rat, and hydrocortisone in that of the guinea pig. After incubation for 180 minutes, the qualitative pattern did not show any change, but the amounts of demonstrable compounds decreased owing to their inactivation by the tissue (Fig. 1).

After incubation of 25 mg hydrocortisone with 10 g liver tissue, the benzene-formamide system revealed 6 new compounds in the dog and rat, and 5 in the cat and guinea pig (Fig. 2).

Among the corticosteroids investigated, hydrocortisone was the most polar compound. The component lying above hydrocortisone showed positive alkaline fluorescence and was reducing tetrazolium, but failed to give the m-dinitrobenzene colour reaction. As judged from its R_f value, this compound was cortisone. The third component had a Δ^4-3-keto structure and reduced tetrazolium; it was identical with 11-desoxy-17-hydroxycorticosterone,

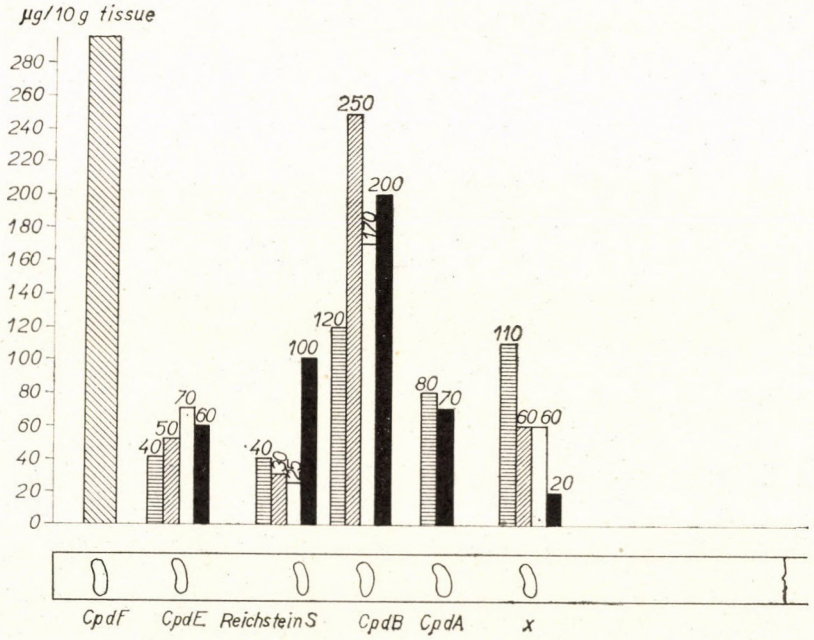

Fig. 2. Incubation of 10 g liver with 25 mg hydrocortisone. Chromatographic pattern in benzene-formamide system.
Thick column with oblique lines shows the ground substrate. For further explanations see Fig. 1

REICHSTEIN's compound S. The fourth compound immediately above this also contained the Δ^4-3-keto, α-ketol side chain; this compound was identical with corticosterone. The fifth component was not found but in the dog and rat; it was 11-dehydrocorticosterone, showing alkaline positivity, reducing tetrazolium but not reacting with m-dinitrobenzene. The sixth component — so far unidentified — was found in all species investigated. It had an R_f value of 0.56, was of the Δ^4-3-keto structure, failed to reduce tetrazolium, and gave no reaction with m-dinitrobenzene.

Analysis of the qualitative pattern described showed that 180 minutes incubation led to complete deacetylation of the hydrocortisone added, as the R_f values obtained after acetylation of either hydrocortisone or other components were not identical with those observed immediately after incubation.

Quantitative determinations revealed the main component arising in the course of hydrocortisone transformation into less polar derivatives to be corticosterone. This compound formed the bulk of the new derivatives, amounting to 30.7 per cent in the dog, 64.1 per cent in the cat, 52.3 per cent in the guinea pig, and 44.4 per cent in the rat.

No qualitative difference in metabolizing of the added hydrocortisone

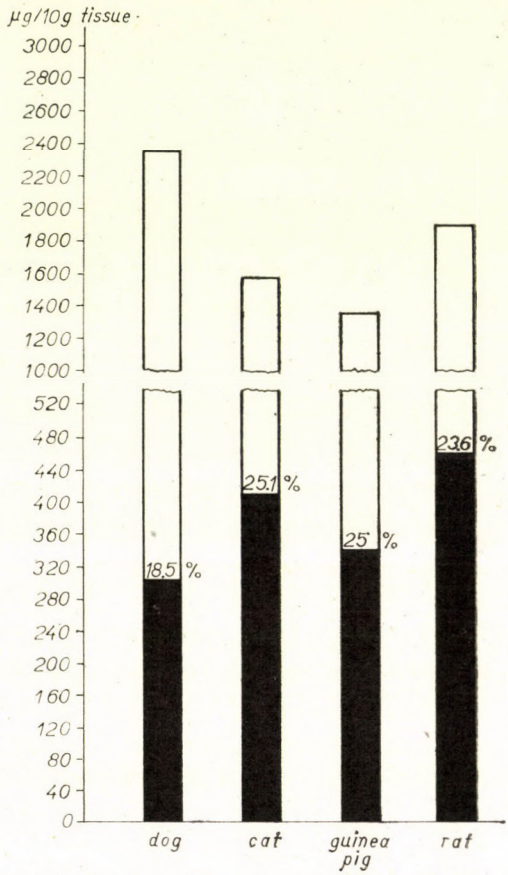

Fig. 3. Ratio of Δ^4-3-keto compounds less polar than hydrocortisone to the total amount of metabolites produced during incubation.
Dark column = less polar Δ^4-3-ketosteroids; white column = other metabolites

was found between liver slices and liver homogenates, nor concerning the quantitative aspects.

After separation in the system B_5 of BUSH, the biologically inactive components were also determined. Comparing the amount of total metabolites found to the amount of the part showing biological activity, about 20 per cent of the total free metabolite content consisted of biologically active derivatives (Fig. 3).

Discussion

Liver tissue is capable not only of inactivating corticosteroids, as it was described in the review of CASPI and HECHTER [11], but also of producing from hydrocortisone some less polar Δ^4-3-keto compounds, among which some biologically active components, too, may occur. According to our studies, essential in this transformation is the 11-β and 17-α-dehydroxylation. Splitting of the 17-α-hydroxyl group of hydrocortisone is shown by the appearance of corticosterone, the 11-β-dehydrogenase-catalysed [12] transformation of

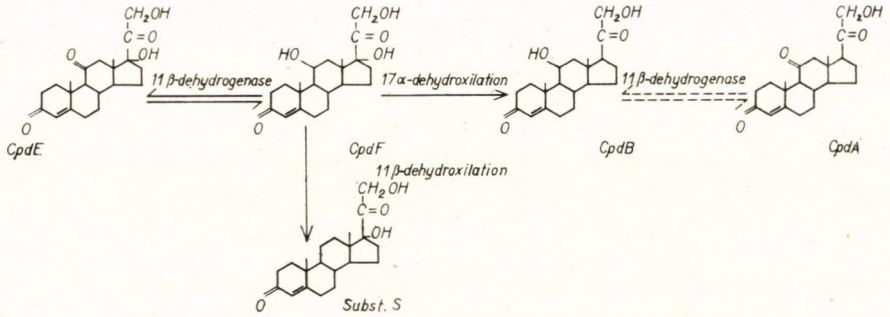

Fig. 4. Transformation of hydrocortisone into less polar free Δ^4-keto derivatives. The conversion shown with dotted line occurs only in dog and rat liver

which into 11-dehydrocorticosterone was demonstrated by several authors [13, 14]. It is remarkable that this transformation is observable only in the rat and the dog, and not in the cat or the guinea pig. The appearance of 11-desoxy-17-hydroxycorticosterone, REICHSTEIN's compound S, is indicative of 11-β-dehydroxylation. Whether these dehydroxylations are due to specific 11-β- and 17-α-dehydroxylating enzymes must be decided by further experiments. Quantitative studies have shown the main direction in the transformation to be the 17-α-dehydroxylation resulting in the appearance of corticosterone. This transformation is especially marked in the cat, where corticosterone amounts up to 64.1 per cent of the biologically active corticosteroids.

Based on the above experimental data, the hepatic transformation of hydrocortisone into less polar, biologically active corticosteroids is supposed to be running in the way illustrated in Fig. 4.

Determination of the free total metabolites indicates the main direction of liver function to be the biological inactivation [15]. However, the above demonstrated metabolites, too, may play a decisive part in the biological action of corticosteroids.

LITERATURE

1. CASPI, E., HECHTER, O.: Arch. Biochem. Biophys. **61,** 299 (1956).
2. ROBBINS, E. D., BURTON, S. D., BYERS, S. O., FRIEDMAN, M., GEORGE, S. S., ISHIDA, T.: J. clin. Endocr. **17,** 111 (1957).
3. BURNSTEIN, S., DORFMAN, R. I., NADEL, E. M.: J. biol. Chem. **213,** 597 (1955).
4. AXELROD, L. R., MILLER, L. L.: Arch. Biochem. Biophys. **60,** 373 (1956).
5. HECHTER, O., FRANK, E., CASPI, E., FRANK, H.: Endocrinology **60,** 705 (1957).
6. MARTIN, J., BATA, G., ENDRŐCZI, E., MOLL, Á.: Acta physiol. hung. **11,** 385 (1957).
7. ENDRŐCZI, E., BATA, G., MARTIN, J.: Endokrinologie **35,** 280 (1958).
8. BURTON, R. B., ZAFFARONI, A., KEUTMANN, E. H.: J. biol. Chem. **188,** 763 (1951).
9. BUSH, I. E.: Biochem. J. **50,** 370 (1952).
10. ENDRŐCZI, E., YANG, T. L.: Acta physiol. hung. In press.
11. CASPI, E., HECHTER, O.: Arch. Biochem. Biophys. **52,** 478 (1954).
12. HURLOCK, B., TALALAY, P.: Arch. Biochem. Biophys. **80,** 468 (1959).
13. HÜBENER, H. J., AMELUNG, D.: Hoppe-Seyler's Z. physiol. Chem. **293,** 126 (1953).
14. RICHARDSON, E. M., TOUCHSTONE, J. C., DOHAN, F. C.: Fed. Proc. **13,** 118 (1954).
15. HECHTER, O., SOLOMON, M. M., CASPI, E.: Endocrinology **53,** 202 (1953).

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METABOLISM IN VITRO OF CORTISONE ACETATE IN LIVER TISSUE OF VARIOUS SPECIES

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Cortisone is metabolized in the liver *in vitro* into less polar, biologically active corticosteroids.

The transformations observed are as follows.

(i) Production of 11-dehydrocorticosterone through 17-α-dehydroxylation.

(ii) Appearance of 11-desoxy-17-hydroxycorticosterone through splitting of oxo-group 11. This conversion occurs only in the dog.

(iii) As an effect of 11-hydrogenase, hydrocortisone is produced, which may enter further transformations.

When given orally, cortisone acetate loses a considerable part of its effectiveness. This therapeutic observation called attention to the decisive part of liver tissue in peripheral cortisone metabolism. As stated by Caspi and Hechter [1, 2], de Courcy *et al.* [3, 4] and Tomkins [5], cortisone is inactivated in the liver, with the concomitant disappearance of its biological activity. The main features in this transformation are (i) saturation of the ring A; (ii) splitting of the side chain 20—21 into 17-keto-group; (iii) hydrogenation of the oxo-group C-20, a change resulting in Δ^4-pregnene-17,20,21-triole-3,11-one (Reichstein's compound U). At the same time it was demonstrated by Martin *et al.* [6] that, within a short period of time, corticosterone — a less polar Δ^4-3-keto derivate — appears during the utilization of cortisone. Our own studies have shown that liver tissue was capable of transforming hydrocortisone into less polar Δ^4-3-keto compounds.

The present paper deals with the metabolism *in vitro* of cortisone acetate n liver tissue of various animals.

Methods

Liver slices and homogenates from dog, cat, guinea pig and rat of both sexes were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) at 37°C for 180 minutes. The incubation medium contained 100 mg per cent glucose, $9 \cdot 10^{-4}$ *M* ATP and $1 \cdot 10^{-2}$ *M* nicotinamide. Cortisone acetate *(Organon)* served as substrate, in an amount of 25 mg per 10 g tissue. Extraction and purification of free corticosteroids was performed according to the procedure previously described [7].

Separation and identification of corticoids was performed by means of paper chromatography. The benzene-formamide system of Burton, Zaffaroni and Keutmann [8] as well as the system B_5 of Bush [9] using benzene-methanol-water were employed.

Identification and quantitative determinations were made as follows.

(i) The R*f* values of individual components were compared with those of crystalline standard preparations.

(ii) Δ^4-3-keto structure was demonstrated by the alkaline fluorescence method of BUSH [9], in UV light of 254 mμ. This method served also for semiquantitative determination.
(iii) The α-ketol side chain was demonstrated by means of tetrazolium-blue reduction.
(iv) Tetrazolium reduction was quantitatively measured after microelution [10] (Pulfrich photometer, wave length 520 mμ).
(v) 17-keto group was demonstrated by means of the m-dinitrobenzene colour reaction.
(vi) Acetylation of extracts and standard compounds was made in a medium containing anhydrous acetic acid and pyridine (DECOURCY *et al.* [3]).

Results

After incubation of 10 g tissue with 25 mg cortisone acetate, four Δ^4-keto derivates appeared in the guinea pig liver, 5 components in the rat and cat liver, and 6 compounds in the dog liver. In these experiments, the benzene-

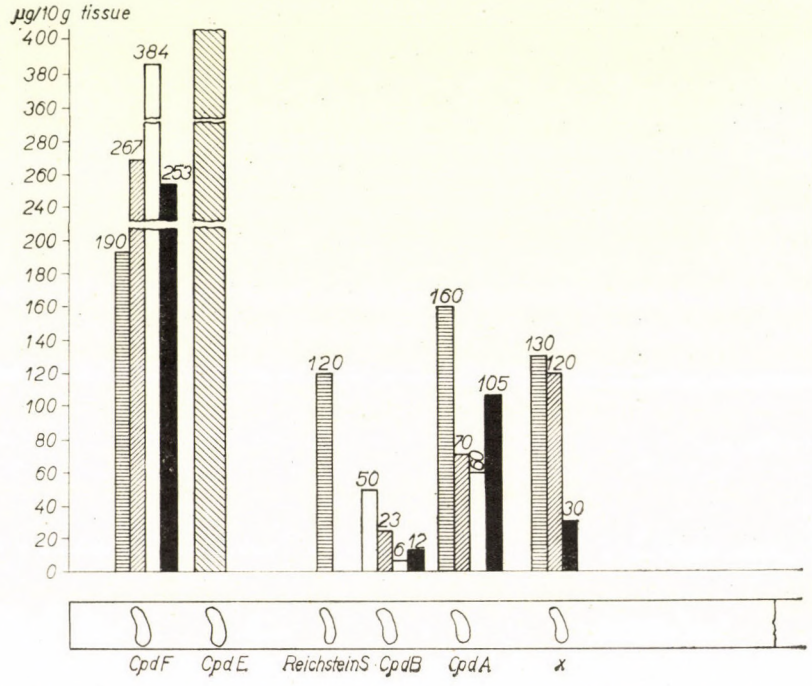

Fig. 1. Incubation of 10 g liver tissue with 25 mg cortisone. Chromatographic pattern in benzene-formamide system

Thick column with oblique lines = ground substrate
Column with horizontal lines = dog
Column with oblique lines = cat
White column = guinea pig
Dark column = rat

formamide system was used (Fig. 1). The most polar compound was hydrocortisone, showing positive alkaline fluorescence and tetrazolium reduction. Immediately above the hydrocortisone, one sees the substrate investigated,

i. e. cortisone. The third compound containing Δ^4-3-keto group and reducing tetrazolium-blue proved, on the basis of the R_f values, identical with 11-desoxy-17-hydroxycorticosterone, the REICHSTEIN's compound S. This derivative occurred only in the dog liver. The component lying above REICHSTEIN's compound S showed alkaline fluorescence and reduced tetrazolium; it was identical with corticosterone. The least polar Δ^4-3-keto derivative of this series was a so far unidentified compound; it failed to reduce tetrazolium and gave no colour reaction with m-dinitrobenzene. This compound did not occur in guinea pig liver.

In order more exactly to identify the compounds demonstrated in the formamide-benzene system, the system B_5 of BUSH was used. It was found that, as far as the R_f values are concerned, the above derivatives behaved exactly as the standards.

Cortisone acetate was completely deacetylated in the course of incubation, as shown by the finding that its R_f value was different from those of the acetylated standard preparations. For control, some of our extracts were subjected to acetylation. The R_f values of these acetylated derivatives were not identical with those of the pure crystalline standards.

As to the quantitative aspects, among the less polar compounds originating from cortisone 11-dehydrocorticosterone appeared in largest amounts. An especially marked predominance of 11-dehydrocorticosterone was observed in guinea pig and rat livers; in these species, the compound amounted to 90.9 and 66.8 per cent, respectively of the total amount of less polar derivatives. The ratio was 34.7 per cent in the dog. In this animal, REICHSTEIN's substance S, too, occurred in great amounts.

In the cat 11-dehydrocorticosterone amounted to 32.9 per cent, but the bulk, 56.3 per cent, of the compounds present, consisted of compound X.

The amount of the compounds produced was practically the same whether tissue slices or tissue homogenates were incubated with cortisone. Neither quantitative nor qualitative differences were noted between male and female animals.

When all hitherto known metabolites of cortisone are considered, the metabolic pathway described by us accounts for some 20 per cent of the total amount of derivatives (Fig. 2).

Discussion

As demonstrated in our studies *in vitro*, in the liver of various animal species cortisone undergoes a change resulting not only in biologically inactive compounds, but also in less polar Δ^4-3-keto derivatives. Enzymatic conversion of cortisone into 17-hydroxycorticosterone was demonstrated by FISH and HÜBENER [11, 12]. This conversion catalyzed by 11-β-hydrogenase calls

attention to the fact that some of the compounds appearing in the course of cortisone metabolism do not originate immediately from cortisone, but from its chief metabolite, hydrocortisone.

11-dehydrocorticosterone is produced in much greater amounts from cortisone than from hydrocortisone. Together with our earlier finding [7] that during hydrocortisone metabolism 11-dehydrocorticosterone appears only

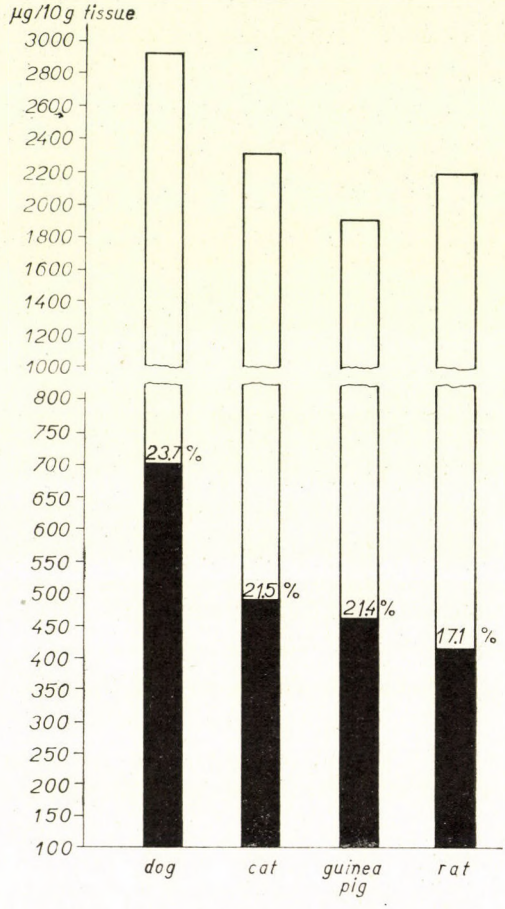

Fig. 2. Ratio of total Δ^4-3-keto corticoid metabolites
Dark column = free Δ^4-3-keto compounds
White column = free metabolites

in dog and rat livers, the above finding indicates that KENDALL's compound A can be produced directly from cortisone through 17-α-dehydroxylation. This is supported by the observation that corticosterone production markedly decreases during cortisone metabolism, a fact indicating that the compound originates partly from dehydrocortisone and partly from 11-dehydrocorticosterone [12].

Production of 11-desoxy-17-hydroxycorticosterone (REICHSTEIN's compound S) occurred only in the dog liver. Accordingly, this type of metabolic conversion, *i. e.* the splitting of the 11-oxo group, represents a transformation characteristic of the species.

Based on the above as well as earlier data [7, 11, 12], the transformation of cortisone into less polar, biologically active Δ^4-3-keto corticoids may be supposed to run in the way shown in Fig. 3.

The results of quantitative tests showed no significant difference between male and female animals. However, the sexual difference found by

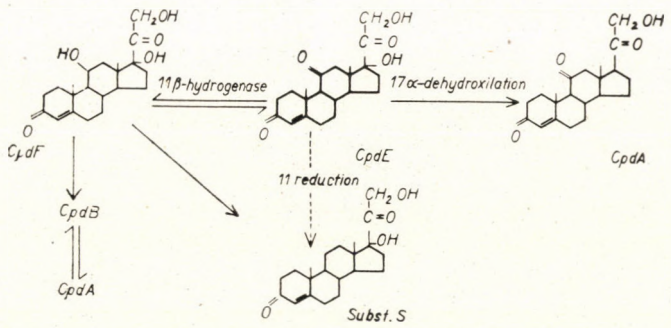

Fig. 3. Transformation of cortisone into less polar Δ^4-3-keto derivatives
The conversion shown with dotted line occurs only in the dog

YATES *et al.* in the grade of inactivation makes further studies necessary, in order to decide whether or not the production of less polar compounds depends on sexual differences.

LITERATURE

1. CASPI, E., HECHTER, O.: Arch. Biochem. Biophys. **52,** 478 (1954).
2. HECHTER, O.: CIBA Foundation Colloquia on Endocrinology **7,** 272 (1953).
3. COURCY, C. DE, GRAY, C. H., BUSH, I. E., LUNNON, J. B.: J. Endocr. **9,** 401 (1953).
4. HOLNESS, N. J., LUNNON, J. B., GRAY, C. H.: J. Endocr. **14,** 138 (1956).
5. TOMKINS, G. M.: Rec. Progr. Horm. Res. **12,** 125 (1956).
6. MARTIN, J., BATA, G., ENDRŐCZI, E., MOLL, Á.: Acta physiol. hung. **11,** 385 (1957).
7. BOHUS, B., ENDRŐCZI, E.: Acta physiol. hung. (In press).
8. BURTON, R. B., ZAFFARONI, A., KEUTMANN, E. H.: J. biol. Chem. **188,** 763 (1951).
9. BUSH, I. E.: Biochem. J. **50,** 370 (1952).
10. ENDRŐCZI, E., YANG, T. L.: Acta physiol. hung. (In press).
11. FISH, C. A., HAYANO, M., PINCUS, G.: Arch. Biochem. Biophys. **42,** 480 (1953).
12. HÜBENER, H. J., AMELUNG, D.: Hoppe-Sey. Z. physiol. Chem. **293,** 126 (1953).
13. YATES, F. E., HERBST, A. L., URQUHART, J.: Endocrinology **63,** 887 (1958).

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THE PHOSPHORYLATION OF ADENOSINE DIPHOSPHATE AND GLUCOSE IN ISOLATED BRAIN MITOCHONDRIA AT DIFFERENT OSMOTIC CONCENTRATIONS

By

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ADP phosphorylation is not demonstrable in isolated brain mitochondria exposed to isotonic incubation. On hypotonic incubation the ADP becomes phosphorylated.

In the preparations in which a lack of phosphate acceptors limits oxidation, respiration is increased by ADP in hypotonic reaction mixtures only.

There is phosphorylation also under isotonic conditions with the hexokinase-glucose trap. When hexokinase is used in limiting quantities, phosphorylation is more intensive under hypotonic than under isotonic conditions. The difference decreases when the amount of hexokinase is increased. Hexokinase increases respiration also under isotonic conditions.*

The isolated mitochondria which have retained their original morphological properties in the course of preparation are in several biochemical reactions inactive compared to those that have undergone a change [1]. Mitochondria, which morphologically are not completely intact, when isolated and incubated in approximately isotonic media become capable of performing certain enzyme reactions only after their structure has been loosened up. Thus, they do not oxidize with phosphorylation the DPNH added externally [2]; moreover, even DPNH oxidation is reduced to a minimum when preparation has been suitably careful [3]. After hypotonic swelling of the mitochondria, oxidative phosphorylation is demonstrable also with external DPNH. This effect is to a certain extent analogous with the "opening phenomenon" observed in various mitochondrial fragments; in the partially intact structure certain added reaction intermediaries do not react until the structure has been broken up further. The *electron transport particle* isolated in GREEN'S laboratory that oxidizes succinate in the presence of oxygen does not reduce the added cytochrome *c* in the presence of succinate, nor does it catalyse oxidation of the reduced cytochrome *c*. After treatment with deoxycholate the extrinsic cytochrome *c* takes part in both reactions [4]. The intermediaries reacting in the bound form do not seem to react in the intact structure.

*The following abbreviations are used in this paper:
DPNH: reduced diphosphopyridine nucleotide
DPN: oxidized diphosphopyridine nucleotide
ATP: adenosine-5'-triphosphate
ADP: adenosine-5'-diphosphate
EDTA: ethylene diamine tetraacetate
Tris: tris (hydroxymethyl) aminomethane

In the oxidative phosphorylation of mitochondria bound adenine nucleotides are taking part [5]. According to our present results, an effect similar to the "opening phenomenon" is observable during the phosphorylation of exogenous ADP.

Methods

Preparation of brain mitochondria

Albino rats were killed by decapitation and the cerebral hemispheres were removed and cooled to 0° C in the homogenizing medium. Four brains were worked up at a time. Homogenization was performed in a plexiglass-glass Potter-Elvehjem apparatus for 90 seconds. The homogenizing medium consisted of 0.25 *M* sucrose, 0.001 *M* EDTA and 0.01 *M* Tris, with a pH between 7.2 and 7.4. The volume of the homogenate was adjusted to 46 *m*l with the medium. Fractionation was carried out by 2 procedures, in a MSE refrigerated centrifuge at 0 to + 2°C (superspeed head).

With either procedure cellular detriments, erythrocytes, nuclei and fibres were removed by centrifugation at 1150 *g* for 10 minutes. The supernatant was centrifuged at 12 650 *g* for 15 minutes, thus causing the mitochondria to settle; they were then fractionated by the following 2 procedures.

The sediment homogenized in about 16 ml of pure 0.25 *M* sucrose was centrifuged for 5 minutes at 16 500 g. In this case the supernatant was definitely turbid and contained lesioned mitochondria, as well as a fraction now under identification. The sediment (mitochondrium fraction "A"), which was still contaminated with the fraction settling at a lower rate, contained the mitochondria. The mitochondria were suspended in 8 to 13 ml of a 0.25 *M* sucrose solution. The suspension contained about 2 mg of total nitrogen per ml.

The other procedure yielded a purer mitochondrium suspension. The sediment centrifuged at 12 650 *g* was homogenized as described above in about 16 ml of 0.25 *M* sucrose and centrifuged at 12 650 *g* for 10 minutes. The loose upper layer of the sediment was washed with sucrose and decanted, the more compact lower portion was homogenized again in sucrose and centrifuged for 10 minutes at 16 500 *g*. The loose upper part was washed again as described above and the compact sediment (mitochondrium "B") was suspended in about 6 ml of 0.25 *M* sucrose. The total nitrogen concentration of the suspension was around 0.6 mg per ml. The mitochondrium yield was less than with the former method, but the preparation was purer.

Oxidation was measured by the usual Warburg method, at 30°C, in air atmosphere, after allowing 10 minutes thermal equilibration period. The standard reaction mixture had the following composition: sodium pyruvate, 10 m*M*; sodium fumarate, 1 m*M*; $MgCl_2$, 5 m*M*; DPN, 0.2 m*M*; NaATP, 1 m*M*; sodium phosphate, 6.6 to 11 m*M*; sucrose. 42 to 250 m*M*. The 250 m*M* sucrose solution was accepted as isotonic.

When measuring phosphorylation, the contents of the Warburg vessels were deproteinized with trichloroacetic acid either after constancy of temperature had been attained or oxidation had been measured. The inorganic phosphate concentration of the filtrate was estimated by the method of LOHMANN and JENDRASSIK [6].

Materials

Pyruvic acid was converted to sodium pyruvate after distillation *in vacuo* and was used after three recrystallisations. Fumaric acid was converted to fumarate after two recrystallisations, immediately before use. Sucrose of analytical purity was used after recrystallisation from ethanol.

The commercial ATP (*Richter*, Budapest) was purified by means of Amberlite IRA 410 ion exchanger; it was stored partly in the form of its sodium salt and partly as Ba-salt, converted to the Na-salt prior to use. Immediately after preparation the compound proved to be pure by paper chromatography; a few months later it contained a small amount of ADP.

The ADP used was partly a product of VEB *Feinchemie*, Berlin, and partly a preparation purified on Dowex 1 ion exchanger and found to be pure by paper chromatography.

DPN was obtained from the *Reanal Laboratories*, Budapest, and was of 70 to 75 per cent purity.

The yeast-hexokinase was prepared by the method of BERGER *et al.*, slightly modified by us. Purification was continued until fraction 3/a had been obtained [7]. The method of assay was that described by BAILEY and WEBB [8]. One unit transferred 1 mg of phosphate from ATP to glucose in 1 hour.

The other chemicals used were of commercial reagent-grade quality. We employed glass-distilled water throughout our experiments.

Results

According to our experiments a net phosphorylation of ADP in brain mitochondrial preparations was demonstrable in the presence of fluoride only. Even under such conditions the value of P/0 was low, ATPase activity not being completely blocked by fluoride.

As visibe in Table I, the more hypotonic the reaction mixture, the larger was the quantity phosphorylated in the presence of ADP. Phosphorylation decreased with the increase of sucrose concentration. There were differences between the single preparations; some of them showed minimum phosphorylation even in the presence of 250 m*M* sucrose, while in others no disappearance of phosphate was any longer demonstrable at a sucrose concentration of 175 m*M*. However, in every experiment the phosphorylated quantity was the greatest when the medium contained 42 m*M* of sucrose, in spite of the fact in that instance the ATPase activity was somewhat higher than at higher concentrations.

The brain mitochondrium preparations were contaminated with variable amounts of ATPase, which could be activated by Mg^{++}. When contamination was heavy, the respiration of fluoride-treated brain mitochondria only could be enhanced with phosphate acceptor. ADP was capable to enhance respiration considerably under definitely hypotonic conditions only; at higher sucrose concentrations there was practically no increase (Fig. 1). When contamination with ATPase was slight (preparation "B"), the ADP acceptor effect was demonstrable similarly even without fluoride (Fig. 2).

The mitochondrium prepared and incubated under isotonic conditions is more likely to approach morphologically intracellular mitochondria than that exposed to hypotonic swelling. It is therefore conceivable that the extramitochondrial free ADP does not react on phosphorylation within the cell, *in vivo*. On swelling the mitochondrium may "open up" also for the extramitochondrial ADP.

Hexokinase-glucose trap for phosphorylation

The question has arisen whether the $\sim$ P generated intramitochondrially under the above experimental conditions could be enzymatically transferred extramitochondrially, when the phosphorylation of free ADP is markedly restricted.

Table I

Sucrose m*M*	42				108				175				250			
Experiment No.	Without ADP		With ADP		Without ADP		With ADP		Without ADP		With ADP		Without ADP		With ADP	
	ΔO	ΔP	ΔO	ΔP	ΔO	ΔP	ΔO	ΔP	ΔO	ΔP	ΔO	ΔP	ΔO	ΔP	ΔO	ΔP
1.	− 2.78	—	− 6.21	− 3.41	—	—	—	—	− 2.93	—	− 3.97	− 2.37	− 1.38	—	− 1.63	− 1.11
2.	− 4.68	− 0.07	− 5.76	− 4.43	− 3.85	− 0.16	− 3.70	− 2.61	− 2.98	+ 0.19	− 2.71	+ 0,60	—	—	—	—
3.	− 3.48	− 0.10	− 5.69	− 4.57	− 3.68	− 0.76	− 3.35	− 2.78	− 2.44	− 0.84	− 2.05	− 1.54	—	—	—	—

Oxidation and phosphorylation of fluoride-treated mitochondria with and without ADP.
Δ O: *µAtom* Δ P: *µMol.* 15-minute measurement.
Reaction mixture: sodium pyruvate, 10 m*M*; sodium fumarate, 1 m*M*; Na-ATP, 1 m*M*; DPN, 0.2 m*M*; $MgCl_2$, 5 m*M*; NaF, 40 m*M*; Na-phosphate, 6.6 m*M*. pH: 7.2.
Sucrose: as indicated. Volume 3.00 ml.
ADP: where indicated, 6.6 m*M*.
Preparation mitochondrium "A", 0.50 ml

Fig. 1. Oxidation of sodium fluoride-treated brain mitochondria after adding ADP

Abscissa: time. Ordinate: O_2-consumption µl; at 20 minutes 20 *µmoles* ADP was added to the mixture from the side vessel. *1*, *3*, without ADP. *2*, *4*, with ADP. *1*, *2*, 250 m*M*; *3*, *4*, 42 m*M* sucrose concentration. Sodium fluoride; 40 m*M*. Mitochondrium preparation "A"

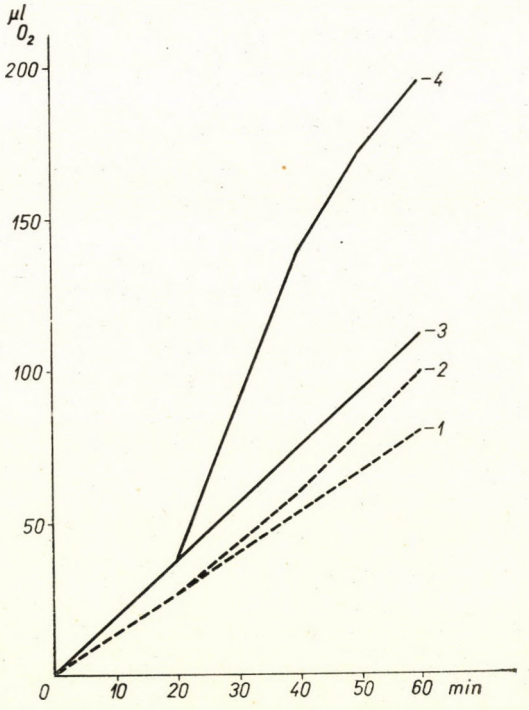

Fig. 2. Effect of ADP on the oxidation of brain mitochondria without treatment with sodium fluoride. Abscissa: time. Ordinate: O_2-comsumption in µl; At the onset of measurement 15 m *moles* ADP were added from the side vessel to the mixture. *1*. *3*, without ADP. *2*, *4*, with ADP; *1*, *2*, 250 m*M*; *3*, *4*, 42 m*M* sucrose concentration. Mitochondrium preparation "B"

When measuring phosphorylation in the presence of hexokinase and glucose, oxidative phosphorylation could be demonstrated also under isotonic conditions. The intensity of oxidation and phosphorylation depends on two factors, *viz.* the osmotic concentration of the test medium and the quantity of hexokinase employed. Under hypotonic conditions even 0.05 ml (about 1 unit) of hexokinase produced maximum oxidation and phosphorylation. The same small amount of hexokinase ensured weaker oxidation and phosphorylation under isotonic conditions. As owing to the presence of ATPase, respiration was not completely controlled, the P/O value was also lower.

Under hypotonic conditions, the twofold or fourfold increase of the initial amount of about 1 U of hexokinase did not increase either oxidation or phosphorylation. On the contrary, it often caused a decrease, apparently due to the complete ATP deficiency produced [9]. When under isotonic conditions the hexokinase concentration was increased in the same measure, both phosphorylation and oxidation increased. The value of P/O was increased simultaneously or, if it had been high, it remained unchanged (Table II).

Discussion

The above results substantiate our hypothesis that *in vivo* it is not the extramitochondrial nucleotides that are phosphorylated in the first place, but the enzymes functioning with $\sim$ P (such as *e.g.* hexokinase) react with the ATP generated in the mitochondrial cristae and transfer its terminal phosphate to outside the mitochondrium. The hypothesis needs further confirmation.

Swelling of the mitochondria both in the isolated state and *in situ* may increase the yield of oxidative phosphorylation. This is due partly to the extramitochondrial nucleotides being phosphorylated directly and partly to the condition favourable for the enzyme taking part in phosphate transfer.

It was remarkable that the hexokinase ensuring optimal phosphorylation under hypotonic conditions did not suffice to cause a transfer of the actually phosphorylated quantity on the basis of analytical data. The endogenous hexokinase of the mitochondria does not explain the difference. According to SIEKEWITZ and POTTER [10], owing to the favourable steric and concentration relations the hexokinase adsorbed onto the mitochondria is more active that the freely-dissolved hexokinase. The presence of mitochondrium-bound hexokinase demonstrated by CRANE and SOLS [11] might have also been due to adsorption. This may play a role in ensuring mitochondrial phosphate transfer.

Adsorption and a loosening-up of the structure make it possible that under hypotonic conditions the hexokinase requirement of the system is mini-

Table II

Sucrose m*M*	42									250								
Experiment No.	hexokinase ml																	
	0.05			0.10			0.20			0.05			0.10			0.20		
	ΔO	ΔP	P/O	ΔO	ΔP	P/O	ΔO	ΔP	P/O	ΔO	ΔP	P/O	ΔO	ΔP	P/O	ΔO	ΔP	P/O
1.	6.40	16.15	2.52	5.90	15.20	2.58	5.66	14.38	2.54	2.25	3.61	1.60	3.32	5.67	1.71	3.73	7.58	2,03
2.	4.29	12.40	2.89	3.44	9.50	2.76	3.56	8.14	2.28	1.44	1.87	1.30	1.93	2.80	1.45	2.24	3.71	1.65
3.	5.73	—	—	—	—	—	5.75	—	—	1.87	4.13	2.20	3.32	7.22	2.18	3.74	8.10	2.17

Oxidation and phosphorylation of mitochondria in the presence of hexokinase-glucose system.
Δ O: μ*Atom* P: μ*Mol.* 15-minute measurement.
Reaction mixture: Sodium pyruvate, 10 m*M*; sodium fumarate, 1 m*M*; Na-ATP, 1 m*M*; DPN, 0.2 m*M*; $MgCl_2$, 5 m*M*;
NaF, 40 m*M*; Na-phosphate, 6.6 to 11 m*M*; glucose, 16 m*M*.
pH 7.2.
Sucrose, hexokinase as indicated.
Mitochondrium preparation "A", 0.50 ml

mal. The site of phosphorylation is not only the outer membrane of the mitochondrium, but also the intramitochondrial cristae, which are of the same structure. The hexokinase must enter the mitochondrium, and this is promoted by the hypotonicity. Under isotonic conditions the hexokinase requirement is higher. Adsorption might be weaker in the intact structure and entering of the hexokinase into the mitochondrium is probably interfered with. A high hexokinase concentration may make up for the defective adsorption and may at the same time create favourable permeability conditions.

Our preparation possessed some endogenous hexokinase activity, but this did not suffice to ensure a significant degree of phosphorylation.

The increase of phosphorylation caused by the increase of hexokinase concentration under isotonic conditions cannot be explained by ATPase competition alone. In the latter case respiration would not change. As respiration increased with the increase of hexokinase, and in some experiments an increase of oxidation and phosphorylation occurred even when the P/O was unchanged, we feel inclined to attribute a primary importance to the effect providing for the phosphate acceptor. At the same time, in some experiments the low and increasing P/O values were suggestive of some ATPase competition.

*

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LITERATURE

1. ZIEGLER D. N, LINNANE A. W.: Biochim. biophys. Acta **30,** 53 (1958).
2. LEHNINGER A. L.: in "Phosphorus Metabolism", ed. by MCELROY W. D., GLASS B. The Johns Hopkins Press, Baltimore, 1951, Vol. 1, p. 344.
3. GREEN D. E., CRANE F. L.: Proc. Intern. Symposium on Enzyme Chemistry, Tokyo-Kyoto 1957, p. 275.
4. MACKLER B., GREEN D. E.: Biochim. biophys. Acta **21,** 1 (1956).
5. SIEKEVITZ P., POTTER V. R.: J. biol. Chem. **215,** 221 (1955).
6. LOHMANN K., JENDRASSIK L.: Biochem. Z. **178,** 419 (1926).
7. BERGER, L., SLEIN, M. W., COLOWICK, S. P., CORI, C. F.: J. gen. Physiol. **29,** 379 (1946).
8. BAILEY K., WEBB E. C.: Biochem. J. **42,** 60 (1948).
9. ERNSTER L.: Exptl. Cell Res. **10,** 704 (1956).
10. SIEKEVITZ P., POTTER V. R.: J. biol. Chem. **215,** 237 (1955).
11. CRANE R. K., SOLS A.: J. biol. Chem. **203,** 273 (1953).

Physiologia

HEXOKINASE ACTIVITY DURING GLUCOSE ABSORPTION

By

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Experiments in nephrectomized rats showed no parallelism between hexokinase activity and the absorbed amount of glucose.

The mechanism of selective sugar absorption is an old and unsolved physiological problem.

According to VERZÁR's phosphorylation theory the absorption rate is determined by the rate of phosphate-ester formation. Thus hexokinase activity would be an important condition of sugar absorption. This hypothesis was supported by the experiments of HELE [3], CSÁKI [2], BISEGGER and LASZT [1] and of others who found a parallelism between the phosphorylation rate of different sugars in the intestinal epithelium, homogenisate and their absorption rate *in vivo*. The increase of phosphor esters in the intestinal epithelium during sugar absorption also supports the phosphorylation theory [4].

SOLS [6, 7] and others, using an improved method of hexokinase determination obtained opposite results. They demonstrated that the most quickly absorbed sugar — galactose — is phosphorylated rather slowly, whereas the slowly absorbed 2-deoxyglucose is phosphorylated rapidly.

In the course of some previous examinations we found hexokinase activity in the intestinal epithelium of nephrectomized rats to vary between very wide ranges. Therefore such animals were considered to be well suited for studying in them the relationship between hexokinase activity and sugar absorption.

Methods

Male and female rats of the same breed, weighing 120 to 150 g each, were used. Feeding was stopped 24 hours prior to operation. Nephrectomy was performed lumbally under nembutal anaesthesia. After further 48 hours' fasting the cardia and the coecum were ligated and the upper and lower ends of the small intestine were cannulated and washed thoroughly with physiological saline solution. Subsequently 5 ml of isotonic glucose solution (275 mg of glucose) was injected into the gut. After 30 minutes the sugar was washed out and the amount of residual sugar was determined. A 10 cm long section of the upper duodenum was removed, placed immediately into ice-cold water, washed, and the mucosa was scraped off. Hexokinase activity was determined by the method of SOLS [6]. In an ice-cold solution containing 0.26 *M* mannitol, 0.005 *M* neutralized EDTA and 0.002 *M* thioglycolic acid, a 10 per cent suspension was made of the mucosa. 0.2 ml of this was put into 0.3 ml of a substrate mixture containing 3 μM glucose dissolved in a pH 8 veronal buffer with 0.01 *M* ATP and 25 μM NaF. After 15 minutes' incubation at 25°C the reaction was stopped by adding 0.5 ml saturated $Ba(OH)_2$. The amount of sugar was determined according to HAGEDORN and JENSEN.

Results

The values for hexokinase activity and glucose absorption obtained from the same animal are recorded in the same row (Table I).

No correlation existed between hexokinase activity and glucose absorption. In some animals hexokinase activity was quite weak but the absorbed amount of glucose was the same, or even more, than in animals with the highest hexokinase activity.

Table I

Hexokinase activity and glucose absorption in nephrectomized rats

Animal No.	Hexokinase activity in μM glucose	Absorped glucose mg/100 g body weight
1	> 0.2	44
2	> 0.2	36
3	> 0.2	58
4	> 0.2	69
5	> 0.2	35
6	0.55	56
7	0.90	55
8	1.30	26
9	1.30	28
10	1.90	100
11	2.30	11
12	2.80	70
13	3.40	57
14	3.40	41
15	3.40	100
16	3.50	89
17	3.70	74
18	4.40	66
19	4.50	35
20	6.60	43
21	8.30	74
22	8.40	75
23	9.10	50
24	12.0	56
25	14.0	10
26	17.4	47
27	18.3	17

Our data support the pertaining recent findings that no direct relationship exists between the phosphorylation and absorption of sugars.

LITERATURE

1. Bisegger, A., Laszt, L.: Helv. physiol. Acta **9**, 60 (1951).
2. Csáki, T. Z.: Science **118**, 253 (1953).
3. Hele, M. P.: Biochem. J. **55**, 857 (1953).
4. Kjerjulf-Jensen, K.: Acta physiol. scand. **4**, 225 (1942).
5. Lüthy, E., Verzár, F.: Biochem J. **57**, 316 (1954).
6. Sols, A.: Biochim. Biophys. Acta **19**, 114 (1956).
7. Sols, A.: Rev. esp. Fisiol. **11**, 277 (1955).

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ANALYSE DER MIT HYPOPHYSENHINTERLAPPEN-EXTRAKT HERBEIGEFÜHRTEN VERÄNDERUNG DER INTESTINALEN WASSERRESORPTION BEI MIT RINDENHORMONEN VORBEHANDELTEN TIEREN

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Mit Hilfe der Horváth—Wixschen Resorptionsmethode wurden die Gesetzmäßigkeiten der Wasserresorption aus dem Dünndarm an mit Cortisonazetat und Desoxycorticosteronglukosid vorbehandelten Rattenmännchen untersucht. Die Untersuchungen erstreckten sich einerseits auf den von den beiden hyaluronidase- bzw. antihyaluronidaseartig wirkenden Substanzen auf die intestinale Grundresorption ausgeübten Effekt, anderseits auf den Effekt, den dieselben Substanzen auf die wasserresorptionssteigernde Wirkung des antidiuretischen Hormons zeigen. Die Ergebnisse lassen sich folgendermaßen zusammenfassen:

1. Von Cortisonazetat und Desoxycorticosteronglukosid wird die konstante Grundresorption der physiologischen Kochsalzlösung aus dem Dünndarm bei vorhandener Nebennierenrindenfunktion in der üblichen pharmakologischen Dosis nicht verändert.

2. Der intravenös eingespritzte Nervenlappenextrakt führt bei den mit Cortisonazetat vorbehandelten Tieren nur geringe Resorptionssteigerung herbei. Durch Vorbehandlung mit Desoxycorticosteronglukosid wird die Resorptionssteigerung nicht signifikant beeinflußt.

Auf Grund dieser Resultate kann die Theorie der Hyaluronidase-Mediation nicht als eindeutig erwiesen angesehen werden.

Methoden

Die Untersuchungen wurden nach dem Horváth—Wixschen Perfusionsverfahren [11], die Hämoglobinbestimmungen nach der Methode von Havemann, Issekutz und Jung [10] mit dem Havemannschen photoelektrischen Kolorimeter nach dem früher von uns beschriebenen Verfahren [6] an 160—220 g schweren Wistar-Ratten vorgenommen, die aus zeitlich einander naheliegenden Würfen stammten.

Der Versuch wurde folgendermaßen durchgeführt: Urethannarkose mit 150 mg/100 g, Operation, Vorbehandlung mit Kochsalz oder dem entsprechenden Hormonpräparat. 30 Minuten Ruhe. Einleitung der Perfusion und Abwarten des Erscheinens der Perfusionslösung in der Ausführungskanüle. Hiernach untersuchten wir die Resorption 5×10 Minuten; die ersten 2×10 Minuten betrachteten wir als Grundresorption, sodann wurde der Hinterlappenextrakt bzw. die Kontrollflüssigkeit in die vorher herauspräparierte V. jugularis infundiert.

Zur Vorbehandlung benutzten wir Adreson *(Organon N. V.*, Oss) und Percorten »wasserlöslich« *(Ciba A. G.*, Basel) intramuskulär in der Dosis von 5 mg/100 g bzw. 1 mg/100 g. Als Hinterlappenextrakt kam Piton *(Organon N. V.*, Oss) in der Dosis von 0,5 mE/100 g und im Gesamtvolumen von 0,5 ml/100 g zur Anwendung.

Die Resorption untersuchten wir an den auf folgende Weise behandelten Gruppen:*

*In der Arbeit kommen folgende Abkürzungen zur Anwendung:

CA = Cortisonazetat;
DCG = Desoxycorticosteronglukosid;
HHL = Hypophysenhinterlappen;
ADH = antidiuretisches Hormon.

	Vorbehandlung i. m.	Eingriff i. v.	Zahl der Tiere
A	Kochsalz	HHL	6
B	CA	Kochsalz	6
C	DCG	Kochsalz	6
D	CA	HHL	6
E	DCG	HHL	6

Die HORVÁTH—WIXsche Originalmethode modifizierten wir insofern, als wir in das Perfusionssystem vor der Einführungskanüle ein zur Messung des hydrostatischen Mitteldrucks geeignetes Manometer einschalteten. Mit dessen Hilfe konnte der in cm H_2O gemessene hydrostatische Druck jederzeit abgelesen und die vollständige Verstopfung der Kanüle mit Schleimpfropfen vermieden werden.

Nach Abschluß des Versuches wurden die Tiere durch Luftembolie getötet, der Darmabschnitt zwischen den beiden Kanülenenden wurde herausgeschnitten, sein Lumen der Länge nach geöffnet und in Formalin fixiert. Nach 48stündiger Formalinfixierung bestimmten wir den Umfang und rechneten die resorbierte Wassermenge auf die Resorptionsfläche um. Auf diese Weise vermochten wir die resorbierte Wassermenge in den Dimensionen $\mu l/cm^2/10'$ anzugeben.

Ergebnisse

Wirkung der Vorbehandlung auf die Grundresorption

Die Grundresorption der mit physiologischer Kochsalzlösung vorbehandelten Kontrolltiere betrug $13{,}56 \pm 4{,}08$ $\mu l/cm^2/10'$ (Durchschnittswert von 12 Versuchen), die der mit CA vorbehandelten Tiere (Durchschnitt von 24 Wer-

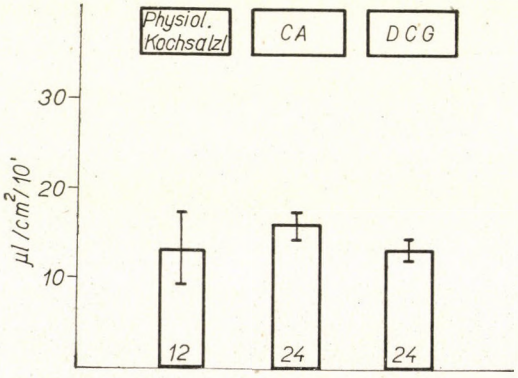

Abb. 1. Die Größe der basalen Wasserresorption aus dem Dünndarm bei den mit Corticoiden vorbehandelten Tieren

Die einzelnen Säulen entsprechen den Durchschnittswerten der mit den angegebenen Substanzen vorbehandelten Tiergruppe mit dem Mittelfehler des Durchschnitts (+ 1 S. E.). Die Anzahl der Tiere ist am Fuß der Säule vermerkt

ten) $16{,}22 \pm 1{,}59$, die der mit DCG vorbehandelten $13{,}72 \pm 1{,}18$ $\mu l/cm^2/10'$ (Durchschnitt von 24 Werten). Die Resultate sind in Abb. 1 graphisch dar-

gestellt. Hiernach wird die Grundresorption von DCG offensichtlich nicht beeinflußt, dagegen durch die Vorbehandlung mit CA offenbar etwas erhöht. Die Erhöhung ist aber derart gering, daß es sich eher nur um eine suggestive und keinesfalls um eine signifikante Erhöhung handelt.

HHL-Wirkung auf die zur Kontrolle der Vorbehandlung dienenden Tiere. Bei den mit physiologischer Kochsalzlösung vorbehandelten Tieren betrugen nach Piton-Verabreichung die Resorptions-Mehrwerte in den drei aufeinanderfolgenden 10-Minuten-Perioden $12{,}17 \pm 1{,}97$, $10{,}21 \pm 1{,}38$ und $3{,}72 \pm 1{,}93$ $\mu l/cm^2/10'$. Nach der Summierung dieser Ergebnisse kam unter Wirkung des HHL-Extraktes bei den zur Kontrolle der Vorbehandlung dienenden Tieren

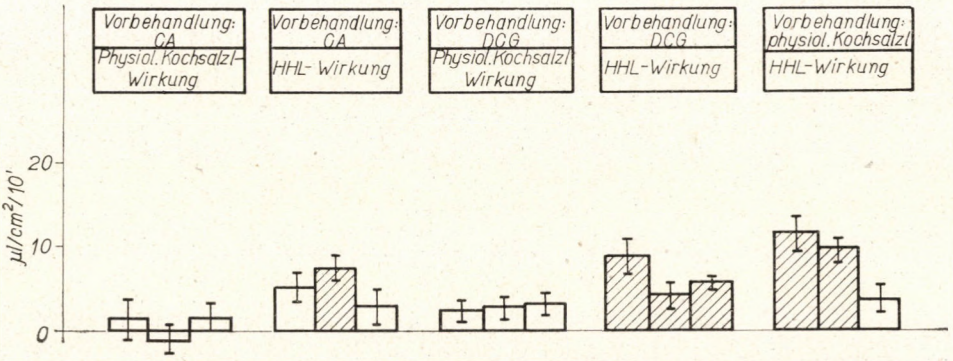

Abb. 2. Die Veränderungen der unter Wirkung von HHL-Extrakt zutage tretenden Mehrresorption aus dem Dünndarm bei den mit Cortisonazetat (CA) und Desoxycorticosteronglukosid (DCG) vorbehandelten und bei den zur Kontrolle der Vorbehandlung dienenden Tieren

Die einzelnen Säulen entsprechen den Mehrresorptionsdurchschnittswerten, während 10' der auf angegebene Weise behandelten Tiergruppen mit dem mittleren Fehler der Mittelwerte ($\pm$ 1 S. E.). Die gestrichelten Säulen zeigen die von Null signifikant abweichenden Werte

in 30' eine Mehrresorption von $26{,}1 \pm 4{,}75$ $\mu l/cm^2$ zustande. Die Abweichung vom 0-Wert der einzelnen 10-Minuten-Durchschnittswerte ist mit Ausnahme des Mittelwertes der letzten 10-Minuten-Periode stark signifikant (Abb. 2 und 3).

HHL-Wirkung auf die mit CA vorbehandelten Tiere. Bei den mit CA vorbehandelten Tieren kam es nach der zur Kontrolle der HHL-Wirkung gegebenen i. v. Einspritzung von physiologischer Kochsalzlösung infolge der geringen Mineralocorticoidaktivität von CA im Vergleich zur Grundresorption zu einer konstanten, aber geringen Resorptionserhöhung, deren Größe verschwindend niedrig und mathematisch nicht signifikant war. Wird HHL-Extrakt vorbehandelten Tieren eingespritzt, so ändert sich die Größe der Resorptionserhöhung im Vergleich zu den nicht vorbehandelten Tieren. Während die Resorptionserhöhung unter Wirkung von ADH bei den nicht vorbehandelten Tieren in 30' insgesamt $26{,}1 \pm 4{,}75$ $\mu l/cm^2$ ausmachte, betrug

dieser Wert bei den vorbehandelten nur $15{,}97 \pm 4{,}08$ $\mu l/cm^2$. Die Werte der einzelnen 10-Minuten-Perioden machten bei den Vorbehandelten unter Wirkung von Normalsalz $+1{,}33 \pm 2{,}08$, $-1{,}0 \pm 1{,}76$ und $+1{,}5 \pm 1{,}54$ $\mu l/cm^2$ aus. Die Abweichung sämtlicher drei Werte von Null ist nicht signifikant. Auch der Durchschnitts-30′-Wert — $+1{,}85 \pm 3{,}63$ $\mu l/cm^2/30'$ — zeigt keine signifikante Abweichung von Null. Nach Zufuhr von HHL betrugen diese Werte $5{,}29 \pm 1{,}86$, $7{,}94 \pm 1{,}76$ und $3{,}19 \pm 2{,}18$ $\mu l/cm^2/10'$. Die Abweichung von Null

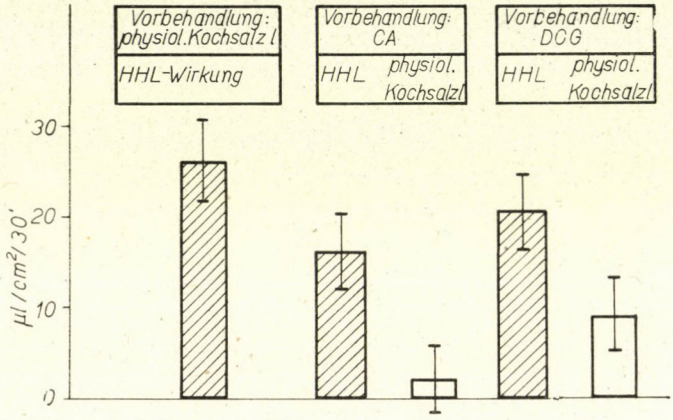

Abb. 3. Die Veränderungen der unter Wirkung von HHL-Extrakt zutage tretenden summierten Mehrresorptionsmengen aus dem Dünndarm bei den mit Cortisonazetat (CA) sowie Desoxycorticosteronglukosid (DCG) vorbehandelten und den zur Kontrolle der Vorbehandlung dienenden Tieren.

Erläuterung wie bei *Abb. 2.* Die einzelnen Säulen zeigen die in 30′ insgesamt zustande gekommene Mehrresorption an

st bei den ersten beiden signifikant, beim letzten Wert nicht. Die Summe dieser Werte, $15{,}97 \pm 4{,}08$ $\mu l/cm^2/30'$, weicht von Null signifikant ab (Abb. 2 und 3).

HHL-Wirkung auf die mit DCG vorbehandelten Tiere. Die Beobachtungen entsprechen ungefähr den im vorigen Abschnitt mitgeteilten Angaben, und die Abweichungen zwischen ihnen sind eher nur quantitativer Natur. Auch nach Vorbehandlung mit DCG kam leere — ohne HHL-Extrakt erfolgte — Resorptionssteigerung zustande, die etwas größer war als nach Anwendung von CA. Ihre Abweichung von Null war indessen mathematisch noch nicht signifikant.

Die 10-Minuten-Werte ohne HHL betrugen der Reihe nach $+2{,}70 \pm 1{,}28$, $+3{,}21 \pm 1{,}33$ bzw. $+3{,}39 \pm 1{,}36$ $\mu l/cm^2/10'$, summiert $+9{,}30 \pm 0{,}00$ $\mu l/cm^2/30'$. Die Abweichung vom Null-Wert ist nicht signifikant. Unter Wirkung des HHL-Extraktes steigen die Werte: $+9{,}13 \pm 2{,}0$, $+4{,}57 \pm 1{,}58$ und $+6{,}63 \pm 0{,}26$ $\mu l/cm^2/10'$ bzw. $+20{,}33 \pm 3{,}84$ $\mu l/cm^2/30'$. Die gewonnenen Werte weichen sämtlich signifikant von Null ab. Gegenüber der unter Wirkung von HHL-

Extrakt eintretenden 192,5%igen Resorptionssteigerung bei den zur Kontrolle der Vorbehandlung dienenden Tieren zeigt diese Gruppe eine 141,6%ige, d. h. etwas niedrigere Resorptionssteigerung (Abb. 2 und 3). Sämtliche gewonnenen Durchschnittswerte sind auch zahlenmäßig in Tabelle I zusammengefaßt

Tabelle I

Wirkung isotonischer Salzlösungen auf die von Hypophysenhinterlappenextrakt verursachte Resorptionssteigerung bei den mit Cortisonazetat und Desoxycorticosteronglukosid vorbehandelten Tieren

Behandlung		Mehrresorption (μl/cm² /t) im Verbältnis zur Grundresorption			
		0—10′	10—20′	20—30′	0—30′
Kochsalz + HHL	6	12,17±1,97 $P < 0{,}01$	10,21±1,38 $P < 0{,}001$	3,72±1,93 nicht sign.	26,1±4,75 $P < 0{,}01$
CA + Kochsalz	6	1,33±2,08 nicht sign.	−1,0±1,76 nicht sign.	1,54±1,54 nicht sign.	1,85±3,63 nicht sign.
CA + HHL	6	5,29±1,61 $P < 0{,}02$	7,49±1,76 $P < 0{,}001$	3,19±2,18 nicht sign.	15,97±4,08 $P < 0{,}02$
DCG + Kochsalz	6	2,70±1,28 nicht sign.	3,21±1,33 nicht sign.	3,39±1,36 nicht sign.	9,30±3,83 nicht sign.
DCG + HHL	6	9,13±2,0 $P < 0{,}01$	4,57±1,58 $0{,}2 < P < 0{,}05$	6,63±0,26 $P < 0{,}001$	20,33±3,84 $P < 0{,}01$

Die Festlegung der gewonnenen Resultate in der Dimension μl/cm²/t und die Registrierung des hydrostatischen Drucks ermöglichten den Vergleich mit den in derselben Dimension ausgedrückten *In-vitro*-Angaben [18]-SMYTH fand bei seinen Versuchen *in vitro*, daß der beobachtete Resorptions′wert bei 20′ cm H_2O Druck 26,6 μl/cm²/10′, bei 45 cm H_2O Druck 35,5 μl/cm²/10′ ausmachte. Dies entspricht annähernd unseren *In-vivo*-Ergebnissen, wo wir im System mit 12—14 cm H_2O Druck einen Durchschnittswert von 13,56 μl/cm²/10′ feststellten. Aus diesem Vergleich schließen wir auf die annähernde Übereinstimmung der Werte *in vivo* und *in vitro*.

Besprechung

In vorangegangenen Untersuchungen hatten wir nachgewiesen [6], daß HHL eine Steigerung der Wasserresorption aus dem Darm bewirkt. Damit bestätigten wir eine extrarenale Wirkung des ADH mit einer objektiven Untersuchungsmethode.

Über diese Tatsache hinaus vermochten wir die Abhängigkeit der Erscheinung von der Dosis festzustellen und die vorher bereits beschriebene Porenhypothese an der Haut und Niere zu beweisen. Wir beobachteten nämlich, daß im Falle der Perfusion des Dünndarms mit physiologischer Kochsalzlösung das ADH imstande ist, die definitionsartig eintretende Wasser-

abgabe zu steigern, und aus dieser experimentellen Tatsache schlossen wir, daß die Öffnung der Poren unter Wirkung des ADH in jedem Fall eintritt, aber die Wasserströmung nur in der den osmotischen Erfordernissen entsprechenden Richtung erfolgt.

Laut GINESCHINSKI [9] übt das antidiuretische Hormon seine renale Wirkung über die Hyaluronidase aus. In der Antidiurese, d. h. wenn der ADH-Spiegel im Blut steigt, nimmt die Hyaluronidaseproduktion der Tubuluszellen zu, und dies bewirkt durch die Depolymerisation der interzellulären Bindegewebsgrundsubstanz die Resorption des osmotisch freien Wassers. Nach anderen Angaben wird die wasserresorptionssteigernde Wirkung des HHL-Extraktes von Hyaluronidase an der Amphibienhaut gehemmt [19].

Vom Gesichtspunkt unserer weiteren Untersuchungen schien die Klarstellung der Fragen interessant, ob einerseits das ADH auch im Darm nach demselben Mechanismus wirkt, und ob anderseits der ADH-Effekt durch das von den im Organismus erzeugten Nebennierensteroiden herbeigeführte aktuelle endokrine Milieu verändert wird.

Über die erste Frage gaben unsere früheren Untersuchungen Auskunft, während wir zur Klärung der zweiten Frage bei den vorliegenden Untersuchungen die Wirkung von Hyaluronidase und Antihyaluronidase auf mit Steroiden vorbehandelte Tiere unter Vergleich mit Kontrolltieren, die nur Kochsalz erhielten, ermittelten. Bekanntlich wird die Permeabilität gewisser Membrane von DOCA ebenso wie von Hyaluronidase bedeutend gesteigert, von Cortison jedoch verringert [16, 17]. Ebenso setzt Cortison die von Hyaluronidase verursachte Kapillarpermeabilitätssteigerung [7, 8] und das dermale Spreading herab [13, 15, 20].

In Kenntnis dieser Tatsachen untersuchten wir die etwaige diesbezügliche Beeinflußbarkeit des Verlaufs der ADH-Wirkung. Zugleich damit erhalten wir — indem die Frage auf andere Weise gestellt wird — auch Antwort darauf, inwieweit der ADH-Effekt von der Anwesenheit der an der Peripherie erreichbaren Corticosteroide abhängt. NATZSCHKA hatte beobachtet, daß von Prednison die Größe der durch kleinere ADH-Gaben verursachten Antidiurese herabgesetzt wird, während Aldosteron diese nicht beeinflußt [14].

Wie die Ergebnisse zeigen, wird die Größe der Grundresorption durch die Vorbehandlung — bei erhaltener Nebennierenrindenfunktion — nicht beeinflußt. Nach DENNIS und anderen [5] sind Nebennierenrindenextrakte imstande, die verringerte Grundresorption von andrenalektomierten Hunden wiederherzustellen. Eine ähnliche Angabe über die Erhöhung der Grundresorption fanden wir bei CAPPELLI [3, 4], der nachwies, daß die infolge Adrenalektomie von 100% auf 22% gesunkene intestinale Wasserresorption von Cortexextrakt auf 44, von Cortison auf 88 und von DOCA auf 42% erhöht wird.

Nach Vergleich der Angaben vorerwähnter Autoren mit den eigenen Resultaten muß daher festgestellt werden, daß die die Grundresorption er-

höhende Wirkung der Rindensteroide bei adrenalektomierten Tieren nur soweit zur Geltung kommt, daß die normale Grundresorption annähernd erreicht wird, so daß zwischen dem Dosiseffekt der Rindensteroide und der intestinalen Wasserresorption kein im engeren Sinne verstandener Zusammenhang besteht.

Unsere Beobachtungen über die Corticoid-HHL-Interferenz zeigen, daß CA eine ziemlich ausgeprägte Senkung des HHL-Effektes bewirkt. Dies steht im Einklang mit den vorhin erwähnten Angaben und mit der Theorie der Hyaluronidase-Mediation des ADH-Effektes, doch muß festgestellt werden, daß sich die Wirkung der Corticosteroide — nach den gegenwärtigen Untersuchungen — offenbar nur auf einen gewissen Teil der Erscheinungen erstreckt. Die mit DCG gewonnenen Resultate wichen von den erwarteten ab, weil sie im Gegensatz zu ihrer spreadingsteigernden Hyaluronidasewirkung die nicht signifikante Senkung der ADH-Wirkung verursachten, die also unverändert blieb. Auf Grund unserer Untersuchungsergebnisse scheint es, daß DCG auf die HHL-Wirkung keinen Einfluß ausübt und daher, obschon es das dermale Spreading steigert, an der Darmmembran nicht wie Hyaluronidase wirkt.

Im Endergebnis lieferten demnach unsere vorliegenden Untersuchungen keine Resultate, die dem Prinzip der Hyaluronidase-Mediation in jeder Hinsicht entsprechen. Eine indirekte CA-Wirkung ist anzunehmen, aber die mit DCG gewonnenen Ergebnisse lassen sich in diese Theorie nur dann einfügen, wenn wir in Ermangelung weiterer Angaben einstweilen voraussetzen, daß der Mechanismus der hyaluronidaseartigen Desoxycorticosteronwirkung nur im Falle bestimmter potentieller und räumlicher Gegebenheiten gilt.

LITERATUR

1. Beneditt, E. P., Schiller, S., Wong, H., Dorfman, A.: Proc. Soc. exp. Biol. (N. Y.) **75,** 782 (1950).
2. Berlyne, G. M.: Nature (Lond.) **185,** 389 (1960).
3. Cappelli, V., Rossi, M. L.: Boll. Soc. ital. Biol. sper. **30,** 545 (1954).
4. Cappelli, V., Rossi, M. L.: Boll. Soc. ital. Biol. sper. **30,** 548 (1954).
5. Dennis, C., Wood, E. H.: Amer. J. Physiol. **129,** 182 (1940).
6. Dombrádi, G., Krizsa, F., Jancsó, T.: Acta physiol. hung. **17.** 301 (1960).
7. Duran-Reynals, F.: Bact. Rev. **6,** 197 (1942).
8. Elster, S. K., Freeman, M. E., Dorfman, A.: Amer. J. Physiol. **156,** 429 (1949).
9. Ginetschinski, A. G. (Гинечинский, А. Г.): Физиол. Ж. **45,** 761 (1959).
10. Havemann, R., Jung, F., Issekutz, B. jun.: Biochem. Z. **50,** 116 (1939).
11. Horváth, L., Wix, G.: Acta physiol. hung. **2,** 435 (1951).
12. Iwanowa, L. N. (Иванова, Л. Н.): Бюл. експ. биол. мед. **45,** 22 (1958).
13. Menkin, V.: Amer. J. Physiol. **129,** 691 (1940).
14. Natzschka, J., Senft, G.: Arch. exp. Path. Pharmak. **237,** 334 (1959).
15. Opsahl, J.: Yale J. Biol. Med. **21,** 225 (1949).
16. Seifter, J., Baeder, D. H., Dervinis, A.: Proc. soc. exp. Biol. (N. Y.) **72,** 136 (1949).
17. Seifert, J., Baeder, D. H., Begany, A. J.: Proc. soc. exp. Biol. (N. Y.) **72,** 277 (1949).
18. Smyth, D. H., Taylor, C. B.: J. Physiol. **136,** 632 (1957).
19. Vartiainen, Ch., Venho, E. V., Eckert, D.: Ann. Med. exp. Biol. fenn. **29,** 69 (1951).
20. Weinstein, L.: Yale J. Biol. Med. **12,** 549 (1940).
21. Wirz, H.: Helv. physiol. Acta **14,** 353 (1956).

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ADRENOCORTICAL CORTICOID SECRETION IN THE GUINEA PIG

By

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The adrenal cortex of the guinea pig was found to contain five corticoids: (i) 2-α-hydrocortisole or 6-β-hydroxycortisole; (ii) hydrocortisone; (iii) cortisone; (iv) ticorcosterone; (v) 11-dehydrocorticosterone.

Hydrocortisone occurred in the greatest amount. The other corticoids were not present in every case. ACTH elicited a characteristic rise in total secretion.

As to adrenal weight, no significant difference was found between left and right organ, or between male and female animals. ACTH treatment increased adrenal weight.

There are few data in the literature concerning adrenocortical corticoid secretion in the guinea pig. Only BUSH [2] discussed this question, while the other data refer mainly to peripheral blood (DONE, ELY, ROULE and KELLY, [5], COHEN and KLEINBERG [4]), urine (PERON and DORFMAN [11], NADEL, YOUNG and HILGAR [12]), or bile (EIK-NES, DEMETRION, MAYNE and JONES [6]).

In previous experiments we demonstrated that placental tissue of the guinea pig was capable of synthesizing corticoids both *in vivo* and *in vitro* (TELEGDY, ENDRŐCZI and HUSZÁR [13]). Those data gave information mainly concerning the metabolism and transformation of corticoids, not permitting, however, any final conclusion as to adrenal secretion.

Methods

A total of 80 guinea pigs of both sexes weighing 250 to 900 g each was used. Adrenal venous blood was collected by means of a cannula inserted into the renal vein. The animals were anaesthetized with 0.01 g/100 g Thialbarbitone and pretreated with 2 I. U./100 g heparin.

Qualitative estimations were made in the pooled blood from 10 to 15 animals.

Blood for quantitative determinations was collected from individual animals for 10 to 15 minutes. ACTH-loading was made by the administration of 5 I. U. Exacthin *(Richter*, Budapest) daily for three days. The values are expressed in μg corticoid/kg body weight/hour.

Corticoid analysis was performed by paper chromatography, using both the benzene-formamide system of BURTON, ZAFFARONI and KEUTMAN [1] and the system BUSH B_5 [3]. The corticoids were extracted as described previously [7, 8, 9]. Identification of individual corticoids in both systems was made (i) by comparison of the R_f values with those of standard preparations *(Organon*, Oss), (ii) by the alkaline fluorescence method, (iii) by the tetrazolium reduction test, (iv) by the acetylation reaction.

The adrenals were weighed on torsion balance with an accuracy of 1 mg, and the weights were expressed in mg/100 g body weight. Comparison was made between the right and left adrenals, male and female animals, and normal and ACTH-treated guinea pigs.

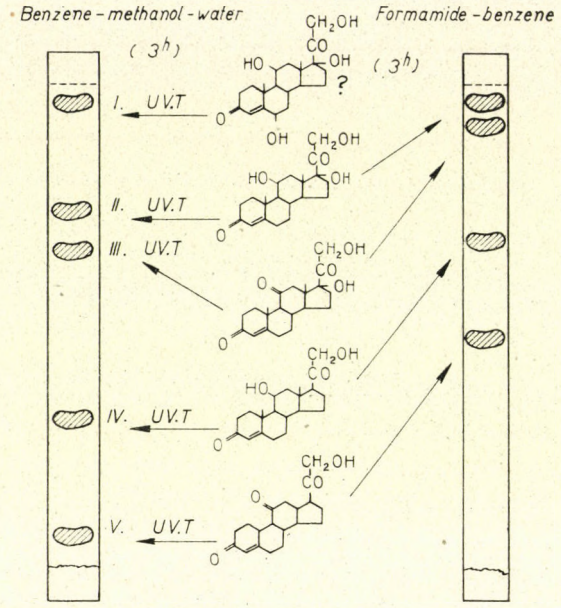

Fig. 1. Schematic drawing demonstrating separation of corticoids found in the venous blood of guinea pig adrenal. T = tetrazolium reduction. UV = NaOH fluorescence.

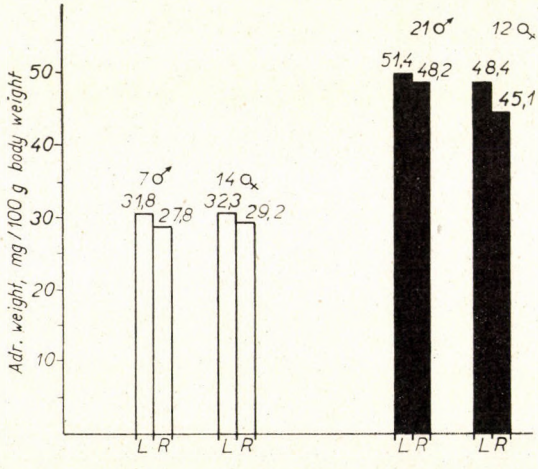

Fig. 2. Adrenal weights expressed in mg/100 g body weight. White column = normal; dark column = ACTH-treated animals; R = right adrenal; L = left adrenal; ♂ male animals; ♀ = female animals. Figures refer to the number of the animals used.

Results

Qualitatively, the following corticoids were observed to occur in the adrenal blood (Fig. 1).

(i) 2-α-hydroxycortisole or 6-β-hydroxycortisole. These derivatives appeared in the benzene-methanol-water system (system BUSH B_5), gave UV fluorescence with sodium hydroxyde and reduced tetrazolium. Their small amount did not permit more exact identification.

(ii) Hydrocortisone. It occurred in both sexes, reduced tetrazolium, gave fluorescence with NaOH, and exhibited a R_f value identical with that of the standard hydrocortisone preparation.

(iii) Cortisone.

(iv) Corticosterone.

(v) 11-dehydrocorticosterone.

The quantitative secretion pattern (Table I) revealed that in all animals hydrocortisone occurred in the greatest amount. Secretion of other compounds was irregular. The total adrenocortical secretion, expressed in μg'/kg'/h, was less in the guinea pig then in other species [10].

Table I

Adrenocortical secretion in normal animals, expressed in μg/kg/h. Cpd F = hydrocortisone; Cpd E = cortisone; Cpd B = corticosterone; Cpd A = 11-dehydrocorticosterone

	Cpd F	Cpd E	Cpd B	Cpd A	Total amount:
♂	9,8	—	3,2	—	13,0
♂	17,0	—	4.0	—	21,0
♂	26,0	—	15,0	26,0	67,0
♂	30,0	10,0	1,8	—	41,8
♂	19,0	4,7	—	57,0	80,7
♂	22,0	11,0	—	—	33,0
♀	21,0	—	—	—	21,0
♀	48,0	19,0	—	4,7	71,7
♀	30,0	—	—	—	30,0
♀	69,0	23,0	3,0	—	95,0
♀	38,0	19,0	3,0	—	60,0
♀	50,0	10,0	2,4	—	62,4
♀	18,0	9,0	1,8	—	28,8
♀	14,0	—	2,8	—	16,8
♀	12,0	—	1,5	—	13,5
♀	13,3	—	1,9	—	15,2
				Average	41,9

Table II

Adrenocortical secretion after treatment of the animals with 5 I. U. ACTH daily for three days. Cpd F = hydrocortisone; Cpd E = cortisone; Cpd B = corticosterone; Cpd A = 11-dehydrocorticosterone.

	Cpd F	Cpd E	Cpd B	Cpd A	Total amount:
♂	42,0	—	—	54,0	96,0
♂	82,0	—	—	—	82,0
♂	99,0	19,0	4,0	—	122,0
♀	61,0	—	10,0	—	71,0
♀	62,0	—	10,0	—	73,0
♀	44,0	—	—	23,0	67,0
♀	33,0	11,0	—	—	44,0
♀	63,0	—	—	—	63,0
♀	64,0	—	—	—	64,0
♀	54,0	—	—	—	54,0
				Average	73,6

ACTH treatment elicited an increase in the total secretion (Table II). This manifested itself mainly with an elevated hydrocortisone secretion, while other derivatives behaved irregularly.

As to the weight of the adrenals, there was no difference between right and left organs, nor between male or female animals (Fig. 4). ACTH was found to increase adrenal weight.

Discussion

Unlike in other animal species, in the guinea pig the adrenals are secreting five corticoids. Most polar of these compounds was 2-α-hydrocortisole or 6-β-hydroxycortisole. Occurrence of these derivatives in the urine was demonstrated also by PERON and DORFMAN [12], as well as NADEL, YOUNG and HILGAR [13]. The substance next in polarity was hydrocortisone, representing the bulk of the hormones secreted. The presence of cortisone, corticosterone and 11-dehydrocorticosterone indicated that the adrenals are richer in enzyme systems in the guinea pig than in other species. However, the occurrence of these derivatives was not regular, and we know little of the factors determining their secretion and of the way in which the interrelationship of these factors is influencing the specific reactivity of the guinea pig.

ACTH load increased mainly the secretion of hydrocortisone, with the concomitant elevation in the total amount secreted. Augmentation of

other derivatives was not regular. The 4 to 10-fold increase in corticoid secretion found by other authors [4, 12] could not be confirmed in these experiments. This discrepancy can be probably accounted for by differences both in the preparations used and the route of administration.

As an effect of ACTH treatment, the adrenal weight showed some inerease. No side or sex difference occurred in the adrenal weights.

LITERATURE

1. Burton R. B., Zaffaroni A., Keutman E. H.: J. biol. Chem. **188,** 763 (1951).
2. Bush I. E.: J. Physiol. (Lond.) **115,** 12 (1951).
3. Bush I. E.: Biochem. J. **50,** 370 (1952).
4. Cohen H., Kleinberg W.: Proc. Soc. expl. Biol. (N. Y.) **100,** 375 (1959).
5. Done A. K., Ely R. S., Roule R. B., Kelly V. C.: Proc. Soc. exp. Biol. (N. Y.) **81,** 667 (1952).
6. Eik-Nes K., Demetriou J. A., Mayne Y. C., Jones R. S.: Proc. Soc. exp. Biol. (N. Y.) **96,** 409 (1957).
7. Endrőczi E., Bata G., Martin J.: Endokrinologie **35,** 117 (1958).
8. Endrőczi E., Telegdy Gy., Bata G.: Endokrinologie **36,** 324 (1958).
9. Endrőczi E., Telegdy Gy., Martin J.: Acta physiol. hung. **14,** 311 (1958).
10. Endrőczi E., Lissák K.: Acta physiol. hung. **15,** 25 (1959).
11. Peron F. G., Dorfman R. I.: Endocrinology **62,** 1 (1958).
12. Nadel E. M., Young B. G., Hilgar A. G.: Amer. J. Physiol. **196,** 273 (1959).
13. Telegdy Gy., Endrőczi E., Huszár L.: Acta physiol. hung. **17,** 57 (1960).

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Pathophysiologia

METHIONINE-^{35}S UPTAKE OF RATS FED ON A METHIONINE-DEFICIENT DIET

By

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Two hours after the intravenous administration of ^{35}S-methionine the radioactivity of the liver and brain was +30 and +40 per cent, respectively, higher in rats previously kept on a methionine-deficient diet than in the normal controls. There was no difference between the two groups of rats, in the radioactivity of muscles, kidney, and serum. Thus, binding of methionine, a sign of biochemical regeneration, occurred first in the liver and the brain.

In previous experiments in rats we determined the sequelae of the pathologic process induced by methionine deficiency [9, 11, 12, 13]. Through loss of weight, methionine deficiency led to rapid and severe dystrophy, as it could be expected, since it had been demonstrated [6, 7, 16, 17] that methionine was an essential amino acid the deficiency of which would lead to a breakdown of nitrogen balance in the rat and in man alike. The best-known change in the pathological process is the liver lesion [1, 2, 3, 4, 5, 8], but other changes are also observable [9, 11, 12, 13].

Although the sequelae to methionine deficiency had been identified, it still remained questionable whether or not the changes produced were reversible. To elucidate this problem, we performed experiments with ^{35}S labelled methionine.

Methods

A group of 12 rats was maintained on a semisynthetic, methionine-deficient diet, composed of glue, 18 per cent; dried yeast, 3 per cent; fats, 10 per cent; normal salt mixture (Sós [10]), 4 per cent; cod liver oil, 1 per cent; starch, 64 per cent. The control group of 12 rats was fed a similar diet which instead of the glue contained an equal quantity of casein.

The active amino acid containing 95 per cent methionine and 5 per cent sulphide contamination, with a specific activity of 390 mc/g, was dissolved in 10 ml of physiological saline. The stock solution thus obtained had a specific activity of 820 μc/ml. This was diluted 2.5-fold, to obtain a specific activity of 328 μc/ml. The single dose was 0.5 ml, *i. e.* 164 μc, corresponding to 0.4 mg of methionine. The 5 per cent sulphide contamination was ignored.

The experiments were made 21, 28 and 31 days, respectively, after starting the methionine-deficient diet. The rats were anaesthesized with urethane. 0.5 ml of the labelled methionine solution was injected into the femoral vein and the animals were killed by decapitation 2 hours later. The blood was centrifuged, and the serum was pipetted off. The brain (cerebrum, diencephalon and mesencephalon, pons and medulla oblong.), the liver, kidneys and a specimen of the gastrocnemius muscle were removed for study. The organs were weighed, the aliquots of the liver, kidney and brain were weighed on analytical scales. The organs were homogenized with 2 ml of distilled water in a Potter homogenizer, and the volume was adjusted to 5 ml.

Likewise, the 0.5 ml specimens of plasma were adjusted to 5.0 ml. Of each homogenate amounts of 0.5 ml were measured into aluminium dishes and dried carefully so as to obtain a homogeneous preparation. Thus the specimen contained more than 30 mg/square cm and the measurement was made on the basis of the infinitely thick layer principle. The impulses were counted by means of a GM counter with a thin end window.

From the activity and weight of the sample the activity taken up by 1 g and by the whole organ was computed. From the activity injected and from the total activity taken up by the organ the percentage uptake of methionine by the organs in 2 hours was computed.

Brain, kidney and liver were weighed. The quantity of muscle and plasma was determined by computation, on the basis of averages for other data, taking for muscle weight 40 per cent of the body weight, and for the average circulating plasma volume, 2.6 ml/100 g.

Results

The methionine uptake values for both the controls and for the rats fed a methionine-deficient diet showed little scattering, a fact indicating the reliability of the method employed.

There was no difference in the total ^{35}S-methionine content of the organs between the control and the methionine-deficient rats (Table I). The organs

Table I

^{35}S uptake by organs.
Means and deviations from the mean for group of 12 animals

	Mean activity, 1000 counts/g		Percentage uptake from injected activity	
	Controls	Methionine-deficient animals	Controls	Methionine-deficient animals
Liver	31 ± 5	42 ± 6	4.8 ± 0.8	6.7 ± 0.8
Brain	4.5 ± 0.6	5.8 ± 0.7	0.25 ± 0.04	0.34 ± 0.05
Kidney	54 ± 7	45 ± 9	2.1 ± 0.3	1.8 ± 0.3
Muscle	2.75 ± 0.4	2.49 ± 0.4	4.44 ± 0.6	3.54 ± 0.5
Serum	24 ± 4	21 ± 6	2.5 ± 0.4	2.2 ± 0.5
Total	116	116	14.2	14.2

of the controls contained a total of 14.2 per cent of the amount injected, those of the methionine-deficient rats 14.6 per cent. There was no difference between the control and methionine-deficient rats in the ^{35}S-methionine content of muscle, kidney and serum either, but there was a significant difference in the uptake by liver and brain.

The control livers took up 4.8 per cent, the methionine-deficient ones 6.7 per cent of the activity injected. The differences are even sharper if we analyse the active methionine concentration of the organs. The pertaining values were 31 000 counts/min/g for the controls and 42 000 counts/min/g for the methionine-deficient rats. The mean of 31 000 counts/min/g was

exceeded by five of the controls and by 10 of the methionine-deficient rats (Fig. 1). Statistical analysis showed the difference to be significant ($P < 0.01$).

The methionine uptake by the control brains was 0.25 per cent, as compared with the 0.34 per cent for the deficient animals. The significance of this

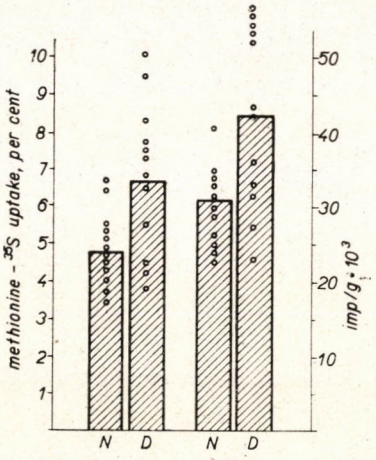

Fig. 1. ^{35}S-Methionine uptake by the liver. *N :* normal rats. *D :* methionine-deficient rats. The first two columns show the uptake by the whole livers, the other two the activity per 1 g liver tissue. The columns represent the means, the dots the individual values

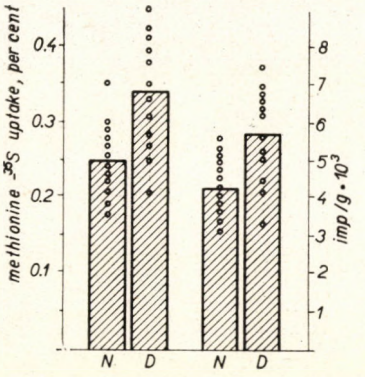

Fig. 2. ^{35}S-Methionine uptake by the brain. *N :* rats fed a normal diet. *D :* rats fed a methionine-deficient diet. The first two columns show the uptake per whole brain, the second two the activity per 1 g brain tissue. The columns represent the means, the dots the individual results

difference was best shown by the ^{35}S-methionine concentration of the brains. The mean specific activity for the controls was 4400 counts/min/g, as compared with the 5800 counts/min/g value for the experimental group. None of the controls reached a concentration of 6000 counts/min/g, while 6 of the methionine-deficient rats showed even higher values (Fig. 2). The difference between the groups was statistically significant ($P < 0.01$).

It may be concluded that the ^{35}S-methionine uptake by the brain and liver is increased by a methionine-deficient diet, a fact pointing to the occurrence of biochemical regeneration as early as 2 hours following the administration of methionine.

Discussion

It is common knowledge that methionine deficiency leads to liver lesion and that methionine protects the liver [1, 2, 3, 4, 5, 8]. It could be anticipated that after feeding a methionine-deficient diet the organism will take up methionine at an increased rate, in order to compensate the loss. As seen, after a methionine deficiency lasting from 20 to 31 days (a loss of body weight of 25 to 35 per cent) the changes in the liver were possible reversible, because that organ took up increased amounts of methionine. It is possible that the regeneration of the liver was followed later by an increased methionine uptake by the muscles and kidney, but this could not be demonstrated in our experiments which lasted only 2 hours.

The increased ^{35}S-methionine uptake by the brain was remarkable since in other experiments [14, 15] it was found that cerebral cortical function did not regenerate even when the other organs were histologically restored to normal. In the present series of experiments the onset of biochemical regeneration was evident 2 hours after the administration of methionine. It seems that the restoration of function lags far behind this.

LITERATURE

1. Aschkenasy, A., Mignot, J.: C. R. Soc. Biol. **141,** 19 (1947).
2. Glynn, L. E., Himsworth, H. P., Neuberger, A.: Brit. J. exp. Path. **26,** 326 (1945).
3. György, P.: Experimental Hepatic Injury. Amer. J. Clin. Path. **14,** 67 (1944).
4. György, P., Goldblatt, H.: J. exp. Med. **90,** 73 (1949).
5. Knowlton Hall, W., Sydenstricker, V. P.: Arch. Biochem. **12,** 147 (1947).
6. Rose, W. C., Haines, W. J., Johnson, J. E.: J. biol. Chem. **146,** 683 (1942).
7. Rose, W. C., Haines, W. J., Warner, D. T., Johnson, J. E.: J. biol. Chem. **188,** 49 (1951).
8. Sims, F. H.: Brit. J. exp. Path. **32,** 481 (1951).
9. Sós, J., Tóth, F., Kemény, T.: Kísérl. Orvostud. (Budapest) **4,** 284 (1952).
10. Sós, J.: Kísérl. Orvostud. (Budapest) **3,** 67 (1951).
11. Sós, J., Kemény, T.: Virchows Arch. path. Anat. **328,** 421 (1956).
12. Sós, J., Kemény, T., Schnell, M.: Acta physiol. hung. **4,** 211 (1953).
13. Véghelyi, P. V., Kemény, T. T., Pozsonyi, J., Sós, J.: Amer. J. Dis. Child. **79,** 658 (1950).
14. Weisz, P., Sós, J., Gáti, T., Harmos, G., Rigó, J.: Ideggyógy. Szle. (Budapest) **8,** 139 (1955).
15. Weisz, P., Sós, J., Gáti, T., Harmos, G., Rigó, J. (Вейс, П., Шош, И., Гати, Т., Хармош, Г., Риго, Я.): Вопросы питания **1,** 15 (1956).
16. Womack, M., Kemmerer, K. S., Rose, W. C.: J. biol. Chem. **121,** 403 (1932).
17. Womack, M., Rose, W. C.: J. biol. Chem. **141,** 375 (1941).

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THE EFFECT OF METHIONINE DEFICIENCY ON HETEROHAEMOTROPIN FORMATION

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A new proof of the role played by methionine in cellular and humoral defence is presented. In addition to the deleterious organic effects of the methionine-deficient diet, inhibition of the heterohaemotropin immune response is also brought about, by an as yet unknown mechanism.

Animals fed on low-calorie and low-vitamin diets are known to have a diminished resistance to infections. After the antibodies had been shown to be of protein character, increased attention was devoted to the relation of protein metabolism to resistance, natural or acquired. In our earlier studies the intravenous administration of protein hydrolysates was found to enhance the phagocytosis of bacteria by leucocytes both in man and in experimental animals [1]. Preparations containing amino acids increased leucocytic phagocytosis also *in vitro*. The amino acids containing sulfur proved to be particularly active [2]. On the other hand, it is also known that leucocytic phagocytosis and antibody production significantly diminish in animals fed on a diet poor in protein [3—15].

It has been observed that the humoral defensive mechanism is much more sensitive to protein deficiency than the cellular one [16]. There is unequivocal evidence of the profound influence exerted by a protein-deficient diet on systemic defence. In possession of these data we examined the effect of a diet deficient in a single amino acid on the immune response of the organism. Numerous experiments have called attention to the deleterious effects of a deficiency in sulfur-containing amino acids, of which methionine seems to play a role of decisive importance [17, 18, 19]. We have therefore made the following experiments.

Methods

Rats were fed on the methionine-deficient diet described by Sós [20] and were tested for heterohaemotropin production by the *in vivo* method described by one of us [21]. A total of 56 albino rats of either sex, weighing about 100 g, were used, in three groups.

Group 1. 18 rats were fed a methionine-containing diet and were injected 1 ml doses intraperitoneally of washed sheep erythrocytes.

Group 2. 19 rats were fed the methionine-deficient diet and immunized with sheep erythorcytes as above.

Group 3. 19 rats before immunization showed the spontaneous phagocytosis values. Three weeks after immunisation each rat was injected 5 ml of a 5 per cent suspension of

sheep erythrocytes intraperitoneally. The control rats were treated in the same way. Three hours later smears were made of the abdominal exudates and were stained according to May-Grünwald and Giemsa. Four hundred leucocytes were counted in every case and the hetero-haemotropin value was expressed in terms of the percentage of phagocytosed erythrocytes among the exudate cells.

Table I

No.	Hetero-haemotropin *in vivo*		Spontaneous phagocytosis in rats not immunized with sheep erythrocytes
	Control rats	Methionine-deficient rats	
1.	15.25%	3.75%	*0.20%*
2.	16.75%	8.25%	*2.75%*
3.	13.75%	6.00%	0.50%
4.	10.25%	3.25%	1.20%
5.	9.50%	12.25%	0.75%
6.	10.25%	2.00%	1.00%
7.	23.50%	4.50%	0.50%
8.	12.75%	5.75%	2.50%
9.	21.25%	7.25%	0.50%
10.	13.00%	2.50%	1.00%
11.	18.00%	6.75%	0.70%
12.	14.00%	6.00%	2.50%
13.	23.00%	*13.25%*	1.50%
14.	8.50%	6.00%	0.20%
15.	10.50%	4.25%	0.70%
16.	8.25%	3.25%	1.75%
17.	9.00%	*1.50%*	1.00%
18.	*4.00%*	1.50%	0.75%
19.	—	8.25%	1.50%
Mean	13.41%	5.48%	1.13%

Results

In the third column in Table I are the heterohaemotropin values for the rats fed on the methionine-deficient diet. The range was 1.5 to 13.25 per cent, with a mean of 5.48 per cent, exceeding significantly the spontaneous phagocytosis value of 1.13 per cent. However, as compared with the values for the rats fed on the complete diet (range 4 to 23.5 per cent, mean 13.41 per cent) this was a significant decrease in immune response. These data are supported

by the earlier experiments of RAUGAM and CHANDRA [22], who found a marked decrease in complement titre in guinea pigs fed a diet poor in methionine and cystine. A similar decrease is noted if we add up the values for the phagocytosed erythrocytes in our present experiments. It will be found namely that while the leucocytes of the control animals phagocytosed a total of 2547 erythrocytes, those of the methionine-deficient rats phagocytosed merely 1065.

Further studies are required to give the answers to such questions as for example: does a deficiency in other sulfur-containing amino acids cause a similar defect in immune phagocytosis? What is the underlying mechanism? etc.

LITERATURE

1. LUDÁNY GY., SÓS J., TÓTH E., VAJDA GY.: Orv. Hetil. (Budapest) **94,** 8 (1953).
2. LUDÁNY GY., PERÉNYI L., SÓS J., VAJDA GY.: Arch. int. Pharmacodyn. **4,** 176 (1955).
3. CANNON P. R., CHASE W. E., WISSLER R. W.: J. Immunol. **47,** 133 (1943).
4. CANNON P. R., WISSLER R. W., WOOLRIDGE R. L., BENEDITT E. P.: Ann. Surg. **120,** 514 (1944).
5. WISSLER R. W., WOOLRIDGE R. L., STEFFEE C. H., CANNON P. R.: J. Immunol. **52,** 267 (1946).
6. WISSLER R. W.: J. infect. Dis. **80,** 250 (1947).
7. WISSLER R. W.: J. infect. Dis. **80,** 264 (1947).
8. WOOLRIDGE R. L.: Fed. Proc. **8,** 376 (1949).
9. TALIAFERRO W. H., WOOLRIDGE R. L., BENEDITT E. P.: Science **109,** 443 (1949).
10. BENEDITT E. P., WISSLER R. W., WOOLRIDGE R. L., ROWLEY D. A., STEFFEE C. H.: Proc. Soc. exp. Biol. (N. Y.) **70,** 240 (1949).
11. STEFFEE C. H.: J. infect. Dis. **86,** 12 (1950).
12. VERSHILOVA P. A. *(Вершилова, П. А.):* Вопр. Инфект. Патол. Иммунол. Москва, 1954.
13. KLIMENTOVA A. A. *(Климентова, А. А.):* Вопр. Инфект. Патол. Иммунол. Москва, 1954.
14. TRNKA Z.: Folia Biol. **5,** 306 (1956).
15. LA VIA M. F., BARKER P. A., WISSLER R. W.: J. Lab. clin. Med. **48,** 237 (1956).
16. GUGGENHEIM K., BUECHLER E.: J. Immunol. **58,** 133 (1948).
17. Sós J.: Kisérl. Orvostud. (Budapest) **3,** 67 (1951).
18. Sós J.: Kisérl. Orvostud. (Budapest) **2,** 306 (1950).
19. VÉGHELYI P., KEMÉNY T., POZSONYI T., Sós J.: Amer. J. Dis. Child. **73,** 685 (1950).
20. Sós J.: MTA. Orvostud. Oszt. Közl. (Budapest) **3,** 273 (1952).
21. VAJDA GY.: Thesis, Budapest, 1957.
22. RAUGAM AND CHANDRA: J. Indian med. Ass. **28,** 2 (1957).

Pharmacologia

STUDIES ON A NEW ANTIFUNGAL ANTIBIOTIC

By

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The biological actions of a new antifungal antibiotic (antibiotic T) previously isolated from *Cephalosporium sp.* have been investigated. As a consequence of the resemblance in chemical structure, the effects of antibiotic T were very similar to those of trichotecin. Its antibiotic activity *in vitro* was relatively low and fungistatic in character. Its toxicity was very slight, but reliable pharmacological investigation was made difficult by the drug's very poor solubility in water. An effective blood level after administration of antibiotic T could not be demonstrated either in the mouse, or in the rat. This finding can be partly accounted for by the inactivation of the drug in the blood. *Candida* infection in mice could not be overcome by antibiotic T. When applied on human or animal skin, it caused irritation. The number of yeast cells present in the faeces of mice fed with an oxytetracycline containing diet was decreased by antibiotic T given orally in high doses. The alcohol part resulting from the hydrolysis of antibiotic T showed properties indicating that the preparation of some derivatives of this alcohol may lead to compounds of more favourable antifungal properties.

In a previous paper we have described a new antifungal antibiotic isolated from the culture of a *Cephalosporium sp.* strain [1]. The substance is different from every antifungal antibiotic known until yet. Chemically, the crystalline compound is an ester containing only the elements C, H and O. Its solubility is poor in water (0.1 mg/ml with boiling), medium in alcohol and in sunflower oil, good in organic solvents. When dissolved in water, the substance withstands boiling and can be stored for 2 months at 37°C without change in activity. In oil solution, at room temperature, it is stable for 5 months, and in propylene glycol for more than three weeks. Chemically, it is closely related to trichotecin from which it can be separated by paper chromatography. The slight difference in the infrared spectra of the two compounds points to the fact that, unlike trichotecin, the new antibiotic contains no ketone group. The chemical structure of trichotecin has been clarified recently by FREEMAN [2], who demonstrated this antibiotic to consist of a special alcoholic part (trichotecolone) and of isocrotonic acid. We succeeded in identifying isocrotonic acid on the paper chromatogram among the splitting products of the hydrolyzed new antibiotic [1], but the alcoholic component obtained was not identical with trichotecolone [6]. Until the final elucidation of its chemical structure, the substance is called antibiotic T. The present paper deals with the actions *in vitro* and *in vivo* of antibiotic T.

Methods

Antifungal activity was investigated by means of the serial agar-dilution method. The medium used (agar Pg) consisted of 1 per cent peptone, 0.5 per cent glucose, 0.5 per cent sodium chloride, 2 per cent agar-agar, and was adjusted to pH 7.0 The tubes were incubated at 25° C. Antibiotic T dissolved in alcohol was added to the first tube.

The solutions containing antibiotic T were tested against *Candida albicans*. For this purpose 20 ml of agar Pg was poured into a Petri-dish and the surface was inoculated with a 1 to 100 dilution of a laboratory strain of *Candida albicans* previously cultured in Pg solution for 24 hours. 0.1 ml was pipetted into 8 mm agar holes and the dishes were incubated at 37°C for 18 hours, when the diameter of the inhibited area was measured. The margin of the inhibitory ring produced by antibiotic T is not sharp: indeed, it is a double ring. The data to be presented refer to the diameter of the entire inhibition, the evaluation of which could be made only with approximate accuracy. Sensitivity of the method changed with the amount of the inoculum, the time of measuring, as well as with other factors. Under suitable conditions, the lower limit of the sensitivity was 10 μg/ml.

Inactivation by blood or bile of the antibiotic was investigated as follows. The substance was dissolved in propylene glycol and added to the mixture in an amount that the final solvent concentration should not exceed 2 per cent, in order to avoid blood clotting. The blood or bile containing the antibiotic was then incubated at 37°C and the inhibitory capacity of samples taken at appropriate intervals was measured.

The influence on the yeast cells present in the gastrointestinal tract of mice was investigated as follows. The animals were kept ten days prior to and during the experiments on a diet described by Sós [3] with the modification that the meat meal prescribed was substituted with caseine. The food was supplemented with 1 mg/g oxytetracycline citrate and the daily portion (3 to 4 g per mouse) was kneaded by means of hot water. Fresh drinking water was given to the animals daily. After ten days, the yeast cell count of the faeces was counted. The substance suspended in 5 per cent gum arabic was then administered by a stomach tube in a volume of 0.2 ml daily for three days. 24 hours as well as 3 to 4 days after the last dose the yeast cells were counted again. The count was expressed in terms of cells in mg faeces (wet weight): a freshly obtained faeces pellet was suspended in water, and after suitable dilution, spread with a glass rod over the surface of a broth-agar of pH 6.0, containing 0.5 per cent glucose and 0.1 mg/ml chloramphenicol. After drying the dishes were kept at 37°C for the first 24 hours and at room temperature for another 24 hours. Thereafter, the colonies grown were counted.

Infection of mice was carried out by using a laboratory strain of *Candida albicans* isolated from a patient suffering from systemic mycosis. The strain was cultivated on Pg agar slants for 48 hours; after suspending under aseptic conditions, the cells were centrifuged and the suspension was diluted to give the desired cell count in a volume of 0.2 ml. This was intraperitoneally injected to mice weighing 18 to 22 g. Treatment of the animals was performed by the subcutaneous route.

The nystatin samples used for comparison contained 3300 units/mg.

Results

The antifungal activity of antibiotic T is summarized in Table I. The fungistatic action was temporary in character, *i. e.* gradually decreased from day to day. While some pathogenic fungi were inhibited strongly, others only slightly. Hence, the characteristics of the inhibition greatly resembled those of trichotecin. Antibiotic T proved to be 3 to 4 times weaker than trichotecin against *Candida albicans* and 2 to 10 times weaker agains plant-pathogenic fungi (not included in Table I). As established after 24 hours on *Candida albicans*, 0.01 per cent antibiotic T was of identical activity as 0.0033 per cent (=100 U/ml) nystatin. In the experiments of FREEMAN [4] trichotecin was also more active than antibiotic T in our own experiments.

Table I

Inhibitory action of antibiotic T on various fungi

	Incubation, days				
	1	2	5	8	14
	inhibitory concentration, μg/ml				
Cryptococcus neoformans		2.5	10.0	15.5	40.0
Candida albicans	4.0	11.0	45.0	90.0	
Saccharomyces cerevisiae	5.0	10.0	20.0	37.0	
Aspergillus niger		100.0			
Trichophyton mentagrophytes		2.5	40.0	90.0	
Epidermophyton inguinale				4.0	20.0
Microsporon Audouini			1.2	2.5	5.0

The very poor solubility of antibiotic T in water rendered the pharmacological investigations somewhat difficult. The LD_{50}, which could be determined only by using a suspension in 10 per cent gum arabic, was 810 mg/kg on intraperitoneal administration to mice, and over 1000 mg/kg after oral administration. Doses lower than the LD_{50} caused transient dyspnoae, paralyses, collapse and, occasionally, convulsions, phenomena highly resembling those observed by FREEMAN after trichotecin administration [4]. When given to mice subcutaneously, antibiotic T suspended in oil was lethal at the 500 mg/kg dose; occasional deaths occurred after doses between 100 and 500 mg/kg. For the rat, the suspension in oil was more toxic, causing death over 100 mg/kg. A similar difference between the susceptibility of rats and mice was observed by FREEMAN in the case of trichotecin. Antibiotic T was administered also in 20 per cent methanol, 250 mg/kg subcutaneously was not lethal; higher doses could not be given because of the toxic effects of alcohol. 20 mg/kg antibiotic T in 10 per cent ethanol produced a slight blood pressure fall in the cat. This solution was prepared immediately before injection and contained 5 mg antibiotic T in each ml. Higher concentrations could not be administered, as antibiotic T was precipitated. The dose of 20 mg/kg given 3 to 4 times successively showed an identical result. Antibiotic T in concentrations of 10^{-4} to 10^{-5} *M* irreversibly weakened the function of the isolated frog heart, impaired conductivity and caused atrio-ventricular dissociation and the heart finally stopped.

Next, an attempt was made to measure the serum level of antibiotic T in mice and rats after subcutaneous, oral, intraperitoneal resp. intravenous administration. Propylene glycol, diluted methanol, oil or gum arabic were used as solvents. The animals were killed between 5 minutes and 24 hours after the administration of 25 to 50 mg/kg antibiotic T, and the inhibitory

effect on *Candida* of the blood taken from the heart (mouse) or the tail (rat) was determined. A great number of attempts to demonstrate a measurable serum level was unsuccessful, even if the antibiotic was administered intravenously in doses (0.2 to 0.4 mg per mouse) enough to be certainly present in serum if distributed in the whole fluid space. Iv. injection of 2 to 4 mg per mouse resulted in a serum level ten times lower than expected. This, too, persisted only for 10 to 30 minutes, after which period there was no more antibiotic demonstrable in the serum. Traces of antibiotic T were found in the urine of mice treated with 200 to 400 mg/kg intravenously, but none in the liver, spleen or lungs, which organs were homogenized and tested either directly or after extraction with organic solvents (the tissues were homogenized with methanol, or the acetone-dried tissue-powder with benzene).

What is the cause of the drug's disappearance is not yet clear. Dilution with water of a solution of antibiotic T in propylene glycol resulted in precipitation of the drug. When diluted with serum, on the other hand, antibiotic T was kept in solution in amounts up to several hundred μg/ml. Even if antibiotic T is precipitated in the tissues, the amount soluble in water (0.1 mg/ml) ought to have been demonstrable. It was therefore supposed that antibiotic T underwent inactivation in the blood or tissues. This assumption was substantiated in the experiments to be reported below, but even this fact could not explain the absence of any measurable serum level 5 to 10 minutes after the intravenous injection, since inactivation takes more time than a few minutes.

Inactivation of antibiotic T in blood was investigated as follows. Human blood as well as cat, rabbit, guinea pig and rat blood were mixed with citrate or heparin, and 0.1 mg/ml antibiotic T was added. Inhibitory action was measured immediately, as well as after incubation at 37 °C. In spite of, that the antibiotic activity immediately after mixing was identical with that of the water control, it decreased as soon as 2 to 3 hours after incubation and disappeared after 24 to 48 hours. (A representative experiment is shown in Table II.)

Table II

Decrease in activity of antibiotic T after incubation with blood

Solution containing 0.1 mg/ml antibiotic T	Diameter of *Candida*-inhibition, mm; incubation time			
	5 min.	3h	24h	bile*
Physiological saline	20	18	18	16
Human citrated blood	19	15	0	—
Cat citrated blood	19	15	0	0

* Addition of bile-B to blood incubated for 24 hours.

Though slower, this phenomenon took place also in human or mouse serum. When antibiotic T was mixed with blood, the inhibitory activities in serum and in the whole blood were roughly identical, a fact indicating that antibiotic T had diffused into the red cells. No active principle could be extracted with benzene from blood which had lost its antibiotic property after incubation for 48 hours. Similarly, we were unable to recover antibiotic T, even if the protein was precipitated either by heating or with a 5-fold volume of ethanol, and the residue was analysed. It was supposed that perhaps antibiotic T was bound to erythrocytes, and this binding prevented detection. Antibiotic activity was, however, absent even after treating the 24 hour incubate with 2 to 3 drops/ml of bile producing haemolysis (Table II). Since immediately after mixing antibiotic T was recovered from the blood both when extracted with benzene and after deproteinization with ethanol, our failure to demonstrate the active principle after incubation indicate that antibiotic T was not only bound to, but also inactivated by blood. Antibiotic T being an ester, an ester-cleavage was supposed. Experiments with prostigmine or diisopropylfluorophosphate used in concentrations between 10^{-3} and 10^{-6} *M* failed, however, to substantiate the above assumption, a fact pointing to the possibility of another type of inactivation.

The resemblance of antibiotic T to trichotecin was further supported by the finding that trichotecin, too, is inactivated by blood, and this inactivation is not inhibited by prostigmine or diisopropylfluorophosphate. The serum level of trichotecin behaved also in a similar fashion: after the intravenous injection to mice of 200 mg/kg trichotecin, a measurable serum level persisted only for 10 minutes.

Inactivation of antibiotic T proceeded in liver suspension even more rapidly than in serum. Rat or mouse liver was homogenized and diluted with an antibiotic solution in physiological saline to give a final volume three times the wet weight of the organ. An inhibitory effect on *Candida* was absent even immediately after mixing. When the suspension was extracted with benzene the active principle was recovered 1 hour after incubation, but not after two hours. The absence of inhibitory action in the first hour — without extraction — was due to the fact that the liver suspension had split the antibiotic before this latter would have diffused into the agar.

As no blood level was found in small animals, antibiotic T does not promise to be suitable for treatment of systemic mycoses. But antimycotics have two further therapeutical indications. One of these is their local application in infections of the skin, the other is to inhibit the multiplication of yeast cells in the gastro-intestinal tract after treatment with broad-spectrum antibiotics.

Local administration is only possible if the substance does not cause pathological manifestations on the skin. Unfortunately, this is not the case

with antibiotic T. When a 0.1 to 1 per cent solution of the antibiotic in oil, propylene glycol or 50 per cent ethanol was applied on the epilated skin of guinea pigs, on the inner surface of the rabbit ear, or on the volar surface of the human forearm, it caused within a few days pruritic erythema or even sloughing. In humans, these symptoms often occurred as soon as following the first painting. The symptoms healed within 1 to 2 weeks, depending on their extent. Trichotecin also irritated the skin [4]. When applied on the rabbit eye in 0.01 per cent solution in water or in a 1 per cent solution in oil, anti-

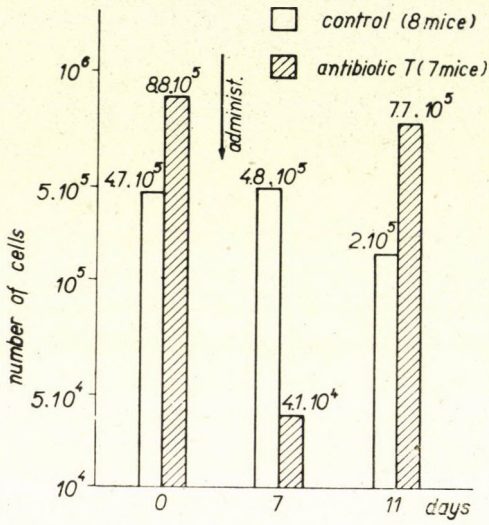

Fig. 1

biotic T caused no irritation. Its 0.1 per cent solution in 10 per cent methanol and 5 per cent glucose gave rise to one drop of thick discharge, but no further change.

Fig. 1 shows the effect of antibiotic T on the yeast cell count in the faeces of mice fed with an oxytetracycline containing diet. As seen, 250 mg/kg of antibiotic T decreased the yeast-cell count by 95.4 per cent. A lower dose (50 mg/kg) was ineffective. In other experiments performed for comparison, 99 per cent reduction in the yeast-cell count was produced by 100 mg/kg (= 33 000 U/kg) nystatin and 100 mg/kg trichotecin, resp. — 250 mg/kg of antibiotic T is, however, such a high dose as making impossible the use of the drug in human therapy.

Even though no blood level count could be demonstrated after the administration of antibiotic T, an attempt was made to examine its action on experimental *Candida* infection, considering that some antibiotics, although failing to show a blood level, are effective against infections of the mice (mycamycine, [7]). Table III summarizes the results obtained in one group

Table III

Effect of antibiotic T and nystatin on the experimental Candida-infection in the mouse

Treatment	No. of mice	Exit, hours						Mean survival time, hours	Growth of *Candida alb.* from			
		40	45	48	63	266	384		At time of death, hours	perito-neum	kidney	heart blood
—	4	1	2	—	1			48.2	63	+	+	+
75 mg/kg antibiotic T	4	2	—	—	2			51.4	63	+	+	+
75 mg/kg nystatin	5	1	—	1	—	1	—	224.4	40	+	—	—

of infected mice. Antibiotic T was administered dissolved in oil, nystatin suspended in water. The control mice received sunflower oil, only. Antibiotic T was either absorbed from, or inactivated *in situ*, as the oil aspirated from the site of injection 24 hours following treatment was not more effective.

The animals were treated by the subcutaneous route both immediately after infection and at 24 hours intervals thereafter. Antibiotic T and the oil were given twice, while nystatin 6 times. Inoculation was made by the intraperitoneal injection of 75 million *Candida* cells, the lowest dose which caused 100 per cent mortality in preliminary experiments. The animals were observed for 16 days following infection, as it is known from the paper of BROWN and HAZEN [5] that mice succomb to the infection in 1 to 2 weeks. Samples taken from the organs of the died animals were inoculated on Pg slant agar containing chloramphenicol. This was done to ascertain the *Candida* infection. In other experiments, the mice were infected with 100 million cells and treated with 100 mg/kg antibiotic T. These animals died even sooner than did the controls, while mice treated with the same amount of nystatin survived longer.

Unlike antibiotic T, the alcohol-component obtained from it after hydrolysis is easily soluble in water. When applied in a 1 per cent aqueous solution, the alcohol did not cause irritation on human skin, and produced but slight irritation even when dissolved in 50 per cent alcohol. The antifungal effect of the alcohol-component is not inactivated by the blood. Hence, it possesses properties more favourable than does the original compound. Unfortunately, its antifungal effectiveness is 200 times less than that of antibiotic T. In the hope to obtain antifungal substances with more favourable properties, attempts will be made to esterify the alcohol with other acids and also to prepare some other derivatives.

LITERATURE

1. GLÁZ, E. T. *et al.*: Nature (Lond.) **184**, 908 (1959).
2. FREEMAN, G. G., GILL, J. E., WARING, W. S.: J. chem. Soc. 1105 (1959).
3. SÓS, J.: cit. in Kovách, A.: A Kísérletes Orvostudomány Vizsgáló Módszerei, Akadémiai Kiadó. Budapest, 1957, *Vol. III.* p. 230.
4. FREEMAN, G. G.: J. gen. Microbiol. **12**, 213 (1955).
5. BROWN, R., HAZEN, E. L., MASON, A.: Science, **117**, 609 (1953).
6. GYIMESI, J.: to be published.
7. TANAKA, N.: J. Antibiot. **11**, 128 (1958).

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Orvostudományi Egyetem Gyógyszertani Intézete. Budapest VIII. Üllői út 26.

UNTERSUCHUNG DER ÄTHEREMPFINDLICHKEIT BEI JUNGEN UND VOLLENTWICKELTEN TIEREN

Von

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1. Die Wirksamkeit des Äthers wurde im Narkosegefäß an kleinen 30—40 g schweren sowie an großen 350 g schweren Ratten untersucht und festgestellt, daß die kleinen Tiere empfindlicher als die großen sind, denn sie fallen bei einem niedrigeren Vol.% des in der Luft bestimmten Äthers in Schlaf als die großen Tiere. Die bis zu ihrem Erwachen aus der tiefen Narkose verstrichene Zeit ist gleichfalls länger und schließlich ist für die kleinen Tiere bereits ein Äther-Vol.% tödlich, den die großen Tiere noch überleben.

2. Die Ursache dieses Unterschiedes in der Ätherempfindlichkeit liegt vermutlich darin, daß der Wassergehalt im Körper der kleinen Tiere größer ist und daher der Äther sich vermutlich besser als im Körper der großen Tiere lösen kann. Teilen wir die in mg% gemessene Äthermenge des im Körper der Tiere absorbierten Äthers durch den mg% des in der Gefäß-Luft verbleibenden Äthers, dann ist der so erhaltene Verbrauchskoeffizient bei den kleineren Tieren größer als bei den großen.

3. Der bei tödlicher Ätherkonzentration gemessene Verbrauchskoeffizient ist vermutlich deshalb niedrig, weil die Tiere schon eingehen, bevor noch die Lösung und gleichmäßige Verteilung des Äthers in ihrem Körper erfolgen konnte.

Im Laufe der Untersuchungen von der narkotischen und toxischen Wirkung der peroxydhaltigen Äther (worüber an anderer Stelle berichtet wird) mußte die Frage erörtert werden, inwiefern das Alter und das Körpergewicht der Tiere ihre Ätherempfindlichkeit beeinflußt. Besondere Aktualität erhielt dieses Problem durch die überraschende Behauptung von J. SAJNER [1], daß junge, 6 g schwere Mäuse bzw. 30 g schwere Ratten ungefähr fünfmal weniger empfindlich gegenüber Äther sind als vollentwickelte 20 bzw. 150—400 g schwere Tiere.

Methoden

In meinen Versuchen gebrauchte ich das Präparat Aether ad narcosim Ph. Hung. V. mit welchem 24 Stunden hungernde Ratten narkotisiert wurden. Auf dem gut schließenden Plexiglasdeckel des 10 Liter Glasgefäßes waren drei Öffnungen. Durch die mittlere Öffnung reichte in den Luftraum des Gefäßes die Achse eines Mischmotors, an deren Ende in einer Höhe von ungefähr 15 cm vom Boden des Gefäßes eine Filtrierpapierscheibe befestigt war. Durch das Drehen dieser Scheibe wurde die gleichmäßige Verteilung des Äthers im Luftraum gesichert. Die zweite, mit einem kleinen Korken verschließbare Öffnung diente zum Einspritzen des Äthers auf die Filtrierpapierscheibe und zwar so viel, bis die gewünschte Konzentration erreicht wurde. Durch die dritte, mit einem Hahn versehene Öffnung des Deckels reichte ein Kapillarröhrchen in das Gefäß, bis in den Luftraum unter dem Filtrierpapier. Durch dieses Röhrchen wurde am Ende des Experimentes mit Hilfe von zwei 20,00 ml fassenden, mit Quecksilber gefüllten Gasbüretten die Luftprobe zwecks der Bestimmung des Äthervolumenprozentes entnommen. Die Bestimmungen erfolgten nach der Methode von G. KÄRBER [2].

Zur Untersuchung der Ätherempfindlichkeit von jungen 30—40 g schweren und von beinahe zehnmal schwereren erwachsenen Ratten wurden gleichzeitig 2 junge und 2 erwachsene Tiere in das Gefäß gelegt. Auf diese Weise wirkte jeweils die gleiche Ätherkonzentration auf beide Tiergruppen. Die Experimente begannen mit einer ganz niedrigen Konzentration, die danach stufenweise bis zur tödlichen Konzentration erhöht wurde. Nach 30 Minuten lang dauernder Narkose erfolgte von zwei entnommenen Luftproben die Bestimmung der Ätherkonzentration. Zur Messung der Tiefe der Narkose erwies sich die Bestimmung der bis zum Erwachen der Tiere erforderliche Zeit als sehr geeignet, denn offensichtlich ist das der im Tierkörper angehäuften Äthermenge proportional, denn je mehr Äther das Tier ausatmen muß, um so längere Zeit wird bis zu seinem Erwachen vergehen. Deshalb wurden nach Abschluß des Experimentes die Tiere aus dem Narkosegefäß herausgenommen, und mit Hilfe einer Stoppuhr die Zeitspanne gemessen, bis die Tiere an frischer Luft von selbst, oder auf schwaches Kneifen, aufstanden.

Ergebnisse

Die Experimente wurden der Tabelle I gemäß auf Grund der verwendeten Ätherkonzentration in acht Klassen eingeteilt. Innerhalb der einzelnen Klassen waren selbstverständlich die Ätherkonzentrationen nicht genau die gleichen, daher sind auch die mittleren und die zwei Grenzwerte angegeben. Aus Tabelle I ist ersichtlich, daß die Ätherempfindlichkeit von kleinen Ratten größer als die Empfindlichkeit der großen Tiere ist. Das beweist der Umstand, daß 2,84 Vol.% Äther bei allen 14 kleinen Ratten eine Narkose hervorrief, während von 14 großen Ratten nur 9 einschliefen, 5 blieben wach. Die Zeit bis zum Erwachen war bei den kleinen Tieren 1′48,4″, bei den großen 1′41,7″. Demnach kann in dieser Beziehung kein signifikanter Unterschied wahrgenommen werden.

In der nachfolgenden Klasse (3,5 Vol.%) schlief von 18 Tieren bloß eine große Ratte nicht ein. Die Zeit bis zum Erwachen betrug bei den kleinen Tieren 2′ 28″, bei den großen dagegen 1′13,1″, der Unterschied ist demnach ziemlich bedeutend. In der III. Klasse (5,21 Vol.%) verschwindet jedoch dieser Unterschied wieder, denn beide Tiergruppen erwachten in einer Zeit von 7′31″ bzw. 7′25″.

In der folgenden IV. Klasse (6,97 Vol.%) ging bereits 41,5% der kleinen Ratten ein, während die großen Tiere selbst noch in der V. und VI. Klasse (8,3 bzw. 9,3 Vol.%) die Narkose bis auf ein einziges Tier (10%) überlebten, obwohl diese Konzentration bei 70% der kleinen Tiere tödlich war. In der VII. Klasse gingen alle kleinen Tiere, von den großen Ratten jedoch bloß 50% ein.

Demnach ist die Ätherempfindlichkeit der jungen Tiere größer als die der vollentwickelten älteren Tiere. Eines der Ursachen hierfür könnte in dem Umstand liegen, daß während der Narkose der Äther im Körper der jungen Tiere eine höhere Konzentration erreicht als im Körper der älteren Tiere.

Das zu untersuchen habe ich eine Methode ausgearbeitet, mit deren Hilfe verhältnismäßig einfach die gesamte, von den Tieren inhalierte Äther-

Tabelle I

Vergleich der Ätherempfindlichkeit von kleinen und großen Ratten

Klasse	Ätherluftgemisch Vol. %	Durchschnittsgewicht der Ratten 37 g					Durchschnittsgewicht der Ratten 350 g				
		Zahl der Tiere	Wach	Narkose	Zeit bis zum Erwachen	Eingegangen %	Zahl der Tiere	Wach	Narkose	Zeit bis zum Erwachen	Eingegangen %
I.	2,84 (2,58—3,06)	14	—	14	1′48,4″ ±11,9″	0	14	5	9	1′41,7″ ±14,7″	0
II.	3,5 (3,27—3,61)	9	—	9	2′28″ ±18,8″	0	9	1	8	1′13,1″ ±13,5″	0
III.	5,21 (4,7—5,69)	16	—	16	7′31,5″ ±42,3″	0	16	—	16	7′25″ ±19″	0
IV.	6,97 (6,4—7,46)	12	—	12	12′39″ ±1′51″	41,5 (5)	12	—	12	14′23″ ±1′22″	0
V.	8,3 (8,19—8,48)	6	—	6	16′48″ ±1′38,5″	50 (3)	6	—	6	17′30″ ±57,6″	0
VI.	9,3 (8,73—9,88)	10	—	10	18′20″ ±1′21,4″	70 (7)	10	—	10	24′54″ ±57,2″	10 (1)
VII.	11,1 (10,1—11,8)	8	—	8	—	100 (8)	8	—	8	26′5″ ±2′23″	50 (4)
VIII.	13,6 (12,2—14,53)						8	—	8	24′40″ ±	87,5 (7)

menge gemessen worden konnte. Die in unserem Institut seit mehreren Jahrzehnten gebrauchten Meßapparate für Gaswechsel erwiesen sich zu diesem Zweck als sehr geeignet. Der Apparat besteht aus einem 2 l fassenden luftdicht verschloßenen Metallzylinder, der mit einem Warburg-Manometer verbunden ist. Das vom Versuchstier erzeugte CO_2 wird vom Natronkalk im Zylinder absorbiert, während der Verbrauch an O_2 aus der, vom Manometer gemessenen Druckverminderung berechnet werden kann. In der Regel waren zwei Apparate in Betrieb, deren Behälter sich in einem Wasserthermostat von 30° C befanden. In den einen Apparat wurden zwei kleine Ratten und in den zweiten eine große Ratte untergebracht, dessen Gewicht dem Gesamtgewicht der beiden kleinen Ratten ungefähr entsprach. Nach 10′ dauernder Durchströmung der Behälter mit O_2 wurden die Hähne verschlossen, und in 10′ Intervallen der O_2-Verbrauch der Tiere gemessen. Als der Verbrauch bereits gleichmäßig geworden war, erfolgte durch den Metallhahn des Stoffwechselgefäßes aus einer zum Konus des letzteren genau angepaßten, kalibrierten

Tuberkulinspritze die Einspritzung der gewünschten Äthermenge, während an den zum Manometer führenden Gummischlauch eine Péansche Klemme angelegt wurde. Dadurch gelang es zu verhindern, daß der durch die Ätherverdampfung hervorgerufene Überdruck die Brodielösung aus dem Manometer heraustreibt. Das Äther-Luftgemisch im Gefäß wurde durch den Metallhahn mit Hilfe einer 20 ml Spritze mehrmals aufgesogen und auf diese Weise durchgemischt. In 20—30 Minuten war der Überdruck ausgeglichen, weil die Tierkörper einen Teil der Ätherdämpfe aufgenommen hatten, und von diesem Zeitpunkt an konnte der O_2-Verbrauch der narkotisierten Tiere gemessen werden, der sich als nicht wesentlich weniger als der O_2-Verbrauch vor der Anwendung des Äthers erwies. Das Aufhören des O_2-Verbrauchs zeigte das Eingehen der Tiere sofort an. Die Narkose wurde während 60 Minuten aufrechterhalten. Danach folgte die Entnahme von je zwei Luftproben aus den Stoffwechselgefäßen, worauf die Tiere aus dem Apparat herausgenommen, an die frische Luft gebracht, und die Zeit bis zu ihrem Erwachen gemessen wurde.

Nachdem zu den Experimenten immer die gleiche Tuberkulinspritze verwendet wurde, konnte ich die Äther milligramm-Mengen, nach Eichung, aus den milliliter-Mengen ausrechnen, z. B. 0,5 ml — im Mittelwert der Messungen 400 mg $\pm 1\%$. In Kontrollversuchen erfolgte nach Einspritzen von 0,5 ml Äther Kontrolle der Äthermenge in der Luft des Behälters, wobei in diesem Fall die Verdampfung des Äthers ohne Anwesenheit der Tiere vorgenommen wurde. Die Titrierung ergab folgende Resultate, z. B.: 402, 400, 398, 397, 393 mg; im Mittelwert 396 $\pm 3{,}5$ mg Äther, was einer Bestimmung einer Genauigkeit von 0,87% entspricht.

In Tabelle II sind die an 52 kleinen 40—65 g schweren Ratten, in Tabelle III jene an 34 größeren, 95—145 g schweren Ratten durchgeführten Experimente zusammengefaßt. In dieser Serie wurden die Tiere auch der Ätherkonzentration gemäß in 5 bzw. 6 Klassen eingeteilt. Aus der Bestimmung des Vol.% des Äthers in der Luftprobe erfolgte die Berechnung der Menge des in der Luft des Apparates gebliebenen gesamten Äthers. Nach Abzug der erhaltenen Menge aus der mit Hilfe der genau geeichten Tuberkulinspritze eingespritzten Äthermenge, wurde die, durch die Tiere verbrauchte Äthermenge in mg erhalten, was in den Tabellen auf 100 g Körpergewicht im Mittelwert angegeben ist. Ferner wurde die mit Hilfe der Formel $\sqrt{\frac{E(x-x)^2}{n(n-1)}}$ berechnete, Streuung dieses Mittelwertes sowie der Probitwert der Unterschiede zwischen den verschiedenen Tiergruppen angeführt (Tabelle IV).

In der Klasse Ia war die Ätherkonzentration 3,18 Vol.% = 10,5 mg%. Die Tiere schliefen alle ein und nach einstündiger Narkose erwachten sie in 2′57″. Im Organismus der Tiere befand sich 176,6 mg% Äther, also ist der Verbrauchskoeffizient der Ätherkonzentration im Tier und in der Luft K = 16,7. Das Ergebnis der Gruppe Ib der großen Tiere war ähnlich, doch erfolgte das

Tabelle II

Klasse	Ätherluftgem.		Tiere		Zeit bis zum Erwachen	Eingegangen %	Ätherverbrauch mg/100 g	Verbrauchskoeff. K.
	Vol. %	mg %	Zahl	Gew.				
I. a.	3,18 (2—4,05)	10,5	12	57 (50—65)	2′57″ ±30,6″	0	176,6 ±16,42	16,7
II. a.	5,38 (5—5,78)	17,7	6	46,2 (40—55)	8′20″ ±1′13,2″	0	283 ±17,9	15,9
III. a.	6,33 (6—6,57)	20,9	14	52 (44—60)	15′24″ ±1′30,6″	15	277 ±24,6	13,2
IV. a.	7,48 (7,03—7,96)	24,7	14	48,8 (40—65)	19′12″ ±1′09,6″	64,4	223 ±25,4	9,03
V. a.	9,16 (8,95—9,61)	30,2	6	47,5 (40—55)		100	126 ±15,8	4,17

Tabelle III

Klasse	Ätherluftgem.		Tiere		Zeit bis zum Erwachen	Eingegangen %	Ätherverbrauch mg/100 g	Verbrauchskoeff. K.
	Vol. %	mg %	Zahl	Gew.				
I. b.	3,32 (2,4—3,99)	10,96	5	112 (95—120)	6′30″ ±2′10,2″	0	165 ±11,4	15,05
II. b.	5,65 (5,23—5,87)	18,6	3	120 (105—140)	14′	0	176 ±47,6	9,4
III. b.	6,49 (6,25—6,78)	21,4	8	135,6 (125—140)	21′30″ ±6′49,2″	0	214 ±22,7	10,0
IV. b.	7,42 (7,1—7,73)	24,5	5	130 (115—145)	20′48″ ±1′59″	0	161 ±24,4	6,5
V. b.	8,64 (8,03—8,93)	28,5	6	118 (100—140)		66	193 ±30,5	6,9
VI. b.	10,33 (9,16—11,5)	34,1	7	131,4 (100—140)		100	209 ±26,1	6,13

Erwachen langsamer. In der Klasse II erwachten auf Wirkung von 5,38 bzw. 5,65 Vol.% Äther die kleinen Tiere in 8′20″, die großen in 14′, obwohl der Körper der letzteren bedeutend weniger Äther enthielt.

In den Klassen III und IV gingen von 6,33 bzw. 7,42 Vol.% Äther 15 bzw. 64,4% der kleinen Tiere ein, während alle großen Ratten am Leben blieben. Es ist interessant, daß im Körper der kleinen Tiere in den Klassen II

Tabelle IV

Vergleich des in Tab. II und III angeführten verbrauchten Äthers in mg/100 g Ratte mit Probitberechnung

Tiergruppen	t	P	Freiheitsgrad
I. a.—III. a.	3,251	$<0{,}001$	11
III. a.—IV. a.	1,524	$<0{,}1$	12
IV. a.—V. a.	2,344	$<0{,}05$	8
I. b.—III. b.	1,583	$<0{,}1$	11
I. a.—I. b.	0,556	$<0{,}5$	15
III. a.—III. b.	1,88	$<0{,}05$	13
IV. a.—IV. b.	4,956	$<0{,}001$	10

und III wesentlich mehr Äther erhalten war als im Körper der großen Tiere. Dies ist auch deutlich aus dem Unterschied zwischen den Verbrauchskoeffizienten ersichtlich. Der letztere ist bei den kleinen Tieren um 6,5 bzw. 3,2 größer, woraus hervorgeht, daß der Äther sich in ihrem Körper infolge des größeren Wassergehaltes besser löst als im Körper der größeren Tiere. Dies dürfte auch die Ursache dafür sein, daß die kleinen Tiere bereits bei einer etwas niedrigeren Luftätherkonzentration eingehen als die großen. Obwohl wurden in dieser Versuchsserie keine so signifikanten Unterschiede gefunden wie in der ersten Serie. Wahrscheinlich wohl aus dem Grunde, daß in der zweiten Serie nicht 350 g schwere, sondern bloß 120 g schwere Tiere verwendet wurden.

Es war bemerkenswert, daß bei Anwendung der tödlichen Ätherkonzentration der Verbrauchskoeffizient des Äthers eine starke Verminderung zeigte. Vermutlich kann das mit dem Umstand erklärt werden, daß sich die Blutzirkulation und die Atmung der Tiere verschlechtert, wodurch der Äther sich nicht im ganzen Körper gleichmäßig verteilt, und auf diese Weise kein vollkommener Ausgleich stattfindet. Infolge der hohen Ätherkonzentration setzt die Lähmung des zentralen Nervensystems bereits zu einem Zeitpunkt ein, wo die übrigen Organe noch nicht in einem dem Verbrauchskoeffizienten entsprechenden Maße mit Äther saturiert sind. Dies geht deutlich aus den Angaben der Tabelle II hervor, denn je mehr Tiere eingingen, um so geringer war die Äthermenge im Körper der eingegangenen Tiere. In der Klasse Va waren die Tiere sehr schnell eingegangen und deshalb konnten sie die, dem Verbrauchskoeffizienten entsprechende, Äthermenge noch nicht aufnehmen. Die größeren Tiere waren offenbar resistenter, gingen langsamer ein, und hatten daher Zeit zur Aufnahme einer größeren Äthermenge.

In dieser Versuchsserie dünkte der Äther toxischer, denn die Narkose währte 60′, und daher stand zur Aufnahme des Äthers mehr Zeit zur Verfügung als in der ersten Serie, wo die Narkose bloß 30′ dauerte. Eine ähnliche

Tabelle V

Toxizität des Aether ad narcosim im Falle von 30 bzw. 60 Minuten während der Narkose bei Ratten von verschiedenem Körpergewicht

Ätherluftkonzentration Vol. %	Zahl und % der eingegangenen Tiere					
	in 30 Minuten im Glasgefäß			in 60 Minuten		
				Glasgefäß	Stoffwechselapparat	
	Durchschnittsgewicht der Tiere in g					
	37	192	350	137	52	124
5,3	0 0/16	—	—	—	0 0/6	—
6,3	—	—	—	0 0/6	15 2/14	—
7,3	41,5 5,14	—	—	—	64,4 9/14	0 0/5
8,3	50 3/6	0 0/6	0 0/6	33 2/6	—	66 0/6
9,2	70 7/10	—	10 1/10	—	100 6/6	—
10,0	— 	33 0/6	— 	100 6/6	 	100 7/7
11,1	100 8/8	—	50 4/8	 	 	
13,6	 	—	87 7/8	 	 	
14,0	 	66 4/6	 	 	 	
15,9	 	100 6/6	 	 	 	

Toxizität zeigte sich als die Narkose auch im Glasgefäß 60′ lang aufrechterhalten wurde.

In Tabelle V sind sämtliche in bezug auf die Äthertoxizität durchgeführten Versuche zusammengefaßt. Der Nenner der Bruchzahlen zeigt die Anzahl der während 30 bzw. 60 Minuten eingegangenen Tiere. Es ist auch ersichtlich, daß die Toxizität des Äthers mit der Erhöhung des Körpergewichts der Tiere parallel abnimmt.

Besprechung der Ergebnisse

Die beiden Versuchsreihen beweisen eindeutig, daß die Ätherempfindlichkeit von kleinen, 30—50 g schweren Tieren größer ist als die Empfindlichkeit von vollentwickelten, 120 bzw. 350 g schweren Tieren. Die Ursache hierfür liegt darin, daß bei gleichem Partialdruck des Äthers im Körper von kleinen Tieren eine größere Äthermenge gelöst wird als im Körper von großen Tieren, d. h. der Verbrauchskoeffizient des Äthers, der in mg% anzeigt, wievielmal größer die Ätherkonzentration im Körper des Tieres als in der Luft ist, bei den kleinen Tieren wesentlich höhere Werte zeigt. Diese Beobachtung ist auch für jene Fälle gültig in denen das Tier im Laufe einer 60 Minuten währenden Narkose nicht eingeht und daher der Äther Zeit hat sich im ganzen Körper gleichmäßig zu verteilen, sich vollkommen auszugleichen.

Infolge des größeren Verbrauchskoeffizienten fallen die kleineren Tiere bereits bei einem solchen Partialdruck des Äthers in Schlaf, der bei großen Tieren noch keine totale Narkose hervorruft. Ein ausgeprägter Unterschied kann auch hinsichtlich der tödlichen Ätherkonzentration festgestellt werden.

Diese Ergebnisse stehen im vollkommenen Widerspruch zu den Versuchsergebnissen von J. SAJNER [1]. Die Ursache hierfür liegt meiner Meinung nach teils darin, daß die von J. SAJNER verwendete Methode zur quantitativen Erfassung der narkotischen Wirkung des Äthers kaum geeignet ist, und hauptsächlich darin, daß die von J. SAJNER zur Berechnung des »Verbrauchskoeffizienten« »K« gebrauchte Formel vollkommen falsch ist.

J. SAJNER legte seine Versuchstiere in einen Exsikkator, durch den er pro Minute 250 ml Äther-Luftgemisch durchströmen ließ. Mit Hilfe einer kalibrierten Bürette bestimmte er die Menge des bis zum Beginn der Narkose verdampften Äthers. Mit der Formel $K = \frac{\text{Zeit} \times \text{Verbrauch}}{\text{Gewicht}}$ berechnete er den Verbrauchskoeffizienten, den er bei kleinen Ratten im Durchschnitt fünfmal höher fand als bei großen Tieren.

Diese Formel wäre aber nur in dem Falle annehmbar, wenn die gesamte Menge des während des Experimentes verdampften Äthers in den Tierkörper gelangt wäre, genau so wie bei peroraler Einführung oder Injizierung. Nur in diesem Falle kann man die Menge des eingeführten Pharmakons durch das Gewicht der Tiere teilen und die Dosis z. B. pro kg Körpergewicht berechnen. In den Versuchen von J. SAJNER kann jedoch hiervon keine Rede sein, denn laut den genauen Messungen von A. C. GUYTON [3] macht das Minutenvolumen der Atmung des Tieres in ml ausgedrückt $^3/_4$ des Körpergewichtes aus. Laut seinen Messungen atmet z. B. eine 63 g schwere Ratte in der Minute 56 ml, eine 171 g schwere Ratte 102 ml Luft ein. Folglich, wenn J. SAJNER bei seinen Experimenten durch das die Tiere enthaltende Gefäß pro Minute 250 ml Äther-Luftgemisch durchströmen ließ, konnten die kleinen Tiere bloß

$^1/_5$—$^1/_{10}$ Teil dieser Luft einatmen, die großen dagegen sogar ½—1 Teil. Somit konnte bloß $^1/_5$—$^1/_{10}$ Teil des verdampften Äthers in den Körper der kleinen Tiere gelangt sein. Es ist also völlig unmotiviert, die gesamte Menge des verdampften Äthers durch das Körpergewicht der Tiere zu teilen. Je geringer das Körpergewicht des Tieres ist, um so größer wird natürlich der Wert des auf diese Weise berechneten »K» sein. Dies ist jedoch zur Bestimmung der Effektivität des Äthers oder zum Vergleichen der Ätherempfindlichkeit von kleinen und großen Tieren ungeeignet.

Bekanntlich ist die Wirkung der Inhalationsnarkotika von ihrem Partialdruck in der Luft, d. h. von ihrem Vol.% abhängig. Zur Vergleichung ihrer Wirksamkeit wird in der Regel die Bestimmung der narkotischen und tödlichen Dosis verwendet. Außerdem kann auch das Gewichtsprozent im Blut oder im ganzen Körper als Vergleichungsgrundlage dienen. Während bei einer Gegenüberstellung aus den obigen Gesichtspunkten die kleinen Tiere sich empfindlicher als die großen erwiesen, konnte bei einem Vergleich der Ätherkonzentration in ihrem Körper dieser Unterschied in der Empfindlichkeit gerade auf dem Gebiet der narkotisch wirkenden Konzentration nicht erwiesen werden.

LITERATUR

1. SAJNER, J.: Arch. int. Pharmacodyn. **113,** 76 (1957).
2. KÄRBER, G.: Arch. exp. Path. Pharmak. **160,** 428 (1931).
3. GUYTON, A. C.: Amer. J. Physiol. **150,** 70 (1947).

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Orvostudományi Egyetem Gyógyszertani Intézete. Budapest VIII. Üllői út 26.

RECENSIO

K. LISSÁK—E. ENDRŐCZI:

Die neuroendokrine Steuerung der Adaptationstätigkeit

161 pages, 13 tables, 45 figures. (The Neuroendocrine Control of Adaptation.) The Publishing House of the Hungarian Academy of Sciences, Budapest, 1960.

The axis of the adaptative mechanism in response to stimuli directly or indirectly threatening life had been considered by CANNON to be the sympato-adrenal system. Later, SELYE placed two endocrine glands, the pituitary and the adrenal cortex, in the centre of that mechanism. Both theories are valuable and have supplied new evidence as to the physiology of functions connected with adaptation, but both ignored the role of the central nervous system in the mechanisms aimed at maintaining or restoring the balance between the organism and its environment. This shortcoming is particularly obvious in SELYE's theory concerning the so-called diseases of adaptation, which he explains solely by a peripheral event, notably by the failure of an endocrine response. However, it has been known for long from the work of I. P. PAVLOV and his school that the stimuli causing a functional overloading of the central nervous system may become the source of such conditions as have been listed by SELYE in the category of diseases of adaptation.

As early as in the twenties, Hungarian authors (F. VERZÁR, A. BEZNÁK and their co-workers) observed that the adrenal cortex increased in size in response to intense physical work (muscle activity) and to intensive environmental stimulation. These results were undoubtedly the overture to the "stress" theory of SELYE, that aroused great interest some 15 years later and which is still the subject of extensive research by hundreds of investigators.

The authors of this book had been engaged in studies on the neuroendocrine control of adaptation for the past decade; these investigations yielded interesting results placing the whole problem in a new light. LISSÁK and ENDRŐCZI started from the view that it was incorrect to approach the problem of adaptation by placing the activity of some organ or system of organs (*e. g.* the pituitary adrenocortical system) into the focus of research, ignoring their connections with the other endocrine organs and with the central apparatus controlling the function of all organs. This view is emphasized throughout the book.

The first two chapters deal with the functional morphology of the anterior pituitary lobe and the adrenal cortex, as well as with cortical secretion during rest and stress. Chapter 3 discusses the biosynthesis of corticoids, the individual and species differences in hormone secretion, the interglandular effects on cortical activity and on the effects of the tranquillizers. In Chapter 4 the authors discuss the methods of ACTH assay, the factors controlling hormone secretion. Chapter 5 deals with the role played in the regulation of ACTH secretion by the connections between the hypothalamus and anterior pituitary. In Chapter 6 the changes in the ACTH secretion by the adenohypophysis in the course of ontogenetical development are treated.

The most original material is to be found in Chapters 7 and 8. These chapters supplement the earlier "decapitated" theories concerning the mechanism of adaptation to strain and stress, and deficiencies of earlier theories by placing the role of the central nervous system in

the mechanism of neuroendocrine adaptation in a light it undoubtedly deserves.

The rather heterogeneous, but throughout interesting Chapter 7 deals with the changes in neuroendocrine responses following the severing of the fronto-orbito-hypothalamic connections and the removal of the orbito-frontal cortex. It is in this chapter that a description is given of the experiments in which the authors succeeded in eliciting different forms of behaviour associated with a depletion of adrenal ascorbic acid by the use of electrodes built in the fronto-orbital cortex of the rat. The rest of the chapter is devoted to the stress reactions elicited by the stimulation of the hypothalamus, thalamus, hippocampus and nucl. amygdalae, preponderantly on the basis of the authors' own experiments. Stimulation of the hippocampus inhibits pituitary-adrenal activity, that of the nucl. amygdalae causes an increase of secretion. Thus, hippocampus and amygdala consitute a functional unit, connected with the system of hypothalamic nuclei through the fornix and septum. The system as a whole plays an important role in the forming of behaviour and in the integration of different automatisms. A further evidence derived from the stimulation experiments is that stimulation of the lower part of the hypothalamus and of the tegmentum gives rise to a merely quantitative increase of cortical hormone secretion, while by the simtulation of the archistriatum and pyriform gyrus the composition of the secretion is also changed.

In Chapter 8 are discussed the psychoses resulting from the administration of cortical hormones, the central nervous hypersensitivity resulting from treatment with such hormones, and the enhancing effect of the pituitary-adrenocortical system on epileptic seizures. The authors emphasize the importance of a decreased pituitary-adrenocortical sensitivity in the genesis of schizophrenia. In schizophrenics namely the hypothalamic-pituitary-adrenocortical system is less responsive to stressor effects, on the one hand; on the other hand, the interventions favourably influencing the disease (prefrontal lobotomy, insulin hypoglycaemia, cardiazol convulsions) are all pituitary-adrenal activators. — Also in this chapter are discussed the effects of the anterior pituitary and adrenocortical hormones on the higher nervous activity of animals. It is a remarkable observation that in dogs of the phlegmatic and melancholic neurotypes ACTH administration strongly intensifies the conditioned defensive and alimentary reflexes, while it hardly affects the conditioned reflexes, in dogs of choleric and sanguinic types. The duration of external inhibition caused by pain goes parallel with the amount of hydrocortisone secreted; the inhibition lasts longer when hydrocortisone output is increased. Correspondingly, the duration of this inhibition of the conditioned reflex may be prolonged by the administration of hydrocortisone. From this the authors conclude that hydrocortisone acts on a system in the brain which is involved in establishing temporary connections. It is also in this chapter that the authors describe their observation in rats that ascorbic acid depletion in response to stressor effects is proportionate to the duration of the inhibition of the conditioned alimentary reflex.

It is impossible to discuss in detail the wealth of the interesting, novel data and their original interpretation. Ultimately, a new synthesis is taking shape in this book, that elevates to a higher plane our former conception of the neuroendocrine control of adaptation. One or another of the views put forward by the authors, especially those in the last chapter, may remain controversial for some time, but it is unquestionable that the experiments and the conclusions courageously derived from them are of fundamental importance and will certainly serve as a guide in further research concerned with the neuroendocrine control of adaptation.

The quality of the book and the printing praise the publisher and the printers alike.

I. WENT

S. R. M. REYNOLDS, B. W. ZWEIFACH:

The Microcirculation

(Symposium on Factors Influencing Exchange of Substances Across Capillary Wall). Proceedings of the Fifth Conference on Microcirculatory Physiology and Pathology, Buffalo, April 1958. — The University of Illinois Press, Urbana, 1950 (170 p.).

In the volume the papers and discussions of the Conference are briefly presented.

D. W. FAWCETT discusses the electromicroscopic histology of the vessels on the basis of data in the literature and personal observations. In contrast to the findings with light microscopy, no appreciable morphological sign of an intercellular space between endothelial cells occupied by intercellular cement could be detected. — E. M. RENKIN, concluding from experiments and calculations considers the diameter of the hypothetical capillary pores 60—70 Å in mammalian skeletal muscle. — According to L. SAPIRSTEIN, plasma volume appears to be definitely higher after estimation by labelled protein, compared with labelled red cells and haematocrit values. Obviously, capillary wall and basal membrane must be permeable for plasma proteins, and the capillaries thus be surrounded by barriers of plasma. — J. ARNOLD discusses the properties of artificial membrane corresponding to the model of capillary wall. The technique of production of appropriate membranes is presented. — J. DITZEL summarizes the problems of diabetic angiopathy. — J. W. IRWIN and co-workers observed during passive anaphylaxis "hyaline" emboli and definite vasoconstriction in the pulmonary arteries of living rabbits. — The paper of W. H. KINSLEY and M. S. MAHALEY JR. is concerned with the problem of tumour metastases and microcirculation. — S. BAEZ presents a technique developed for the *in vivo* microscopic analysis of the intestinal vascular bed in rats. It is a remarkable finding that submucous arterial arcades flow is two-directional blood. During haemorrhagic hypotension the muscular coat may be entirely excluded from the circulation, while mucosal arteries exhibit continuous blood flow. — After injecting albumin labelled with ^{131}I into a cannulated leg lymphatic duct of the dog, H. S. MAYERSON observed that lymphatic proteins of the extremity in question return without loss and apparently only *via* the thoracic duct into the vascular system. M. C. BROWN and L. WARNER describe their reflecting microscope supplied with a colour television system. The method is suitable for the visualization, *e. g.* of the retinal vessels. — B. W. ZWEIFACH produced a special preparation of the skin of the rat to study the cutaneous circulation from the undersurface. He demonstrated the great sensitivity of the venous network to temperature changes; a fall of 1—2°C increased the response to epinephrine as much as tenfold. The sensitivity of skin capillaries to 5-hydroxytryptamine is also marked, producing changes at concentrations as low as 10^{-10} μg/ml.

The presentation of the volume must be considered excellent, the figures are technically first rate; the electronmicroscopic pictures and microphotograms are instructive.

The book is warmly recommended to all those concerned with the problems of microcirculation and permeability.

P. GÖMÖRI

HENRIK LUNDEGARDH, Penningby (Schweden):

Pflanzenphysiologie

VEB Gustav Fischer Verlag, Jena, 1960. 717 Seiten mit 283 Figuren im Text.

LUNDEGARDHs Name ist bekannt. Besonders populär ist seine Arbeit »Klima und Boden«. Die »Pflanzenphysiologie« ist die gründlich umgearbeitete deutsche Ausgabe seines vor 10 Jahren in schwedischer Sprache erschienenen Werkes Lehrbuch des »Pflanzenphysiologie und Pflanzenanatomie«. In der ganzen Welt gibt es nur wenige umfassende pflanzenphysiologische Arbeiten, und unter diesen zählt LUNDEGARDHs Buch zu den besten.

Zur Erhellung der grundlegenden Lebensprozesse der pflanzlichen Zellen und des pflanzlichen Organismus nimmt Verfasser auf breiter Basis die modernen biophysikalischen und biochemischen Kenntnisse in Anspruch. Dieses Bestreben äußert sich bisweilen so stark, daß die speziellen pflanzenphysiologischen Gesichtspunkte ein wenig in den Hintergrund gedrängt werden.

Die ersten beiden Kapitel des Werkes befassen sich mit dem morphologischen und chemischen Aufbau der Zelle und in diesem Rahmen unter entsprechender Betonung mit den Protoplasmamembranen sowie den physikochemischen Prozessen, die sich an die Membrane knüpfen. Im dritten Kapitel werden die anatomischen Kenntnisse, welche die Grundlagen der physiologischen Prozesse bilden, in knapper Form zusammengefaßt.

Es ist für den Leser nicht ganz klar, was den Verfasser dazu veranlaßt hat, unter den grundlegenden Lebensprozessen an erster Stelle die Fragen der Photosynthese zu behandeln, noch bevor er die Fragen des Wasserhaushalts und der Mineralernährung besprochen hätte. Diese Einteilung läßt sich auch damit nicht begründen, daß er die enzymatischen Prozesse und Energietransporterscheinungen des Organismus vorher darzustellen wünschte, weil er diese Fragen im folgenden Kapitel »Atmung und Gärung. Enzymchemie« fast als selbständige Einheit erörtert. In diesem Abschnitt lernen wir den modernsten Stand der Beziehungen zwischen Photosynthese und Kohlenhydratbildung sowie -verwertung kennen. Die sehr aktuelle Probleme der photosynthetischen Phosphorylation werden hingegen in diesen Kapiteln nur kurz berührt.

Das sich mit dem Stickstoff-Stoffwechsel befassende 6. Kapitel schließt sich dem vorangegangenen auf der Grundlage an, daß die Atmosphäre die Urquelle auch dieses ausschlaggebend wichtigen organogenen Elementes bildet. Dieser Abschnitt ist besonders abwechslungsreich, und hier tritt die ökophysiologische Betrachtungsweise des Verfassers am deutlichsten zutage.

Für die Kapitel, welche die Mineralernährung und den Wasserumsatz behandeln, ist weniger die ausführliche Erörterung der Themen bezeichnend als die Hinweise auf die neuesten Literaturangaben.

Im 10. Kapitel werden die Keimung, das im engeren Sinne verstandene Wachstum, der Mechanismus der Phytohormone sowie die Wechselwirkungen des Wachstums und der Umweltbedingungen analysiert. Sehr gründlich ausgearbeitet ist das letzte Kapitel, das sich mit den Bewegungen der Pflanzen befaßt.

Es wäre unrichtig zu verschweigen, daß wir in der Arbeit einiges vermissen. Einige theoretische und praktische Fragen der modernen Pflanzenphysiologie werden gar nicht erwähnt, z. B. die der Entwicklungsphysiologie sowie die physiologischen Beziehungen der Vernalisation, der Blütenbildung, Bestaubung, Befruchtung und Fruchtbiludng. Vollständigkeit darf man auch von einer derart umfangreichen und hohen Ansprüchen gerecht werdenden Arbeit nicht erwarten, dennoch meinen wir, daß der Leser im Interesse einer ausführlicheren Besprechung der eben angeführten Themen auf die Zusammenfassung der aus anderen Arbeiten bekannten pflanzenanatomischen, enzymchemischen, ja auch der wiederholt anzutreffenden rein organisch-chemischen Grundlagen gern verzichtet hätte.

Alles in allem stellt LUNDEGARDHs Werk nicht nur für die Studenten, sondern auch für junge Forscher eine nützliche Arbeit zur Einführung in diesen Wissenschaftszweig dar. Das weitere Studium der Pflanzenphysiologie wird durch mehr als 2000 bibliographische Hinweise erleichtert. Ein besonderes Lob verdient die schöne und sorgfältige Ausstattung des Buches.

Á. FALUDI DÁNIEL

A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki szerkesztő: Farkas Sándor
A kézirat nyomdába érkezett: 1960. IX. 5. — Terjedelem: 6,50 (A/5) ív, 22 ábra

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РЕЗЮМЕ

ОЧИСТКА РИБОНУКЛЕИНОВОЙ КИСЛОТЫ, ИНДУКТИРУЮЩЕЙ СИНТЕЗ ПЕНИЦИЛЛИНАЗЫ В КЛЕТКАХ B. CEREUS

В. ЧАНИ, М. КРАМЕР и Ф. Б. ШТРАУБ

Применялись и сопоставлялись два метода очистки рибонуклеиновой кислоты (РНК), полученной из образующих пенициллиназу клеток *Bac. cereus*. Эти очищенные препараты рибонуклеиновой кислоты имеют по существу тот же самый, повышающий образование пенициллиназы в индуктируемых клетках Bac. cereus эффект, что и описанная раньше неочищенная вытяжка.

Полученные этими двумя методами препараты рибонуклеиновой кислоты, как и неочищенная вытяжка, были количественно сравнены в отношении их действия на синтез пенициллиназы.

Было установлено, что уровень насыщения рибонуклеиновой кислоты, имеющий еще полную активность, должен быть порядка 10^{-2}—10^{-3} молекул рибонуклеиновой кислоты на клетку.

Обсуждается предполагаемая роль специфической рибонуклеиновой кислоты и рибонуклеазного воздействия.

ПРЕОБРАЗОВАНИЕ КОРТИЗОН-АЦЕТАТА В ПЕЧЕНОЧНОЙ ТКАНИ РАЗЛИЧНЫХ ВИДОВ ЖИВОТНЫХ В ОПЫТАХ IN VITRO

Б. БОХУШ и Э. ЭНДРОЦИ

В ходе метаболизации in vitro, в печеночной ткани кортизон преобразовывается в менее полярные биологически активные кортикостероиды.

Наблюдались следующие преобразования:

1. Путем дегидроксилирования 17-α-получается 11-дегидрокортикостерон.
2. Отщеплением 11-ой оксо-группы получается у собак 11-дезокси-17-гидрокортикостерон.
3. При действии 11-гидрогеназы образуется гидрокортизон, способный к дальнейшим преобразованиям.

ПРЕОБРАЗОВАНИЕ ГИДРОКОРТИЗОНА В ПЕЧЕНОЧНОЙ ТКАНИ СОБАК, КОШЕК, МОРСКИХ СВИНОК И КРЫС В ОПЫТАХ IN VITRO

Б. БОХУШ и Э. ЭНДРОЦИ

В опытах in vitro было установлено, что печеночная ткань собак, кошек, морских свинок и крыс преобразовывает гидрокортизон в менее полярные свободные 4—3-кетокортикостероиды.

Наблюдались следующие преобразования:

1. Путем дегидроксилирования 17-α — получается кортикостерон.
2. После дегидроксилирования 11-β — получается соединение S Рейхенштейна.
3. При действии 11-β-дегидрогеназы, из кортикостерона получается 11-дегидрокортикостерон; эта реакция не имеет места в печеночной ткани морской свинки и кошки.
4. При действии β-дегидрогеназы из гидрокортизона образуется кортизон.

ИССЛЕДОВАНИЕ ГЕКСОКИНАЗНОЙ АКТИВНОСТИ ПРИ ВСАСЫВАНИИ ГЛЮКОЗЫ

И. ФЕХЕР, И. ДЕШИ и К. САЛАИ

В опытах in vivo у крыс с удаленной почкой, не было обнаружено параллельности между активностью гексокиназы и количеством всасываемой глюкозы.

АНАЛИЗ ИЗМЕНЕНИЯ КИШЕЧНОГО ВСАСЫВАНИЯ ВОДЫ, ВЫЗВАННОГО ВЫТЯЖКОЙ ЗАДНЕЙ ДОЛИ ГИПОФИЗА, У ЖИВОТНЫХ, ПРЕДВАРИТЕЛЬНО ОБРАБОТАННЫХ КОРКОВЫМИ ГОРМОНАМИ

Г. А. ДОМБРАДИ, Ф. КРИЖА, Т. ЯНЧО и Ф. ОБАЛ

С помощью методики всасывания по Хорват—Уйксу исследовались закономерности всасывания воды из тонкой кишки у крыс-самцов, предварительно обработанных ацетатом кортизона и глюкозидом дезоксикортикостерона. Исследования распространялись на действие двух веществ с гиалуронидазным или же антигиалуронидазным эффектом, с одной стороны на кишечное основное всасывание, а с другой, на возможное воздействие этими всасывание, а с другой, на возможное воздействие этими же веществами на повышающий, всасывание воды, эффект антидиуретического гормона. Полученные результаты можно резюмировать следующим образом:

1. В случае применения обычных фармакологических доз, при сохранении функции коры надпочечников, ацетат кортизона и глюкозид дезоксикортикостерона не изменяет постоянного основного всасывания физиологического раствора поваренной соли из тонкой кишки.

2. Внутривенно-введенная вытяжка нервного отростка вызывает у животных, предварительно обработанных ацетатом кортизона, только незначительное повышение всасывания. Предварительная обработка глюкозидом дезоксикортикостерона сигнификантно не изменяет повышения всасывания.

На основе полученных результатов теория о медиации гиалуронидазы не кажется доказанной.

СВЯЗЫВАНИЕ МЕТИОНИНА ^{35}S У КРЫС, СОДЕРЖАННЫХ НА РЕЖИМЕ ПИТАНИЯ С НЕДОСТАТКОМ МЕТИОНИНА

П. КЕРТАИ и Й. ШОШ

У крыс, предварительно содержанных на режиме питания с недостатком метионина, два часа после внутривенного введения метионина ^{35}S, радиоактивность печени и головного мозга, по сравнению с контрольными животными, значительно повышена (+ 30% и + 44%). Активность мышц, почек и сыворотки была у двух групп крыс одинаковой. Среди исследованных органов и тканей связывание метионина, указывающее на биохимическую регенерацию, повышается быстрее всего в печени и в головном мозге.

ИССЛЕДОВАНИЕ НОВОГО ПРОТИВОГРИБКОВОГО АНТИБИОТИКА

Е. ГЛАЗ, Е. ШЕЙБЕР и К. ЙАРФАШ

Исследовались биологические действия нового противогрибкового антибиотика (антибиотика Т), полученного раньше авторами из культуры **Cephalosporium sp.** Было установлено, что эти действия — вследствие химической структуры антибиотика Т — весьма подобны свойствам трихотецина. Противогрибковое действие **in vitro** сравнительно слабое и имеет фунгистатический характер. Он обладает весьма слабой токсичностью, однако, фармакологическое исследование наталкивалось на большие трудности ввиду весьма плохой растворимости этого антибиотика в воде. После его введения мышам

и крысам не удалось выявить эффективного противогрибкового уровня в крови; причиной этого факта является по всей вероятности наблюдаемая инактивация антибиотика в крови. Заражения мышей грибком Candida этим антибиотиком нельзя отражать. На коже животных, как и человека он оказывает раздражающее действие. Число дрожжевых клеток, присутствующих в испражнении мышей после кормления животных окситетрациклином, уменьшается на действие введения per os большей дозы антибиотика Т. Свойства алкоголя, полученного путем гидролиза антибиотика Т указывают на то, что получение производных этого алкоголя может привести к возникновению противогрибковых соединений с благоприятными свойствами.

ИССЛЕДОВАНИЕ ЧУВСТВИТЕЛЬНОСТИ К ЭФИРУ НА МОЛОДЫХ И ВЗРОСЛЫХ ЖИВОТНЫХ

П. ИШШЕКУТЦ

1. В сосуде для наркоза была исследована эффективность эфира на молодых крысах весом в 30—40 г, как и на взрослых крысах, весом в 350 г, и было установлено, что молодые животные более чувствительны чем взрослые, ибо они засыпают при более низком, определенном в воздухе объемном проценте эфира, чем взрослые животные. Время до просыпания из глубокого наркоза также длиннее у молодых животных, и для них эфир уже при таком объемном проценте смертельный, который взрослые животные еще хорошо переносят.

2. Причиной этой разницы в чувствительности является, что в организме молодых животных содержание воды больше, и, следовательно, эфир предположительно лучше растворяется, чем в теле взрослых животных. Если делить количество измеренного в мг% эфира, находящегося в организме животных, на количество оставшегося в воздухе мг% эфира, то полученный коэффициент всасывания больше у молодых, чем у взрослых животных.

3. Измеренный при смертельной концентрации эфира коэффициент поглощения низкий, предположительно по той причине, что животные погибают еще до полного растворения и равномерного распределения эфира в их организме.

ФОСФОРИЛИРОВАНИЕ ПРИ РАЗЛИЧНЫХ ОСМОТИЧЕСКИХ КОНЦЕНТРАЦИЯХ АДЕНОЗИНДИФОСФАТА И ГЛЮКОЗЫ В ИЗОЛИРОВАННЫХ МИТОХОНДРИЯХ ГОЛОВНОГО МОЗГА

А. ФОНЬО и Я. ШОМОДЬИ

Изотонической инкубацией изолированных митохондриев головного мозга не удалось выявить фосфорилирования аденозиндифосфата. В случае гипотонической инкубации аденозиндифосфат фосфорилируется.

В тех препаратах, где окисьление лимитируется недостатком акцепторов росфата, дыхание повышается аденозиидидисросоратом только в гипотонической среде.

С акцептором гексокиназы-глюкозы фосфорилирование состоится также в изотонических условиях. Если применяется гексокиназа в лимитирующем количестве, то фосфорилирование в гипотонических условиях больше, чем в изотонических. Если же повысить количество гексокиназы, то разница становится меньше. Гексокиназа повышает дыхание также в изотонических условиях.

ДАННЫЕ К СЕКРЕЦИИ КОРТИКОИДОВ КОРЫ НАДПОЧЕЧНИКОВ МОРСКОЙ СВИНКИ

Д. ТЕЛЕГДИ, Э. ЭНДРОЦИ и К. ЛИШШАК

В выделениях коры надпочечников морских свинок было обнаружено 5 кортикоидов: I. 2-α-гидроксикортикол или 6-β-гидроксикортизол, II. гидрокортизон, III. кортизон, IV. кортикостерон, V. 11-дегидрокортикостерон.

В выделениях коры надпочечников некоторых животных, гидрокортизон обнаружен в самом большом количестве, а остальные кортикоиды не удалось обнаружить. После обработки адренокортикотропным гормоном (АКТГ) общее выделение значительно повышается.

Вес правого и левого надпочечника как и вес надпочечников самца и самки значительно не отличаются между собой. После введения АКТГ вес надпочечников повышается.

ДЕЙСТВИЕ НЕДОСТАТКА МЕТИОНИНА НА ОБРАЗОВАНИЕ ГЕТЕРОГЕМОТРОПИНА

Д. ВАЙДА, И. РИГО и И. ШОШ

Данные авторов служат новым доказательство того, что метионин играет важную роль в клеточной и гуморальной защите. Кроме описанных до сих пор =*ошем* и сотрудниками вредных действий режима питания с недостатком метионина, необходимо учесть также — до сих пор невыясненное — интенсивное нарушение ответной иммунной реакции (гетеро-гемотропин).

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Biochemia

A SIMPLE AND SENSITIVE METHOD FOR THE ESTIMATION OF INORGANIC PHOSPHORUS

By
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A new method of inorganic phosphorus determination has been developed, consisting of precipitating the phosphorus as the strychnine salt of phosphomolybdic acid, dissolving the washed precipitate and measuring the light absorption at 253 mμ. The minimum amount determined is 0.05 μg/ml. Adenosine-phosphates, Ca^{++}, Mg^{++}, trichloroacetic and perchloric acids are not disturbing. Arsenate must be absent. ATP shows no measurable hydrolysis, phosphocreatine a hydrolysis amounting to about 5 per cent under the conditions prescribed.

Numerous methods for the estimation of inorganic phosphorus are known. The continuous effort to find the most convenient procedure for the molybdenum blue method or for methods extracting the phosphomolybdic acid in organic solvents, shows that in spite of there being very simple and sensitive methods, we are still in want of more satisfying ones. The most serious difficulty lies in the error caused by the hydrolysis of labile phosphorus compounds in the acid mediums used [1], a source of error more or less common to all known methods. Recently Marsh [2] has pointed out that one of the most widely used extraction methods (Allen [3]) is unsatisfactory owing to the hydrolysis of ATP, if one has to know exactly the small amounts of P_i present in ATP preparations. Furthermore the most sensitive and reliable extraction methods known are tedious to carry out in great number.

We have worked out a new method somewhat more sensitive than the most sensitive of the hitherto known extraction methods and technically much more simple than most of them. The method is based on Embden's [4] gravimetric strychnine-molybdate method, by now of a historical interest only. Its turbidimetric modification by Bergold and Pister [5], an ideally simple and sensitive procedure indeed, has been unsatisfactory in our hands. Owing to the inherent lability of turbidimetric methods, we could not get reproducible results, except in the case of pure inorganic phosphorus solutions. On the other hand, we have found that by collecting the strychnine-phosphomolybdate precipitate, followed by dissolution and measurement of light absorption in the ultraviolet, we can estimate phosphorus in a simple and very sensitive way.

Reagent

To 28 ml warm (slightly fuming) sulphuric acid (s.g. 1.82) are given 4 g of ammonium molybdate in small portions. It dissolves readily. When it begins to cool (a slight blue colour does not disturb) it is poured to 450 ml strychnine-nitrate solution containing 1 g of the alkaloid, and made up to 500 ml. If the slightest turbidity is observed, the reagent is filtered through a G4 glass filtre. It is stored in a polyethylene bottle.

Procedure

The samples, if not neutral (or if they are neutral by the buffering action of some component being there in substantial amounts) are brought to about pH 1.5. One may use one drop of thymol blue indicator solution: the solution with the indicator must have an orange hue.

Minimum final concentration of P_i must be at least 0.05 μg/ml, the minimum total amount (carrying out the precipitation in one ml total volume), 0.05 μg.[1] It is advisable to make the precipitation in one series of estimations in equal volumes, *i.e.* to make up every sample to the same volume, giving the same volume of reagent to all of them.

The samples prepared according to these specifications are cooled to 0° C and 0.05 ml of reagent is added to every ml of sample. After standing for 10 minutes (in the case of less than 1 μg, because of some delay in crystallisation, 30 mins) the samples are filtered through small G4 glass filtres. One may conveniently use a series of large tubes, with side tubes for suction, and with placed-in test tubes, for collecting the filtrate. The suction of the filtres may be carried out simultaneously with the aid of a multibranched vacuum.

The precipitate on the filtre is washed with 3 ml ice-cold alcohol, containing 3 μg strychnine/ml, added in three portions, with careful rinsing of the filtres. The washed precipitate is dissolved in 3 ml 0.2 per cent ammonia, in three portions, the filtrate being collected in a test tube bearing a mark at 5 ml. After completion of the volume, the extinction is measured at 254 mμ against a blank prepared from distilled water and reagent and carried with the samples through the whole procedure (including filtration and washing of the filtre).

The measurement of light absorption was carried out with a new type of simple photometer including, between other ultraviolet lines resp. bands, the 254 mμ mercury resonance line.[2] The use of a spectrophotometer allows

[1] The maximum amount is not exactly stated. Above 15 μg P, in a final mixture of 10 ml, the extinction of the appropiately diluted dissolved precipitate tends to decline from linearity.

[2] "Uvifot" photometer of MOM *(Hungarian Optical Works)*, resp. BFK-Photometer of *Kipp*, Delft, Holland.

even greater sensitivity at a shorter wave length, such as 230 mμ, as seen from the absorption spectrum of the dissolved strychnine-molybdate precipitate shown in Fig. 1.

At 254 mμ, 1 μg of phosphorus gives with the volumes given above, 0.197 extinction units.[3]

Neutralized trichloroacetic acid and perchloric acid (up to a final concentration of 5 to 6 per cent) cause no error. AMP, ADP, ATP, and pyrophosphate up to a concentration of 20 mM, Ca^{++} or Mg^{++} up to 10 mM

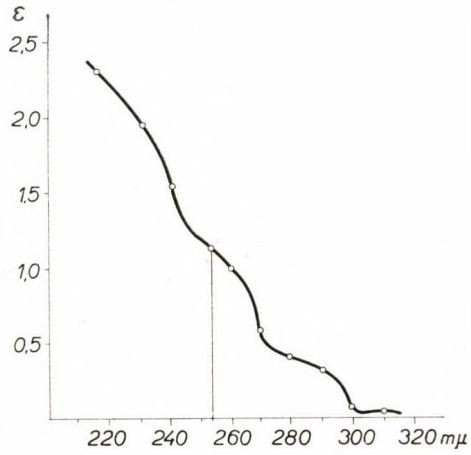

Fig. 1. Absorption spectrum of dissolved strychnine-phosphomolybdate. 4.70 μg P_i precipitated, treated according to the procedure, and dissolved in 4 ml (1 cm cuvette, Beckman Model DU spectrophotometer)

do not disturb either. Higher concentrations were not tested. Arsenate forms a precipitate with the reagent like phosphate does, so that its presence must be avoided.

Experiments and discussion

The solubility of the strychnine salt of phosphomolybdic acid is surprisingly small under the conditions of the estimation procedure given above. With the aid of ^{32}P-labelled phosphorus we found this solubility in order of magnitude of 10^{-6} M/l. Some results are given in Table I. Since the concentration of inorganic phosphorus left in the filtrate is very much dependent on the amount of phosphorus precipitated, the values obtained cannot be considered as those of true solubilities. We suppose that part of the phosphorus found

[3] By the exactly stated experimental conditions as pH and composition and added amount of reagent a change in the composition of the heteropolyacid has been ruled out, so a strict proportionality can be assumed between phosphorus content and extinction (see Table II).

in the filtrate was due to the heterodispersity of the precipitate, a constant small fraction passing through filtres. Fortunately this unprecipitated amount of phosphorus is practically proportional to the total amount precipitated. As seen from Table I, it amounts to 4 per cent of the total, independently whether 2 or 10 μg are precipitated from 10 ml volume.

Table I

Recovery experiments with ^{32}P

The indicated amounts of P_i, counting 10 808 impulses/μg were precipitated from a total volume of 10 ml under the conditions given under "Procedure". The radioactivity of the filtrate of the collected washings and of the dissolved precipitate (made up to 10 ml) was determined. The P_i values were calculated from the radioactivity found in the solutions, "total" P_i being the sum of the phosphorus content of the three solutions. All the other values refer to 10 ml total volume of the respective solutions.

Total P_i μg	Filtrate		Washings		Precipitate dissolved	
	μg	% of total	μg	% of total	μg	% of total
11.39	0.49	4.3	0.60	5.3	10.3	90.4
11.22	0.57	5.1	0.55	4.9	10.1	90.0
11.50	0.45	3.9	0.66	5.7	10.4	90.4
1.802	0.072	4.1	0.110	6.1	1.62	89.8
1.875	0.091	4.9	0.124	6.7	1.66	89.4
1.784	0.104	5.8	1.100	5.6	1.58	88.6

It was difficult to find an appropriate solution to wash the precipitate free from the remaining reagent, which absorbs much light. Cold nitric acid, as applied in the classical gravimetric method of EMBDEN for mg amounts, dissolves the whole precipitate when working in the μg range. After many trials we found that in an alcoholic solution of strychnine nitrate (3 μg/ml) the precipitate is practically insoluble. Although strychnine has some light absorption at the wave length used, it is negligible in this great dilution, even when the amount of phosphorus to be determined is as small as 0.05 μg.

In determinations with ^{32}P-labelled phosphate there were small but measurable losses of phosphorus at washing. As seen in Table I, the loss of phosphorus as washed out radioactivity amounted to some 6 per cent of the total.

Fortunately these losses are also proportional to the phosphorus present.[4] All the data concerning the perfection of precipitation and washing (of which Table I shows only a small fraction) led to the result that under the conditions given the recovery of phosphorus (*i. e.* radioactivity) in the final filtrate is

[4] The proportionality of the radioactivity remaining in solution resp. washed out, may be explained by the presence of some radioactive impurity. In fact, we found in some cases traces of pyrophosphate in our ^{32}P preparations.

Table II

Calibration experiments

The given amounts of phosphorus (from appropriate dilutions of a stock solution carefully prepared by exact weighing of crystalline KH_2PO_4 of analytical purity) are precipitated and treated as given under "Procedure".

Volume	µg phosphorus present	$E^{1\text{ cm}}_{254\text{ m}\mu}/\mu g$ P
2 ml	0.096	0.208
	0.192	0.192
	0.384	0.190
	0.432	0.200
4 ml	1.150	0.196
	1.440	0.194
	1.730	0.194
	2.020	0.205
	2.305	0.194
6 ml	2.59	0.188
	3.17	0.192
	3.74	0.190
	4.32	0.189
8 ml	5.77	0.200
	6.72	0.202
	7.67	0.205
	8.63	0.201
10 ml	9.60	0.198
	11.52	0.189
	12.48	0.201
	13.92	0.205
		Mean 0.197 ±0.005

90 per cent, independently of the total amount of phosphorus precipitated. In accordance with this, the calibration experiments in Table II show a good proportionality between the amount of phosphorus and extinction, in a widely varied range of concentrations.

As mentioned above, the solution used to wash the precipitate free of reagent has some small absorption at the wave length used. Some extinction may result from the insufficiently removed reagent. It is therefore advisable, especially when determining small amounts of phosphorus, to prepare a blank carried through the whole procedure.

We performed some experiments on the hydrolysis of ATP and phosphocreatine by means of our procedure. Considering the rate of hydrolysis of these substances [1], one may expect our procedure to be very favourable. The final concentration of molybdate, which enhances the rate of hydrolysis in most cases, is comparatively small and the applied temperature of 0° C also favours the suppression of hydrolysis.

Table III

Rate of hydrolysis of ATP resp. phosphocreatine under the conditions of the procedure

The amounts of substances given were distributed in several equal samples and the estimation of P_i was carried out as described. Filtration of the precipitate was carried out after addition of reagent as indicated. Total volume: 10 ml.

The ATP was a commercial preparation *(Reanal*, Budapest), the "phosphocreatine" was a frog muscle homogenate deproteinized according to SOMOGYI [6]. Total amount of phosphocreatine phosphorus: increment of P_i after hydrolysis at 65° C in 0.1 *N* HCl and 9 minutes [7].

P_i was estimated in the ATP experiment to 0.140 μM (FISKE and SUBBAROW [8]), in the phosphocreatine experiment to 0.016 μM (BERENBLUM and CHAIN [9]).

Time minutes	ATP 2.5 μmoles $P_{10'}$	*Phosphocreatine* 0.201 μmoles phosphocreatine-P
	P_i found, μmoles	
5	0.121	0.0036
10	0.123	0.0069
20	0.126	0.0079
30	0.134	0.0090
45	0.126	0.0139
60	0.129	—

As shown in Table III, with ATP we did not observe any measurable hydrolysis. In the case of phosphocreatine, at 30 minutes, the time recommended for complete precipitation, hydrolysis amounted to 4.5 per cent of the total, an amount not negligible, but concurring well with the rate of phosphocreatine hydrolysis usual with most of the known phosphorus estimations.

The experiments in Table III show further that the method works well in the case of tissue extracts. The divergence in this case of the results of the classical methods and ours are attributed to differences in hydrolysis with the different methods.

LITERATURE

1. WEIL-MALHERBE, H., GREEN, R. H.: Biochem. J. **49,** 286 (1951).
2. MARSH, B. B.: Biochem. biophys. Acta **32,** 357 (1959).
3. ALLEN, R. J. L.: Biochem. J. **34,** 858 (1940).
4. EMBDEN, G.: Hoppe Seyler's Z. physiol. Chem. **113,** 138 (1921).
5. BERGOLD, G., PISTER, L.: Z. Naturforsch. **3b,** 332 (1948).
6. SOMOGYI, M.: J. biol. Chem. **86,** 655 (1930).
7. ENNOR, A. H., ROSENBERG, H.: Biochem. J. **57,** 203 (1952).
8. FISKE, C. H., SUBBAROW, Y.: J. biol. Chem. **66,** 375 (1925).
9. BERENBLUM, J., CHAIN, E.: Biochem. J. **32,** 205 (1938).

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BIOSYNTHESIS OF CORTICOSTEROIDS IN THE RABBIT ADRENAL

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In the adrenal gland of the rabbit the 11-βOH group of the corticosteroids may be formed also by the reduction of the 11-oxo group. The process requires the presence of molecular oxygen. The coenzyme transferring hydrogen is not DPN. The process is accelerated by the addition of the intermediaries of the Szentgyörgyi—Krebs cycle. The cell structure need not be intact. Aldosterone and cortisol are biosynthesized from 11-deoxycorticosterone. Corticosterone is formed mainly from 11-deoxycorticosterone but may be formed also from 11-dehydrocorticosterone. The former reaction is about twice as intensive as the latter. In the rabbit adrenal, the hydroxylation in position 17 may take place also after the formation of the hydroxyl group in position 21, but only when the compound possesses an 11-oxo group.

The biosynthesis of corticosteroids is composed of known processes, but nevertheless, some aspects are still unclear. The 11-βOH group of corticosteroids is formed by the introduction of a hydroxyl group, under the effect of the enzyme 11-βhydroxylase, described for the first time by Hayano, Dorfman and Prins [9] as occurring in slices and homogenates of bovine adrenals.

However, steroids containing an 11-oxo group (*e.g.* cortisone, 11-dehydrocorticosterone) were also isolated from the adrenals and their venous blood. The presence of these compounds does not fit into the biosynthetic scheme constructed by Hechter and Pincus [10].

According to the investigations of Bush [4], Kass [14] Pankov [17] and Holzbauer [12] the rabbit adrenal secretes mainly corticosterone and in traces aldosterone, cortisol and 11-dehydrocorticosterone. On the other hand, analysis of adrenal extracts showed the cells to contain chiefly 11-dehydrocorticosterone. Since in other investigations [7] it could be observed that when cortical secretion was increased the amount of 11-dehydrocorticosterone also increased in the rabbit adrenal, it was surmised that that compound had an important role to play in the biosynthesis of the secreted steroids. This problem will be analysed in the present paper.

Methods

Preparation and incubation of adrenal slices. Adrenals from 20 rabbits were used. The rabbits weighing from 1500 to 3500 g were killed by air embolism. The adrenals were removed immediately, placed into ice-cold Krebs—Ringer phosphate solution, weighed and cut with a razor blade into slices about ½ to 1 mm thickness. One half of the material served for control

and the other half was incubated with the material to be tested. The adrenal slices thus prepared were placed into Warburg vessels containing 5 ml of Krebs—Ringer phosphate solution, prepared without $CaCl_2$, containing 200 mg per 100 ml of glucose and having a pH of 7.4. Into one vessel we placed 170 to 220 mg of adrenal slices added 200 μg of steroid in 0.05 ml of ethanol, and incubated for 3 hours with continuous shaking (90 to 110/min), at 37° C, in O_2, air or N_2 atmosphere.

Each experiment was made in duplicate. The homogenates were prepared in a glass homogenizer of the Potter—Elvehjem type.

Extraction. After incubation, 10 ml of acetone was added to each sample and the samples were extracted overnight. After filtration, the acetone was removed by evaporation *in vacuo*. The aqueous residue was extracted three times with 1.5 vol. ethyl acetate, the pooled extracts were washed twice with 0.05 vol. of 0.1 *N* K_2CO_3, then with 3×0.05 vol. of distilled water and were dried finally on dry Na_2SO_4. The ethyl acetate was evaporated in vacuo, the contents of the flask solved in a mixture of 10 ml benzene : petrolether, and transferred to a separatory funnel. The flask was then washed again with 10 ml of 70 per cent ethanol and the material was partitioned against benzene : petrolether and after separation partitioned again with 10 ml of 70 per cent ethanol. The pooled alcoholic fractions were evaporated in vacuo. The dry residue dissolved twice in 5 ml ethyl acetate was concentrated in a micro-vacuum distillator and the residue dissolved in 0.05 ml of ethyl acetate was analysed by paper chromatography. All the vacuum distillations were done in a water bath at 45° C. Owing to the high lipid content of the rabbit adrenals, it was necessary to carry out all these processes of purification.

Paper chromatography. The spots dropped on the chromatographic paper were concentrated by using a filtre paper ring [15]. A ring of filtre paper soaked in a 2 : 1 mixture of ethyl acetate : methanol is placed exactly around the spot to be concentrated. The solvent then forces the spot to move inward. After the solvent has reached the centre, the ring is removed and the spot is dried with hot air (40° C). This procedure is repeated several times until the spot has become round, small and even.

The following solvent systems were used.

1. Benzene 1000 ml, methanol 500 ml, water 500 ml (the B_5 system of BUSH).
2. Benzene 400 ml, petrolether 600 ml, methanol 700 ml, water 300 ml (a system with a polarity between BUSH B_3 and B_4).
3. Benzene 300 ml, petrolether 700 ml, methanol 700 ml, water 300 ml (polarity between BUSH B_2 and B_3).

The chromatograms were run at room temperature. With system 1, *Schleicher & Schüll* 2043 Mgl, with systems 2 and 3, *Schleicher & Schüll* 2043 b M papers were used. Equilibration lasted 3 hours. System 1 was used for the separation of complete extracts prepared from incubates; system 2 to isolate more polar steroids and for rechromatography; and system 3 to identify corticosterone acetate.

The chromatograms were studied by the combined tetrazolium blue — NaOH fluorescence test [1]. The chromatograms were pulled through a 9 : 1 mixture of 2 *N* NaOH and 0.1 per cent tetrazolium blue and after gentle heating the tetrazolium blue-positive spots were marked. Then the chromatograms were dried at 90° C in infrared light and were examined in UV light. The steroids were estimated semiquantitatively, by comparing the intensity of UV fluorescence with that shown by standard quantities. Acetylation was performed by the method of DE COURCY [5].

The DPN used was isolated from yeast according to WILLIAMSON [20]. From the formed compounds, corticosterone was identified on the basis of its R_f values (systems 1 and 2), group reaction (Tetrazolium blue, NaOH), as well as by determining the R_f values of its acetylated product (system 3). Cortisol and aldosterone were identified on the basis of their R_f values after repeated chromatography and their group reactions (Tetrazolium blue, NaOH).

Results

Biosynthesis of 11-dehydrocorticosterone. In the rabbit adrenals extracted without incubation, on the average 8 μg/g of 11-dehydrocorticosterone and 0.4 μg/g of corticosterone were found. The slices of adrenal tissue, incubated without adding steroid, synthesized an average of 32 μg/g of 11-dehydrocorticosterone and 2 μg/g of corticosterone. When the adrenal slices were incubated with progesterone, the amount of 11-dehydrocorticosterone produced

averaged 54 μg/g. Thus, the adrenal slices synthesized 11-dehydrocorticosterone with a high intensity; the rate of synthesis was increased by the addition of progesterone.

Biosynthesis of corticosterone. When the slices of rabbit adrenal were incubated with 11-dehydrocorticosterone, corticosterone synthesis increased 22-fold, *i.e.* 45 μg/g was produced, as compared with the 2 μg/g found in the control tissue. Accordingly, the rabbit adrenal is capable of converting the 11-oxo group to 11-βOH. In the next step we investigated the conditions required for this conversion. It has been found that no corticosterone was formed from 11-dehydrocorticosterone when incubated in N_2 atmosphere. At the same time, the adrenal slices incubated in O_2 atmosphere converted the 11-oxo group to 11-βOH. Thus, molecular oxygen and cell respiration are essential for the above process.

Cell respiration being connected with the function of the Szentgyörgyi—Krebs cycle, it has been assumed that this cycle was involved in the above process. To prove this hypothesis, adrenal slices incubated with 11-dehydrocorticosterone in Krebs—Ringer phosphate solution were brought together in the presence of 0.2 ml 10^{-3} *M* ATP with succinic acid (0.2 ml, 10^{-3} *M*), then with malic acid (0.4 ml, 10^{-2} *M*), so that the end volume was 5 ml. The chromatograms showed that the above compounds had markedly increased the biosynthesis of corticosterone, for example malic acid increased it more than twofold (to 110 μg/g). Since the process requires the oxidation of the intermediaries of the Szentgyörgyi—Krebs cycle, the specific dehydrogenases may be supposed to transfer the hydrogens split from their substrates to the 11-oxo group of the steroid molecule with the aid of coenzyme, or coenzymes and an unknown enzyme.

When the slices of adrenal tissue were incubated in a system generating DPNH (one containing 0.2 ml 10^{-3}*M* DPN and 0.5 ml 10^{-2}*M* malic acid), the biosynthesis of corticosterone did not increase, as compared with that in the controls either in O_2 or in N_2 atmosphere. This has made it improbable that in this reaction DPN should play the role of the coenzyme transferring hydrogen.

To study the relationship between the intensity of 11-β hydroxylation and that of the reduction of the 11-oxo group in the rabbit adrenal, slices of adrenal tissue were incubated with 11-deoxycorticosterone. About twice the amount of corticosterone was formed in the slices thus treated than that produced in the slices incubated with 11-dehydrocorticosterone. Similar results were obtained for incubation with progesterone.

Biosynthesis of aldosterone. Incubated without adding steroid, the slices of adrenal tissue synthesized an average of 9 μg/g of aldosterone. In response to 11-dehydrocorticosterone this amount increased to 22 μg/g. In N_2 atmosphere no aldosterone was synthesized from 11-dehydrocorticosterone, while incuba-

tion in O_2 atmosphere in the presence of 0.3 ml 10^{-3} *M* succinic acid and 0.2 ml 10^{-3} *M* ATP increased the amount of aldosterone by about 30 per cent. In response to 0.5 ml 10^{-2} *M* malic acid there was a threefold increase to 62 μg/g. It was most remarkable that in the rabbit adrenal no aldosterone was produced from 11-deoxycorticosterone as it was shown by incubating slices of adrenal tissue with 11-deoxycorticosterone.

Biosynthesis of cortisol. Incubated without steroid the slices of adrenal tissue synthesized 0.5 μg/g cortisol. After adding 11-dehydrocorticosterone this amount increased to 4.5 μg/g. In N_2 atmosphere no cortisol was produced, while in O_2 atmosphere in response to 0.3 ml $10^{-3}M$ succinic acid, in the presence of 0.2 ml 10^{-3} *M* ATP the amount increased by about 25 per cent, and in response to 0.5 ml 10^{-2} *M* malic acid by about 33 per cent, as compared with the controls. No cortisol was formed from 11-deoxycorticosterone.

The rabbit adrenal slices produced from 11-dehydrocorticosterone, as a precursor, other compounds, too. These are shown in Table I.

The compounds No. 1, 2 and 3 react weakly with tetrazolium blue, are more polar than cortisol and possess a Δ^4-3-oxo group. These properties suggest their having an alpha-glycerol side chain. Compound No. 4 has an alpha-ketol side chain because it reacts well with tetrazolium blue, but does not give the NaOH fluorescence reaction of Bush [3] (NaOH) specific for the Δ^4-3-oxo group. Thus this compound is a di- or tetrahydro derivative saturated in ring A. As judged from its R_f value it seems to be allotetrahydrocortisol long known to be present in adrenal extracts [18, 20]. Its amount is

Table I

Corticosteroids synthesized by slices of rabbit adrenals incubated with 11-dehydrocorticosterone. Chromatographed in system No. 1

No.	R_f	BT	NaOH	Compound
1	0.09	weak positive	+	
2	0.14	weak positive	+	
3	0.21	+	+	
4	0.25	+	−	allotetrahydrocortisol?
5	0.30	+	+	cortisol
6	0.43	+	+	aldosterone
7	0.51	+	+	cortisone?
8	0.57	+	+	
9	0.61	+	+	
10	0.73	+	+	
11	0.81	+	+	corticosterone
12	0.88	+	+	11-dehydrocorticosterone

about 15 μg/g, which increases to about 30 μg/g in response to 0.5 ml $10^{-2}M$ malic acid. Compound No. 5 appears to be cortisol, as judged from its R_f value and group reactions, while compound No. 6 is aldosterone. Compounds No. 7, 8, 9 and 10 possess both an α-ketol side chain and a Δ^4-3-oxo group; their amount varies from 0.5 to 3 μg/g, which in response to malic acid (0.5 ml, 10^{-2} M) increases 3 to 4-fold. Compound No. 7 appears to be cortisone; it was found in one case only. Compound No. 11 is corticosterone and No. 12 is 11-dehydrocorticosterone.

Of the compounds mentioned Nos 3, 4, 7, 8 and 9 have never occurred in the control adrenals incubated without 11-dehydrocorticosterone. These compounds are formed exclusively when exogenous 11-dehydrocorticosterone is added in excess, thus are the products of enzymatic reactions normally inactive in the adrenal cells and active only when the substrate concentration is very high. The other compounds are present also in the extracts of adrenals incubated without the addition of steroids. The synthesis of each compound takes place also in homogenate, thus the cell structure need not be intact. Their amount is merely about 20 per cent less, presumably due to mechanical damage to the cell particles.

Discussion

The above results make clear that the rabbit adrenal slices are capable of converting the 11-oxo group of the steroid molecule to a hydroxyl group. This is proved by the fact that corticosterone, aldosterone and cortisol, each having an 11-β OH group, are produced from 11-dehydrocorticosterone. We think that this process involves a reduction of the 11-oxo group. Another possibility would be that 11-deoxycorticosterone is formed first and is then converted to the 11-βOH group by 11-β hydroxylation. However, there is little probability for the latter mechanism partly because we never found 11-deoxycorticosterone in the samples incubated with 11-dehydrocorticosterone and partly because the adrenal slices incubated with the 11-deoxy compound have never produced either aldosterone or cortisol.

There is ample evidence that most of the cortisone introduced into the organism is converted to cortisol by the reduction of its 11-oxo group [2, 8]. This process takes place in the liver and involves an enzyme, named 11-βOH dehydrogenase [2]. According to HUBENER *et al.* [13] the process does not require the presence of molecular oxygen, it takes place also in nitrogen atmosphere and the cofactor transporting hydrogen is unknown. In contrast with this, the process described by us requires the presence of molecular oxygen and its intensity is greatly increased by the addition of the intermediaries of the Szentgyörgyi—Krebs cycle. We therefore think that the intermediaries of the Szentgyörgyi—Krebs cycle play the role of generating the reduced coenzyme.

The slices of rabbit adrenal tissue were found to synthesize from 11-dehydrocorticosterone all the compounds present in the venous adrenal blood of the rabbit, *i.e.* those actually secreted by the adrenal gland [17]. From this it may be concluded that the secreted compounds are synthesized from 11-dehydrocorticosterone, of which large amounts are present in the cells. This, however, is in our opinion only partially true because, as already mentioned, twice as much corticosterone is formed from 11-deoxycorticosterone than

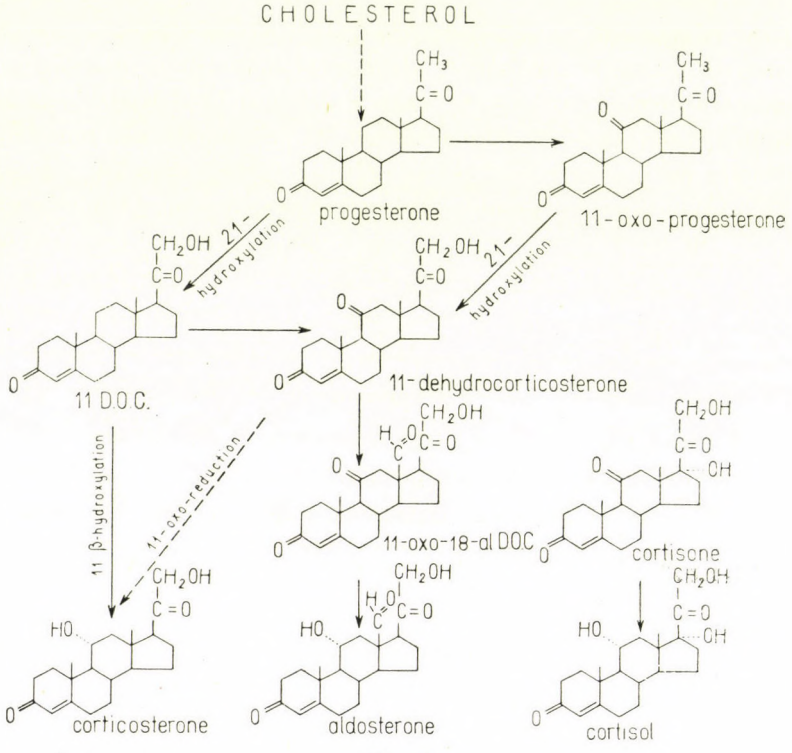

Fig. 1

from 11-dehydrocorticosterone. It appears therefore that the biosynthesis of this compound takes place by 11-β-hydroxylation, although the reduction of the 11-oxo group is also a possible mechanism. On the other hand, the situation is different with aldosterone and cortisol, which are not synthesized from the 11-deoxy compound, only from the 11-dehydrocorticosterone possessing a 11-oxo group.

These results are most interesting from the point of view of the sequence of hydroxylation. HECHTER and PINCUS [10] have namely shown that if a compound possesses a hydroxyl group at position 21, it cannot be hydroxylated at position 17. In the present case, however, cortisol, a compound with a hydroxyl group at position 17 was formed from 11-dehydrocorticosterone,

a compound with a hydroxyl group at position 21. According to HEARD *et al.*[11], and DORFMAN *et al.* [6] there is a limited possibility for hydroxylation at position 17 also in the case of 11-deoxycorticosterone. We found the same for 11-

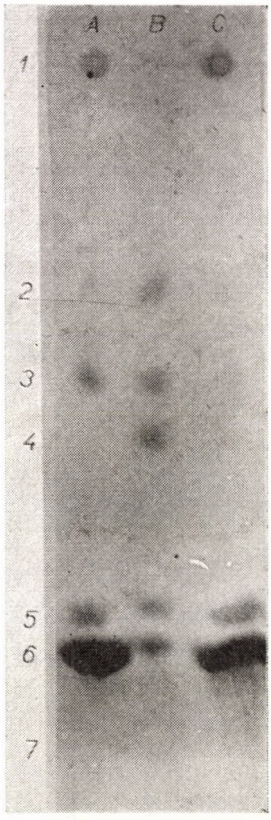

Fig. 2. Chromatogram of rabbit adrenal incubates. *1:* Site of application, *2:* cortisol, *3:* aldosterone, *4:* cortisone, *5:* corticosterone, *6:* 11-dehydrocorticosterone and 11-deoxycorticosterone, resp. *7:* solvent front. Row *A:* steroids produced by the adrenals incubated with 11-dehydrocorticosterone. Row *C:* steroids produced on incubation with 11-deoxycorticosterone. Row *B:* standard steroids. Note that no aldosterone and cortisol are formed from 11-deoxycorticosterone. Run in Bush B_5 system, developed with BT

dehydrocorticosterone, while 11-deoxycorticosterone was not hydroxylated at position 17 in our experiments.

The formation of aldosterone from 11-dehydrocorticosterone is known [16], but this is the first case to be observed that 11-dehydrocorticosterone may play the role of a precursor of cortisol. On the basis of the investigations of KASS *et al.* [14], in the biosynthesis of cortisol a significant role is attributed to the ACTH secretion of the pituitary, owing to its stimulating action on 17-hydroxylation.

As indicated by our results, the biosynthesis of corticosteroids in the rabbit adrenal may take place in the following way:

In Fig. 1 the formation of 11-dehydrocorticosterone is doubtful. As already mentioned, it is formed from progesterone, but this may take place in two different ways, via 11-deoxycorticosterone, or via 11-oxoprogesterone. Further studies are required to solve the problem. Cortisol may be formed exclusively via cortisone, which we have succeeded in demonstrating in one case.

Acknowledgements

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LITERATURE

1. BURTON, R. B., ZAFFARONI, A., KEUTMANN, E. H.: J. biol. Chem. **188,** 763 (1951).
2. BURTON, R. B., KEUTMANN, E. H., WATERHOUSE, C.: J. Clin. Endocrin. **13,** 48 (1953).
3. BUSH, I. E.: Biochem. J. **50,** 370 (1952).
4. BUSH, I. E.: J. Endocr. **9,** 95 (1953).
5. DE COURCY, C., BUSH, I. E., GRAY, C. M., LUNNON, J. B.: J. Endocr. **9,** 401 (1953).
6. DORFMAN, R. I., HAYANO, M., HAYNES, R., SAVARD, K.: Ciba Foundation Colloquia on Endocrinology **7,** 191 (1953).
7. FAZEKAS, Á. GY.: Unpublished results.
8. GRAY, H. G., LUNNON, J. B.: Mem. Soc. Endocr. **2,** 64 (1953).
9. HAYANO, M., DORFMAN, R. I., PRINS, D. A.: Proc. Soc. exp. Biol. (N. Y.) **72,** 700 (1949).
10. HECHTER, O., PINCUS, G.: Physiol. Rev. **34,** 459 (1954).
11. HEARD, R. D. H., JACOBS, R., O'DONNELL, V., PERON, F. G., SAFFRAN, J. C., SOLOMON, S. S., THOMPSON, L. M., WILLOUGHBY, H., YATES, C. H.: Recent Progr. Hormone Res. **9,** 383 (1954).
12. HOLZBAUER, M.: J. Physiol. (Lond.) **139,** 294 (1957).
13. HUBENER, H. J., FUKUSHIMA, D. K., GALLAGHER, T. F.: J. biol. Chem. **220,** 499 (1956).
14. KASS, E. H., HECHTER, O., MACCHI, I. A., MOW, T. W.: Proc. Soc. exp. Biol. (N. Y.) **85,** 583 (1954).
15. KOVÁCS, E., FAZEKAS, A. GY.: Kísérl. Orvostud. (Budapest) **6,** 654 (1960).
16. MULLER, A. F., O'CONNOR, C. M. (Ed.): International Symposium on Aldosterone. Churchill, London, 1958. P. 232.
17. *Панков, Ю. А.:* Пробл. эндокр. и гормонотер. 3, 31, (1957).
18. REICHSTEIN, T.: Helv. chim. Acta **19,** 29 (1936).
19. WINTERSTEINER, O., PFIFFNER, J. J.: J. biol. Chem. **111,** 599 (1935).
20. WILLIAMSON, S., GREEN, D.E.: J. biol. Chem. **135,** 345 (1940).

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A MODIFIED IODOMETRIC METHOD OF PENICILLINASE ASSAY

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The iodometric method of Perret is reliable for determining penicillinase, but unsuitable for serial tests. Applying sodium tungstate and gelatine as inhibitors, the method has been rendered suitable for use in serial work. A few results obtained by the modified method have been presented.

Penicillinase activity can be estimated reliably and rapidly by Perret's iodometric method [1] based on the reaction between penicilloic acid (resulting from the breakdown of penicillin catalysed by penicillinase) and elementary iodine. In a suitable medium penicillinase and penicillin are incubated; penicilloic acid is formed; the reaction is stopped by adding an acetate buffer of pH 4 and iodine to the incubation mixture; the penicilloic acid binds some of the iodine: the excess iodine is titrated back with thiosulphate. Considering that under the given conditions the incubation of the iodine-resistant [2] penicillinase is not complete (40 per cent of its activity resists) [1], it is imperative that the duration of incubation be exactly as specified and titration follow without delay. These requirements mean a certain difficulty when performing serial tests and present a source of error. To assay the exopenicillinase *B. cereus* NRRL 569, a reliable and simple method suitable for use in serial testing was developed.

The above requirements were met by adding 0.05 *M* sodium tungstate and 0.05 per cent gelatine (related to total volume of incubation mixture) to the mixture of acetate buffer and iodine used with Perret's method. This resulted in a 98 to 99 per cent inhibition of the penicillinase activity. By applying more concentrated solutions of iodine and thiosulphate, the method allows the assay of solutions containing more than 20 (but not more than 150) units of penicillinase. It is therefore necessary to know the gross enzyme content in advance. For this purpose we use the rapid, semiquantitative method of Citri [2].

If the amount of enzyme exceeds 20 units, the following method is used. To 1 ml of the test solution in a test tube is added 1 ml of 0.5 per cent gelatine. After heating for 5 minutes in a water bath of 30° C 2 ml of a heated solution of penicillin (penicillin G 60 mg/ml, dissolved in 0.2 *M* of a pH 6.5 sodium

phosphate buffer) is added and the mixture is incubated for 10 minutes. Then the reaction is arrested by adding 5.5 ml of 1.5 *M* acetate buffer of pH 4 and 0.5 ml of 1 *M* sodium tungstate solution. The flocculent precipitate that is formed does not interfere with the further steps in the assay. The reaction mixture is then washed into a titrimetric flask and 10 ml of a 0.05 *N* iodide solution are added. Ten minutes later this is titrated with 0.05 *N* thiosulphate in the presence of starch as the indicator. When the expectable amount of penicillinase is less than 20 units, 0.01 *N* iodine and 0.01 *N* thiosulphate solutions are used and the titration is carried out with a microburette. If the expectable amount of enzyme is less than 3 units, the duration of incubation is prolonged proportionately.

One unit of penicillinase breaks down in one hour one micromole of penicillin [1]. Under the given conditions one molecule of penicilloic acid combines with 8.3 atoms of iodine [3] and correspondingly 1 ml of the 0.05 *N* iodine solution equals 6.220, 1 ml of the 0.01 *N* iodine solution equals 1.204 units of penicillinase.

Composition of the incubation and inhibitor mixtures must be exactly as specified, to ensure maximal inhibition of the enzyme. Sodium tungstate alone, instead of inhibiting, activates the enzyme. Inhibition is weak when gelatine is absent or is present in a concentration different from that specified above. It may be surmised that the gelatine precipitated by the tungstate in acid medium carries with it penicillinase and this may be responsible for inhibition. Similarly as the phosphate buffer, the tungstate lessens the spontaneous breakdown of penicillin.

It is not necessary to titrate without delay, but it should be borne in mind that iodine consumption by the reaction mixture continues somewhat to increase after inhibition (Table II). Two controls should be used, one without penicillin (because the eventually present reducing components in the test-solution may consume iodine) and one without enzyme, to estimate spontaneous penicillin breakdown. The amounts of iodine used in the two control tests are added and their sum is subtracted from the actual amount of iodine consumed at each titration.

If penicillin of suitable quality is used, the penicillinized control must not consume more than 0.2 ml of the 0.01 *N* solution of iodine. The non-specific iodine consumption of the test solution depends on the medium used and is highly variable. A solution not consuming more than 0.5 ml of 0.01 *N* iodine should be used.

The penicillinase values estimated in the supernatant of a culture of *B. cereus* 569/H, containing 0.12 mg cellular dry material/ml, are presented in Table I.

Table I

Volume of test solution ml	Penicillinase units/ml
1.0	64.3
0.8	51.3
0.6	38.6
0.4	25.6
0.2	12.8

Table II shows the results of titrations made at various points of time in 1 ml samples of the same supernatant.

Table II

Minutes after adding iodine	Penicillinase units/ml
0	64.3
15	64.6
30	64.9
45	65.3

LITERATURE

1. PERRET, C. J.: Nature (Lond.) **174,** 1012 (1954).
2. CITRI, N.: Biochim. biophys. Acta **27,** 277 (1958).
3. The Chemistry of Penicillin, p. 1026 Princeton Univ. Press, Princeton, 1949.

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THE FUNCTION OF CALCIUM IN THE REGULATION OF POTASSIUM ACCUMULATION IN GUINEA PIG BRAIN CORTEX SLICES

By

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The presence of Ca ions is required for the accumulation at maximum rate of potassium in guinea pig brain cortex slices. In the absence of Ca ions K-accumulation ceases completely. In the concentrations used the Ca ions have no influence whatever on the carbohydrate metabolism and energy production of the tissue.

Calcium is believed to be an integrant component of the carrier mechanism regulating cation transport.

It has been generally accepted that the adenosine triphosphate (ATP) produced in the course of carbohydrate metabolism is responsible for the maintenance of the cation concentration gradient between the cells and the extracellular fluid. It remains, however, unclear how the energy generated by the breakdown of ATP is utilized for maintaining the ion movement and, also, the question of the mechanism of K-Na transport of cells and tissues. Experiments in which the ion movements are influenced directly, thus not through the high-energy phosphate synthesis, may bring us nearer to a solution of these problems.

SCHATZMANN [21] was the first to investigate the effect of the cardiac glycosides on the ion permeability of erythrocytes and found these substances to inhibit the active cation transport by the cells, without influencing glycolysis. This finding pointed to a direct action on the carrier mechanism. This action of the cardiac glycosides is, however, not restricted to erythrocytes; similar effects have been demonstrated with muscle, skin and brain tissue slices [19. 23, 24, 25].

To gain additional information about the mechanism of K-Na transport, a point was sought at which the carrier mechanism could be influenced directly. This is why we started to study the role of alkaline earth metal ions. In earlier experiments [6, 7, 8] we observed that the Ca ions had a direct influence upon the K-transport of human erythrocytes and the evidence obtained indicated that the Ca ions had an important role to play in the carrier mechanism. In the present paper we shall analyse the function of alkaline earth metal ions (and that of Ca, in the first place) in another tissue, notably in guinea pig brain cortex slices.

We started from the observation of KREBS *et al.* [11, 22] that guinea pig brain cortex slices were able of intensive K-accumulation in the presence of glucose and L-glutamate. The present experiments supplied further evidence as to the mechanism of this active ion accumulation. Some of the results have already been published in a preliminary report [9].

Methods

Preparation of tissue. Guinea pigs were killed by decapitation, the brain was removed without delay and placed on *Whatman* No. 1 filtre paper slightly wetted with physiological NaCl solution. The slices cut from the grey matter by means of a rasor blade were weighed by analytical scales. Samples of about 100 mg wet weight were incubated in 2 ml of a standard medium in Warburg vessels or in small Erlenmeyer flasks, at 37° C, for various lengths of time, mostly for 60 minutes, shaking in pure O_2 or in air. The medium was composed of 0.136 *M* NaCl, 0.006 *M* KCl and 0.03 *M* Tris-HCl buffer (pH 7.4). As substrates, 0.02 *M* glucose and 0.01 *M* L-glutamate were used. After incubation the slices were weighed again and the analytical data were corrected for the tissue swelling.

Analytical methods. The slices were homogenized in trichloroacetic acid, the precipitate was centrifuged and in the suitably diluted supernatant the K contents were determined by flame photometry.

O_2 consumption was determined by the Warburg method. The ATP content was estimated on the basis of the inorganic phosphate (P_0) and acid-soluble phosphate (P_7) determination according to FISKE and SUBBAROW [4]. The lactic acid content of tissues was determined by the method of BARKER and SUMMERSON [2].

Results

In the absence of substrates the brain cortex slices lost 50 to 70 per cent of their K contents in 5 minutes, but in the presence of glucose and L-glutamate they regained most of the K lost. Under such conditions the slices could accumulate not more than 70 per cent of the lost K. However, when 10^{-3} *M* $CaCl_2$ was also added to the medium, the total amount of the lost K was accumulated (Fig. 1). The accumulation of K was promoted also by $MgSO_4$, but only at higher concentrations; in the presence of 10^{-3} *M* $MgSO_4$ the increase of accumulation was 10 per cent compared to the effect of $CaCl_2$ in the same concentration.

When the alkaline earth metal ions were totally removed from the system, the slices were not able to accumulate K even in the presence of glucose and L-glutamate. For the removal of alkaline earth metal ions, ethylenediamine tetraacetate (EDTA) or calgon (Na-hexametaphosphate) were used. The accumulation of K ceased completely after treatment with 10^{-3} *M* EDTA or 10^{-3} *M* calgon, but it started again in the presence of 10^{-3} *M* $CaCl_2$ (Fig. 2). $MgSO_4$ had to be used in a concentration as high as $5 \cdot 10^{-3}$ *M* to produce a similar effect.

To prove that EDTA had acted on the K-transport through removing the alkaline earth metals (Ca, in the first place), we prepared the Ca-complex of EDTA and studied its action on the accumulation of K. The EDTA-Ca

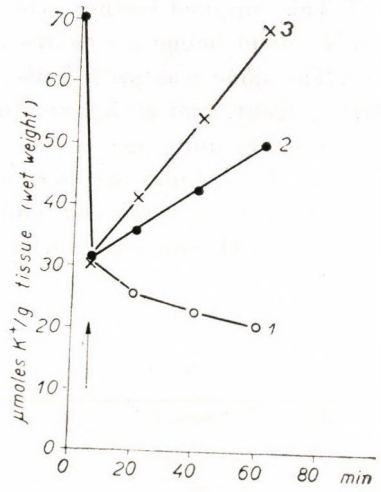

Fig. 1. Changes in the K content of brain cortex slices in the presence of $CaCl_2$

1: Substrate-free control, *2:* glucose and L-glutamate, *3:* glucose, L-glutamate and 10^{-3} *M* $CaCl_2$. The substrates and $CaCl_2$ were added at 5 minutes

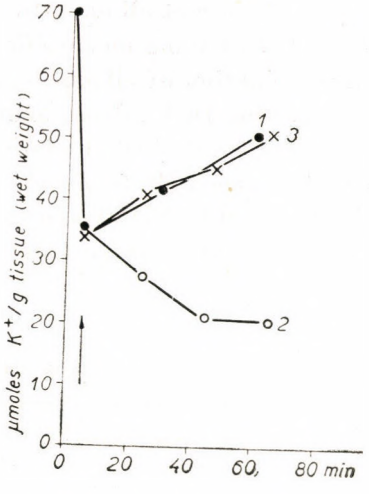

Fig. 2. Changes in the K content of brain cortex slices in the presence of EDTA and $CaCl_2$. *1:* glucose and L-glutamate, *2:* glucose, L-glutamate and 10^{-3} *M* EDTA, *3:* glucose, L-glutamate, 10^{-3} *M* EDTA and 10^{-3} *M* $CaCl_2$

Substrates, EDTA and $CaCl_2$ were added at 5 minutes

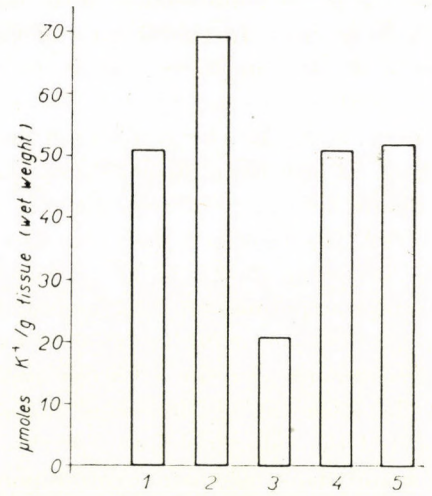

Fig. 3. The effect of $CaCl_2$, EDTA and EDTA-Ca on the K content of brain cortex slices. The columns show the K content of the slices after 60 minutes of incubation

1: glucose and L-glutamate, *2:* glucose, L-glutamate and 10^{-3} *M* $CaCl_2$, *3:* glucose, L-glutamate and 10^{-3}*M* EDTA, *4:* glucose, L-glutamate, 10^{-3} *M* EDTA and 10^{-3} *M* $CaCl_2$, *5:* glucose, L-glutamate and 10^{-3} *M* EDTA-Ca

had no influence at all on K-transport (Fig. 3). This supplied further evidence of the EDTA having no specific action and of its effect being due exclusively to an elimination of alkaline earth metal ions. The same was proved also by demonstrating that calgon, another Ca-chelating agent, had a similar effect.

Next, we studied the influence of the complex-forming agents or $CaCl_2$ upon the carbohydrate metabolism of the tissue. The results are presented in Table I. $CaCl_2$, EDTA and calgon, in the concentrations employed, had no effect on the ATP content, lactic acid production and O_2 consumption of the brain tissue slices.

Table I

Effect of EDTA and $CaCl_2$ on the metabolism of brain cortex slices

Agents added	ΔO_2	lactic acid	ATP
	μ moles/g tissue, after 60 minutes incubation at 37° C		
Glucose, L-glutamate	81.0	52.0	0.96
Glucose, L-glutamate, 10^{-3} *M* EDTA	83.0	48.0	0.95
Glucose, L-glutamate, 10^{-3} *M* EDTA, 10^{-3} *M* $CaCl_2$	84.0	50.0	0.97

Discussion

There is ample evidence in the literature to show the important part of Ca in the regulation of cation transport. In 1952 AEBI [1] reported that the presence of Ca was essential in the maintenance of the normal K concentration in surviving guinea pig liver slices. In the absence of Ca the K-concentration gradient between the cells and medium cannot be maintained. In 1955 GEYER *et al.* [10] showed the same for rat liver slices; in their experiments the presence of Ca was essential for the restoration and maintenance of the K-concentration gradient of rat liver slices. CONN and WOOD [3] demonstrated *in vivo* that calcium deficiency increased the K outflow from the cells in skeletal muscle. REITER [20] described the essencial role of Ca in the maintenance of normal K-Na permeability in heart muscle. FRANKENHAEUSER and HODGKIN [5] presented experimental evidence to show that a deficiency of Ca leads to an increased Na-permeability in nerve tissue. MAIZELS [16, 17] showed for tortoise erythrocytes that in a Ca-free medium an equilibration of K-Na took place between the erythrocytes and serum. More recently, MAIZELS [18] has reported that also in human erythrocytes the presence of Ca is essential for the maintenance of normal cation permeability. In our own experiments with human erythrocytes [6, 7, 8] we, too, showed that in the absence of Ca the erythrocytes showed an abnormal cation permeability. Our results have been confirmed by the experiments of PASSOW *et al.* [12, 13, 14, 15].

In addition to confirming the above data from the literature our present experiments allowed further conclusions. Since Ca has no effect on the energy production of cells, its action must in all probability be localized on the cell membrane or in the carrier mechanism itself. In this respect the action of Ca is comparable to that of the cardiac glycosides, which have no effect on cell metabolism, but appear to act directly on ion transport.

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LITERATURE

1. AEBI, H.: Helv. physiol. Acta **10,** 184 (1952).
2. BARKER, S. B., SUMMERSON, W. H.: J. biol. Chem. **138,** 535 (1941).
3. CONN, H. L., WOOD, J. C.: J. clin. Invest. **36,** 879 (1957).
4. FISKE, C. H., SUBBAROW, Y.: J. biol. Chem. **66,** 375 (1925).
5. FRANKENHAEUSER, B., HODGKIN, A. L.: J. Pyhsiol. (Lond.) **128,** 40 P (1955).
6. GÁRDOS, G.: Biochim. biophys. Acta **30,** 653 (1958).
7. GÁRDOS, G.: Acta physiol. hung. **14,** 1 (1958).
8. GÁRDOS, G.: Acta physiol. hung. **15,** 121 (1959).
9. GÁRDOS, G.: J. Neurochem. **5,** 199 (1960).
10. GEYER, R. P., SHOLTZ, K. J., BOWIE, E. J.: Amer. J. Physiol. **182,** 487 (1955).
11. KREBS, H. A., EGGLESTON, L. V., TERNER, C.: Biochem. J. **48,** 530 (1951).
12. LEPKE, S., PASSOW, H.: Pflügers Arch. ges. Physiol. **271,** 389 (1960).
13. LEPKE, S., PASSOW, H.: Pflügers Arch. ges. Physiol. **271,** 473 (1960).
14. LINDEMANN, B., PASSOW, H.: Pflügers Arch. ges. Physiol. **271,** 488 (1960).
15. LINDEMANN, B., PASSOW, H.: Pflügers Arch. ges. Physiol. **271,** 497 (1960).
16. MAIZELS, M.: J. Physiol. (Lond.) **125,** 263 (1954).
17. MAIZELS, M.: J. Physiol. (Lond.) **132,** 414 (1956).
18. MAIZELS, M.: Nature (Lond.) **184,** 366 (1959).
19. MATCHETT, P. A., JOHNSON, J. A.: Fed. Proc. **13,** 384 (1954).
20. REITER, M.: Arch. exp. Path. Pharmakol. **232,** 298 (1957).
21. SCHATZMANN, H. J.: Helv. physiol. Acta **11,** 346 (1953).
22. TERNER, C., EGGLESTON, L. V., KREBS, H. A.: Biochem. J. **47,** 139 (1950).
23. VICK, R. L., KAHN, J. B.: J. Pharmacol. **121,** 389 (1957).
24. WHITNEY, B. P., WIDDAS, W. F.: J. Physiol. (Lond.) **146,** 35 P (1959).
25. WOLLENBERGER, A., WAHLER, B.: Arch. exp. Path. Pharmakol. **228,** 134 (1956).

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STUDIES ON D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES

XVIII. THE LIPID COMPONENT OF THE ENZYME

(SHORT COMMUNICATION)

By

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In electrophoretic analysis of PGADs* isolated from various species and recrystallized four times it was found that the zone corresponding to protein could be indicated not only with aminoblack or fuchsin, but also with Sudan deep blue, a dye characteristic of lipids. A typical experiment is illustrated in Fig. 1.

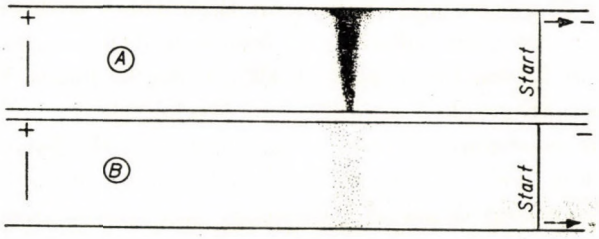

Fig. 1. Paper electrophoretic pattern of PGAD
400 V, 15 mA, 0.1 *M* borate buffer, pH 8.5, 8 to 10 mg of protein
A = stained for protein; *B* = stained for lipid

The component staining with Sudan may be extracted from the protein in the following way. Four times recrystallized PGAD is dissolved in a small volume of water and dialysed against distilled water overnight at 5° C. The protein concentration is adjusted to about 50 mg/ml. Five ml of the solution are placed into a boiling water bath for 10 minutes, the precipitated protein is centrifuged, and washed twice with distilled water, twice with 10 ml acetone: ether (1 : 1), and twice with 10 ml ether, at room temperature. The pooled organic solvents are distilled off in a water bath, the thin film of the residue is weighed and emulsified in 5 ml of a 0.1 *N* glycine buffer, pH 8.5, under continuous stirring with a glass rod.

* The following abbreviations are used: ADH = alcohol dehydrogenase. DPN = diphosphopyridine nucleotide. PCMB = p-chloromercuribenzoate. PGA = D-glyceraldehyde-3-phosphate. PGAD = D-glyceraldehyde-3-phosphate dehydrogenase.

Following extraction the protein was dissolved in 3 ml of 0.1 *N* NaOH. The protein and the extract were separately subjected to electrophoresis in an 0.1 *M* borate buffer, pH 8.5. The protein still migrates towards the cathode, but it does not any more stain with Sudan. The residue of the organic extract does not migrate and stains with Sudan, but does not react with ninhydrin.

Such a lipid component is demonstrable in the PGADs from bovine rabbit, swine [1] and crayfish muscle [2], as well as in PGAD from baker's yeast [3], in amounts of from 0.5 to 1 per cent of protein dry weight.

The lipid component is demonstrable also in the protein recrystallized in the presence of detergent (lauroylsulphonate), and in protein deprived of bound DPN by charcoal treatment or by addition of 15 eq of PCMB. Enzymologic studies showed that after recrystallisation in the presence of lauroylsulphonate the PGAD still contains 2.7 moles DPN per mole of enzyme, thus does not remove the bound coenzyme.

For further analysis the lipid component was dissolved in 0.5 ml of ethyl acetate and subjected to descending electrophoresis in a pyridine-acetic acid buffer pH 5.6, showing a Sudan positive but not migrating zone. It was eluted with ethyl acetate and shaken out three times with equal volumes of water. The organic phase was distilled to dryness and the residue was hydrolysed with 20 per cent hydrochloric acid at 105° C for 16 hours. The hydrolysate was electrochromatographed according to MIKEŠ [5].

As shown by semiquantitative determinations, the hydrolysate contains a large amount of serine, about half as much of glutamic acid and lysine, as well as a small amount of alanine, threonine and leucine. Since before hydrolysis the Sudan positive component does not reactwith ninhydrin, it is assumed that a peptide is bound to the lipid. According to the investigations of TELEGDI and KELETI [6] the lipid component contains no phosphorus, thus it is not a phospholipid, in spite of its high serine content.

Next the effect of the lipid component on the activity of PGAD and ADH was studied, using WARBURG's optical test [4, 7].

The lipid emulsion diluted to a final concentration of 0.002 to 0.2 mg/ml in the assay system gives a perfectly clear solution. As a control a similarly emulsified lipid extracted with ether from the aqueous extract of swine muscle was used. The latter material forms a true emulsion even in the above mentioned range of concentrations. It is supposed that the water solubility of the PGAD lipid is due to the presence of its peptide component.

The lipid component of PGAD inhibits the oxidation of PGA in a measure proportionate to the amounts employed, whereas the control lipid has no inhibitory effect even at much higher concentrations (Table I).

Table I

The effect of PGAD lipid and swine muscle lipid on enzyme activity of PGAD

Substance tested	mg/ml	Inhibition per cent
Lipid component of swine PGAD	0.0014	13
	0.0028	25
	0.0060	64
	0.0120	93
Lipid extracted from swine muscle	0.123	0
	0.246	0
	0.369	0

The reaction mixture contained 1 to 3 μg/ml of enzyme

Table II shows that the PGAD lipid equally inhibits the activity of PGADs, isolated from different species as well as that of ADH [8].

Table II

Inhibitory effect of the lipid components of different kinds of PGAD

Enzyme tested	Lipid fraction (concentration 0.0028 mg/ml) extracted from		
	bovine PGAD	swine PGAD	rabbit PGAD
	Inhibition per cent		
Bovine muscle PGAD	10	10	25
Swine muscle PGAD	42	30	42
Rabbit muscle PGAD	28	35	52
Crayfish muscle PGAD	36	—	—
Baker's yeast PGAD	—	48	—
Baker's yeast ADH	—	15	—

Enzyme concentration 1 to 3 μg/ml. Reaction mixture as described in [4] and [7], respectively.

Accordingly, the inhibitory effect is not specific, neither is it a simple lipid effect, the lipid from swine muscle extract not being inhibitory.

As to the nature of the inhibition, it was found to be not competitive with the substrate, but competitive with the coenzyme. Typical experiments of each type are shown in Fig. 2.

From the fact that both ADH and PGAD require DPN as a coenzyme, as well as from the observation that the inhibition is competitive with DPN, it has been concluded that structural parts near the coenzyme binding areas may play a role in binding the lipid component.

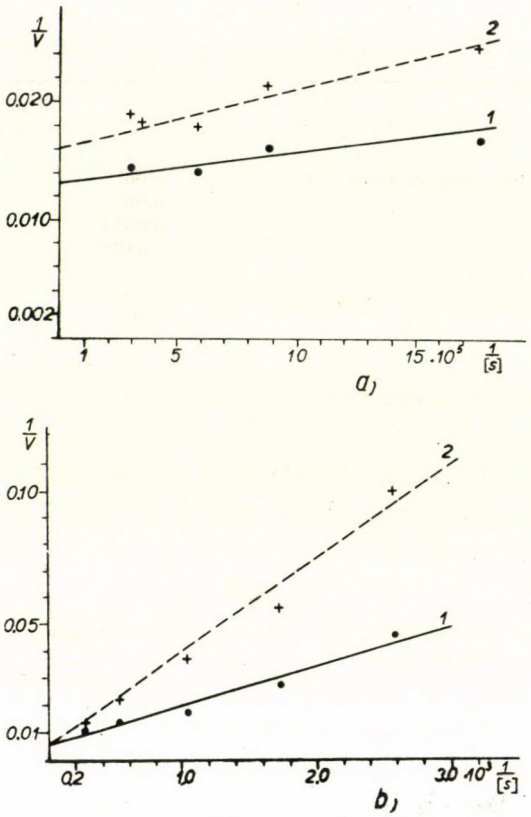

Fig. 2. Lineweaver-Burk plot of the inhibitory action of the lipid component of PGAD a = Competition with substrate; b = Competition with coenzyme; $\frac{1}{V}$ = reciprocal of the reaction rate expressed in $\Delta E_{340m\mu}^{30''}$; $\frac{1}{[S]}$ = reciprocal of substrate and DPN concentration, respectively, in moles. *1:* without inhibitor; *2:* in the presence of 0.0033 mg/ml of lipid. Enzyme concentration in reaction mixture 2 μg/ml

LITERATURE

1. Elődi, P., Szörényi, E.: Acta physiol. hung. **9,** 339 (1956).
2. Szörényi, E., T., Elődi, P. (Соренн, Э, Т., Элоди, П.): Укр. биохим. Ж. **26,** 387 (1954).
3. Keleti, T., Elődi, P., Telegdi, M.: In preparation.
4. Szabolcsi, G., Elődi, P.: Acta physiol. hung. **13,** 207 (1958).
5. Mikeš, O.: Coll. Czech. chem. Commun. **22,** 831 (1957).
6. Telegdi, M., Keleti, T.: In preparation.
7. Keleti, T.: Acta physiol. hung. **13,** 103 (1958).
8. Keleti, T.: Acta physiol. hung. **13,** 239 (1958).

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THE BINDING OF Ca BY ISOLATED MYOFIBRILS

(PRELIMINARY NOTE)

By

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In different muscle model systems (actomyosin gel, myofibrils, glycerinated muscle fibres) the phenomenon analogous to muscle relaxation, *i. e.* the inhibition of ATPase activity, of syneresis, of tension, is abolished by the addition of very small concentrations of Ca. This is true for the inhibition caused by overoptimal substrate concentrations [1], as well as for relaxation caused by not yet exactly identified fractions isolated from muscle, the so-called relaxing factors [2—6]. The similar character of the action of Ca in systems containing relaxing factors and in systems containing the contractile proteins only, allows the assumption that the effect of Ca in the two kinds of system is based on an interaction with one and the same component. The binding constants of ATP with the alkali earth metals involved are well-known (see *e. g.* 7), but data on the binding of Ca to the contractile proteins are scarce and not applicable to the problem envisaged [8, 9], owing to their failure to consider the very small concentrations of metal ions. It seemed therefore interesting to investigate the binding of Ca by isolated myofibrils.

According to our experiments, washed rabbit myofibrils prepared according to Perry [10] contain considerable amounts of Ca. About half of this is easily removed by washing with ethylene diamine tetraacetate (EDTA), in accordance with the results of Hasselbach [11] obtained for the "insoluble muscle substance" the bulk of which are myofibrils. We found the fraction of Ca removable by EDTA to exchange instantaneously with added Ca labelled with ^{45}Ca. This part of the Ca-content, investigation of which has been the aim of the experiments to be reported below, will be termed in the following "dissociable Ca".

We determined the association constant of this dissociable Ca by the following simple procedure. Duplicate samples of the myofibril suspension were incubated with labelled Ca given to the samples at different final concentrations. The protein was separated by centrifugation and the radioactivity remaining in solution was measured. The experiments were carried out at 0° C, pH 7.0 (0.02 *M* borate). The concentration of the myofibrillar protein was 7—10 mg/ml. The specific activity of Ca in the different samples was

calculated by adding the total amount of dissociable Ca of the myofibrils to the amount of Ca present. The constants were computed on the basis of the reciprocal plots [12]. The diagrams and calculations showed clearly that in the binding of Ca two different sites are involved. One gram of myofibrillar protein containd round 10 to 20 micromoles of "strong" binding places, with an association constant of 20 000—30 000, and about 100 micromoles of "weak" ones with a constant of about 100 (Table I). By means of suppression of Ca-

Table I

Association constants and number of binding sites for the binding of Ca by myofibrillar protein

Range of concentrations of free Ca $\mu M/l$	Number of points with different Ca additions	n_1 $\mu g/g$ myofibr. protein	K_1 $1/M$	n_2 $\mu M/g$ myofibr. protein	K_2 $1/M$
60— 80	7	26	18 000	—	—
6—400	9	15	22 000	79	1300
30—400	9	25	28 000	107	1350

binding by the addition of Mg we obtained some indication of the specificity of the "strong" binding sites for Ca as opposed to Mg. We found the affinity for Mg to be very much smaller than for Ca.

The Ca association constant is well-known for several proteins (see *e. g.* [13, 14]) and these constants are all in the range of our "weak" binding. This refers also to myosin, more precisely to the meromyosins [9]. It is therefore possible that the strong binding observed by us is not effected by myosin but by some other component of the myofibril, *e. g.* by actin. Or else, as in the experiments of NANNINGA [9] when chemical methods were used and were consequently not extended to determining the small concentrations of Ca, our strong binding could not be observed.

The identification of the protein component of the myofibril responsible for the strong binding is in progress and will be reported together with a detailed account of these experiments on a later occasion.

As regards the suppressing effect of Ca on the inhibition by overoptimal ATP concentration, the following can be concluded. If using the n and K values for the myofibrillar proteins and the association constants for MgATP or CaATP given by WALAAS [7] we compute the concentrations of the different molecular species, in the case of addition of the reactants in concentrations causing maximum activity, substrate inhibition or abolition of substrate inhibition, we shall find that the greatest part of the bound Ca is removed (by dilution and complexing with ATP) in the first two cases. In the third case, however, the addition of Ca increases the amount of protein bound Ca about 50 times. (In order to elucidate the situation, in Table II we give the results of

Table II

Distribution of the different reactants in a typical experiment on the suppression of substrate inhibiton by addition of Ca
Myofibrillar protein concentration, 1.4 mg/ml. t = 20° C. ATPase activity expressed as μg P split in 5 min. All concentrations expressed in $M/1$

ATPase activity	Maximum activity	Substrate inhibition	Inhibition suppressed by Ca
	70	11	66
[ATP] total	$4 \cdot 10^{-3}$	$8 \cdot 10^{-3}$	$8 \cdot 10^{-3}$
[Ca] added	—	—	$5 \cdot 10^{-4}$
[MgATP]	$3.5 \cdot 10^{-3}$	$4 \cdot 10^{-3}$	$4 \cdot 10^{-3}$
[CaATP]	$1 \cdot 10^{-5}$	$1 \cdot 10^{-5}$	$50 \cdot 10^{-5}$
[Ca] bound to protein	$6 \cdot 10^{-8}$	$2 \cdot 10^{-8}$	$1 \cdot 10^{-6}$(!)
[Ca] free	$1 \cdot 10^{-7}$	$3 \cdot 10^{-8}$	$1 \cdot 8 \cdot 10^{-6}$

calculations in the case of one special experiment.) It is possible that it is this change in the amount of bound Ca which causes the suppression of inhibition, although it involves the somewhat paradoxical assumption that in the absence of free ATP no Ca bound to the myofibril is needed to attain maximal activity while it is necessary in the case of substrate inhibition, *i. e.* in the presence of free ATP. This assumption has in part been supported by our still unpublished experiments in which kinetic analysis has demonstrated the substrate inhibition to be due essentially to a competition of free ATP with MgATP as substrate.

It is still uncertain whether the view suggested here can be applied to the action of Ca in the case when relaxation is caused by relaxing factors. The minimal concentration of free Ca inhibiting the effect of the relaxing substance has been given by PARKER and GERGELY in a recent paper [6] as 10^{-6} $M/1$. As the concentration of free Ca is higher than the concentration of protein-bound Ca, *i. e.* when Ca is added in the order of 10^{-6} $M/1$, it is conceivable that the substrate inhibition is caused by some relaxing substance remaining in the myofibrillar preparations. It is however difficult to reach conclusions before the nature of the relaxing substance has been further elucidated.

LITERATURE

1. PERRY, S. V., GREY, T. C.: Biochem. J. **64,** 184 (1956).
2. MARSH, B. B.: Biochim. biophys. Acta **9,** 247 (1952).
3. BENDALL, J. R.: Proc. roy. Soc. B. **142,** 409 (1954).
4. PORTZEHL, H.: Biochim. biophys. Acta **24,** 474 (1957).
5. EBASHI, S.: Arch. Biochem. Biophys. **76,** 410 (1958).
6. PARKER, C. J., GERGELY, J.: J. biol. Chem. **235,** 3449 (1960).
7. WALAAS, E.: Acta. Chem. Scand. **12,** 528 (1958).
8. GOSH, B. N., MIHÁLYI, E.: Arch. Biochem. Biophys. **70,** 346 (1952).

9. NANNINGA, L. B.: Arch. Biochem. Biophys. **70,** 346 (1957).
10. PERRY, S. V.: Biochem. J. **51,** 495 (1957).
11. HASSELBACH, W.: Biochim. biophys. Acta **25,** 562 (1957).
12. EDSALL, J. T., WYMAN, J.: Biophysical Chemistry. Academic Press, New York, 1958. Vol. 1.
13. CARR, C. W.: Arch. Biochem. Biophys. **46,** 424 (1953).
14. CARR, C. W., WOODS, K. R.: Arch. Biochem. Biophys. **55,** 1 (1955).

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THE PHOTOOXIDATION OF MYOGLOBIN

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Some of the histidine residues participating in the binding of the prosthetic group may have an important role in the formation of the structure and in the functional properties of myoglobin.

According to the investigations of WEIL *et al.* [1] the histidine residues of proteins can be oxidized photocatalytically. On the basis of these data we studied the effect of photooxidation upon the structure and the functional properties of myoglobin.

Ferrimyoglobin, isolated according to the procedure of BOWEN [2] and recrystallized three times, was shaken in a Warburg apparatus in oxygen atmosphere, in the presence of methylene blue. The vessels were irradiated from one side with a light beam of 0.5 W/cm^2 intensity. It was found that while the O_2 uptake of the haem-free protein amounted to 9—11 moles per mole of protein, that of the ferrimyoglobin was only 2 moles. The time course of the oxidation is represented in Fig. 1. It can be seen that under these conditions, in the case of ferrimyoglobin, the oxygen uptake ceased after having reached the value of 2 moles O_2 per mole Mb.

After the photooxidation, the haem was removed from the protein by treatment with acetone-hydrochloric acid — the methylene blue being also removed in this step — and the histidine content of the photooxidized ferrimyoglobin was determined by the method of MACPHERSON [3]. In a parallel experiment the ferrimyoglobin was shaken under the same conditions but in the absence of methylene blue. Here again, the histidine content was determined after removal of the haem group. The oxidized protein was found to contain two histidine residues less than the control protein (Table I).

Table I

Myoglobin	moles histidine/ mole myoglobin
native	9.2
photooxidized	6.9

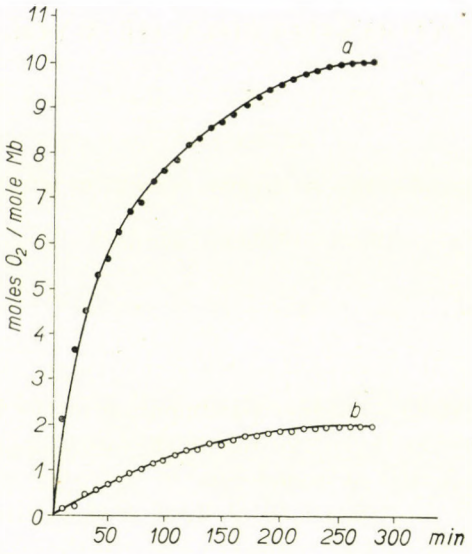

Fig. 1. Time course of oxidation

Reaction mixture: myoglobin, 0.6—2.0 μM; 0.2 ml of phosphate, 0.3 M pH 8.9; 0.5 ml of methylene blue, 0.7 mM. Total volume, 2.3 ml. In the middle vessel 0.2 ml of 20 per cent KOH. Temperature, 37.0° C

a = haem-free protein; b = ferrimyoglobin

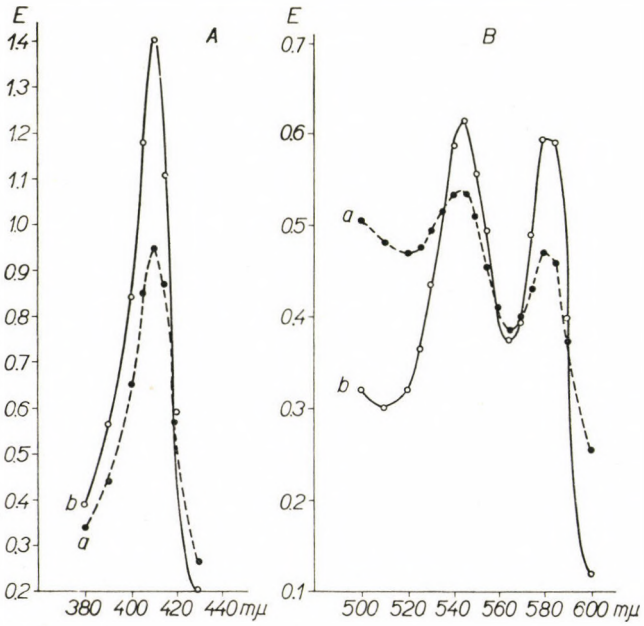

Fig. 2. Effect of photooxidation on the spectrum of myoglobin

A) Spectra of photooxidized and control ferrimyoglobins in the Soret zone

B) Spectra of photooxidized and control myoglobins after reduction and oxygenetion

a = photooxidized myoglobin; b = control myoglobin

Absorption of the photooxidized ferrimyoglobin was substantially decreased in the Soret zone characteristic of the haem-globin bond. The spectrum of the photooxidized protein was measured against a methylene blue solution of the given concentration. The photooxidized and control ferrimyoglobins were first reduced to ferromyoglobin, then shaken with oxygen. In determining the spectrum of the photooxidized protein a methylene blue solution of the concentration given was used as a blank. As seen in Fig. 2*b*, in the presence of oxigen the photooxidized myoglobin did not show the characteristic spectrum of oximyoglobin.

Attempts were made to separate the peptide fraction containing the photooxidized histidine. We intended to trace this modified peptide by comparing the tryptic hydrolysates of native and photooxidized myoglobins. Electrophoretic investigations revealed in the mildly acid fractions of the hydrolysate the appearance of a new peptide absent from the identically-treated control hydrolysates. The isolation of this peptide is in progress.

As the oxidation of the two moles of histidine results in the weakening of the haem—protein bond and in the loss of ability of the protein to bind oxygen, we assume that the new peptide seen in the electrophorogram is identical with the haemopeptide of myoglobin.

After these experiments had been performed we learned about the work of NAKATANI, dealing with the photooxidization of cytochrome *c* [4] and peroxidase [5] concerning the connections between the oxidation of histidine and the functional properties of proteins.

LITERATURE

1. WEIL, L., GORDON, W. G., BUCHERT, A. R.: Arch. Bioch. Biophys. **33,** 90 (1951).
2. BOWEN, W. J.: J. biol. Chem. **176,** 747 (1949).
3. MACPHERSON, H. T.: Biochem. J. **40,** 470 (1946).
4. NAKATANI, M.: J. Biochem. (Tokyo) **48,** 633 (1960).
5. NAKATANI, M.: J. Biochem. (Tokyo) **48,** 640 (1960).

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Physiologia

EFFECT OF THE REMOVAL OF ENDOCRINE GLANDS ON AUDIOGENIC EOSINOPHILIA

By

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The effect of intensive sound stimulus on the eosinophil count was investigated in rats after the extirpation of different endocrine glands. Audiogenic eosinophilia did not occur in adrenalectomized rats kept alive by different treatments, in hypophysectomized rats, nor in those thyroidectomized animals which had audiogenic seizures, whereas the eosinophil count rose in those thyroidectomized rats in which the sound stimulus did not provoke seizures. Prolonged cortisone treatment prevented eosinophilia also in normal animals. It has been concluded that audiogenic eosinophilia cannot develop unless the hormonal supply is well balanced.

It has been observed that strong sound stimuli of short duration increased the number of circulating eosinophils in rats. The intensity of the sound alone sufficed to release in 15 to 30 per cent of the animals an audiogenic seizure characteristic with rodents. The seizure was accompanied by intensive motor activity, but the eosinophil count increased whether or not the attack occurred [2, 3].

While glucocorticoids are known as the principal agents inducing a reduction of the eosinophil count, no specific agents causing acute eosinophilia have so far been discovered.

Several authors observed eosinophilia in adrenalectomized patients and animals [*e. g.* 6, 7, 22, 12, 13, 17, 18], whereas others failed to do so [*e. g.* 10, 21, 26]. After thyroidectomy the eosinophil count was found normal [16] and the eosinopenic effect of ACTH within the usual limits [4, 16], although adrenalin induced a slight but still significant decrease in the eosinophil count [4]. The sequelae of hypophysectomy may become notable not only by a disturbance of interglandular relations but also by the cessation of the usual trophic effect which the pituitary body exerts upon endocrine glands. ACTH, produced by the anterior pituitary, governs the eosinophil level through the adrenal cortex, and it will lastingly rise of the hypophysis is extirpated [17, 18. 23, 24, 25]. There are, however, also authors who did not observed increased eosinophil count in such cases [19].

The purpose of the experiments to be reported was to study the hormonal factors involved in the development of audiogenic eosinophilia. We investigated the effect of sound stimulation on the eosinophil level of adrenalectomized, thyroidectomized and hypophysectomized animals.

Methods

Inbred male rats were used in the experiments. Previous treatment at intervals of 4 to 5 days during 2 to 3 weeks served to accustom the animals to the non-specific conditions of the experiment (handling, cutting of tail, *etc.)*. The adrenals were extirpated in 90, the thyroid was extirpated in 36 and the pituitary in 21 rats. As a substitution therapy, cortisone in daily doses of 0.15 to 0.25 mg per 100 g body weight was administered to part of the adrenalectomized animals, while the rest of these were given to drink a 1 per cent solution of NaCl *ad libitum*. The last cortisone injection was administered 40 to 50 hours before the experiment. A separate group had not been subjected to adrenalectomy but was nevertheless treated with cortisone quite like the adrenalectomized animals; the hormonal supply was cut off immediately before the experiment also in this group.

The thyroidectomized animals received no substitution.

The hypophysectomized rats were given 5 mg of cortisone immediately after the operation; subsequently, no drug was administered for 15 to 20 days, when 1—2 I. U. of ACTH were applied immediately after each experiment. We kept these animals on a diet enriched with cane sugar and edible oil. We started the experiment 6 to 9 days after removal of the adrenals and the thyroid, and 3 to 4 days after hypophysectomy.

Non-adrenalectomized rats received cortisone at least for 12 days before the first experiment.

The experiment was performed as follows. Blood was drawn from the tail of the animals, and each of them was then placed in a separate small glass-walled box into which an electric bell had been mounted. The bell was then rung for 2 minutes. The animals which responded with audiogenic seizure will be termed positive, those which remained quiet are to be called negative in the following. A second blood sample was taken 4 hours after the audiogenic stimulation. The same animals were employed several times at weekly intervals. The controls were not exposed to sound stimuli.

Blood samples were stained with a mixture of methylene blue and eosin [1], and an eosinophil count was made.

Statistical analyses were made by the "t" test on the basis of the differences expressed in per cents of the initial values.

Results

1. *Adrenalectomy*

The initial eosinophil level was 739/mm³ in the adrenalectomized animals treated with cortisone, against 473/mm³ for the untreated intact animals

Table I

Eosinophil count in the various experimental groups

	n	Eosinophil count/mm³
Normal*	206	473 ± 21
Normal + chronic cortisone treatment	230	356 ± 17
Adrenalectomy + 1% NaCl in drinking water	97	1061 ± 77
Adrenalectomy + chronic cortisone treatment	185	739 ± 40
Thyroidectomy	292	502 ± 24
Hypophysectomy	220	719 ± 55

n = number of examinations;
* = inclusive of data determined in earlier experiments.

(Table I). The change caused by ringing the bell was a decrease of 5.2 per cent in the positive and of 2.9 per cent in the negative animals. This means an average decrease of 3.8 per cent in the test animals, as against a decrease of 3.0 per cent in the corresponding controls (Table II). This shows that audiogenic stimulation does not give rise to an increase of the eosinophil count in cortisone-treated adrenalectomized rats.

The initial eosinophil level was 1061/mm^3 in those adrenalectomized animals which had drunk the salt solution before the experiment (Table I). The change caused by the audiogenic stimulation was a decrease of 7.0 per cent in the positive, and an increase of 27.0 per cent in the negative animals, thus an average increase of 20.0 per cent as against 20.1 per cent in the controls (Table II). Adrenalectomy prevented audiogenic eosinophilia also under such conditions.

The initial eosinophil count amounted to 356 cmm in normal animals pretreated with cortisone (Table I). The sound stimulus induced a rise of 13.8 per cent in the positive, and one of 15.9 per cent in the negative animals, thus an average increase of 15.4 per cent, as against one of 32.7 per cent in the corresponding controls. Thus, following chronic treatment with cortisone the sound stimulus produced a not significant decrease in the number of circulating eosinophils (Table II).

2. *Thyroidectomy*

An audiogenic stimulation of 2 minutes decreased the eosinophil count by 4.8 per cent in the positive and increased it by 30.4 per cent in the negative thyroidectomized animals (average, + 20.0 per cent), as against an increase of 14.6 per cent in the controls. The values of the negative group were statistically different and the other two values statistically not different from those of the controls. This would prove that — at least, on the evidence of negative animals — the thyroid plays no significant role in the development of audiogenic eosinophilia.

3. *Hypophysectomy*

The initial eosinophil count following removal of the pituitary was 719/mm^3, much higher than that found in normal animals (473/mm^3) (Table I). Ringing the bell for 2 minutes produced an increase of 40.0 per cent in the positive, and one of 31.2 per cent in the negative animals, *i.e.* an average increase of 32.7 per cent, as against one of 38.8 per cent in the controls. These values were thus not different (Table II).

Table II

Effect of interventions on audiogenic eosinophilia

	Positive [a]		Negative [b]		Positive and negative [c]		Controls [d]		Statistical analysis		
	n	difference %	n	difference %	n	difference %	n	difference %		t	*P*
Normal*	69	+29.2 ± 7.6	101	+25.1 ± 4.7	170	+26.7 ± 4.0	41	− 0.2 ± 0.7	a−b	0.51439	70 > *P* > 60
									a−d	2.84926	1 > *P* > 0.1
									b−d	2.93791	1 > *P* > 0.1
									c−d	3.10341	1 > *P* > 0.1
Normal + chronic cortisone treatment	15	+13.8 ± 10.7	46	+15.9 ± 7.9	61	+15.4 ± 6.3	43	+32.7 ± 8.2	a−b	0.14164	90 > *P* > 80
									a−d	1.23687	30 > *P* > 20
									b−d	1.49443	20 > *P* > 10
									c−d	1.69406	10 > *P* > 5
Adrenalectomy + 1% NaCl in drinking water	9	−7.7 ± 19.5	42	+27.0 ± 8.9	51	+20.8 ± 8.2	44	+20.1 ± 9.6	a−b	1.63305	20 > *P* > 10
									a−d	1.20810	30 > *P* > 20
									b−d	0.52555	70 > *P* > 60
									c−d	0.05567	*P* > 90
Adrenalectomy + chronic cortisone treatment	37	−5.2 ± 8.0	60	−2.9 ± 7.6	97	−3.8 ± 5.5	81	−3.0 ± 4.5	a−b	0.20146	90 > *P* > 80
									a−d	0.25672	80 > *P* > 70
									b−d	0.01207	*P* > 90
									c−d	0.10952	*P* > 90
Thyroidectomy	39	−4.8 ± 8.9	93	+30.4 ± 5.9	132	+20.0 ± 5.1	104	+14.6 ± 5.4	a−b	3.27236	1 > *P* > 0.1
									a−d	1.87848	10 > *P* > 5
									b−d	1.98324	5 > *P* > 2
									c−d	0.72288	50 > *P* > 40
Hypophysectomy	8	+40.6 ± 22.8	41	+31.2 ± 8.7	49	+32.7 ± 8.3	46	+38.8 ± 7.7	a−b	0.35501	80 > *P* > 70
									a−d	0.05826	*P* > 90
									b−d	0.58133	60 > *P* > 50
									c−d	0.53994	60 > *P* > 50

a, b, c, d = Symbols used in statistical analyses − * = Based also on earlier results − n = Number of experiments

Discussion

Part of the adrenalectomized rats were kept alive by cortisone. A daily dose of 0.15 to 0.25 mg/100 g body weight was sufficient not only to ensure survival but also for growth at a diminished rate. Large doses of cortisone give rise to suprarenal atrophy by the inhibition of ACTH secretion *(e. g.* [8, 14, 15, 20]) and, according to certain authors [5, 9, 28], they may even induce morphological changes in the hypothalamic nuclei, although such changes were not observed by other authors [11, 29]. We therefore not only avoided to apply high doses, but with a view to eliminating the eosinopenic effect of the glucocorticoid, administration of even the small doses was discontinued 40 to 50 hours prior to the experiments. We thought of a substitution treatment by desoxycorticosterone to ensure survival without (or only slightly) influencing the eosinophil count. Being, however, aware of reports according to which desoxycorticosterone considerably reduces the excitability of the central nervous system, while cortisone produces the opposite effect [30, 31], we decided in favour of the latter. In doing so, we were chiefly led by the consideration that the excitability of the nervous system is generally reduced in animals with adrenocortical insufficiency (see below). We then tried to determine the eventual disturbing effect of cortisone by other experiments. — It is evident from Table II that audigenic stimulation does not induce an increase in the eosinophil count in adrenalectomized rats treated with cortisone.

Since, however, adrenalectomized animals were treated with cortisone as a substitute, the experimental conditions were not suitable for furnishing reliable information about the role of the adrenals. To investigate this point we performed two kinds of experiments. The first consisted in substituting the cortisone treatment with 1 per cent salt in the drinking water in a group of the adrenalectomized animals; audiogenic eosinophilia failed to occur also under such conditions (Table II).

The second kind of experiment consisted in treating intact animals with cortisone quite like in the case of the adrenalectomized rats. Table II shows that audiogenic stimulation rather reduced the eosinophil level in these animals.

These results justify the conclusion that, in the absence of the adrenals, sound stimuli do not provoke prompt eosinophilia even if the animals are treated with cortisone or are given salt in the drinking water. The negative response of normal animals pretreated with cortisone seems to prove that audiogenic eosinophilia may be prevented not only by the absence of adrenocortical hormones but also by the slight preponderance of glucocorticoids induced by the pretreatment so that a disturbance of the normal hormon equilibrium already suffices to prevent the reaction. We assumed that the decisive factor was really the absence or excess of adrenocortical hormones

i. e. that the development of audiogenic eosinophilia depends on the presence of a definite amount of corticoids.

Eosinophil counts at various intervals, sometimes several months, after thyroidectomy gave an average of 502/mm³ (Table I), a value near to that of normal animals (473/mm³). Accordingly, as regards the chronic regulation of eosinophils, removal of the thyroid gland did not influence our experiments. Although thyroidectomy usually does not interfere with the normal regulation of the eosinophil count, audiogenic stimulation fails to give rise to eosinophilia in thyroidectomized rats (Table II). Explanation of the difference observed between the negative and positive animals requires further investigations.

Removal of the pituitary also prevented the development of audiogenic eosinophilia in our experiments.

It should be noted that the eosinophil count following hypophysectomy (719/mm³) was practically the same as that observed in adrenalectomized animals kept alive by means of cortisone (739/mm³) (Table I). Considering it solely on the evidence of the eosinophil count, this phenomenon seems to indicate that in the hypophysectomized rats the adrenals must have secreted an amount of corticoids which corresponds to 0.15 to 0.25 mg/100 g body weight of cortisone. Audiogenic stimulation did not increase the eosinophil count in either of these two groups.

In our opinion it is improbable that the absence of audiogenic eosinophilia should be due to a definite kind of shift in hormonal equilibrium. Although the ringing of the bell failed to increase the eosinophil count in the cortisone-treated adrenalectomized as also in the hypophysectomized animals, and although the basal eosinophil count was equal in both groups, we do not think that the disturbances of regulation (or rather those of eosinophil regulation) are the same in both groups. It seems more plausible that some kind of disturbance of hormonal equilibrium is capable of preventing the reaction in general. This supposition seems to be corroborated by the fact that, although the basal eosinophil count was identical in the intact and the thyroidectomized animals (473 and 502/mm³ respectively) (Table I), audiogenic stimulation was followed by eosinophilia in the first and not followed by eosinophilia in the second group. This experiment does not, however, reveal whether changes in the release of hormones by the thyroid or any other endocrine gland suffice for causing a disturbance capable of inhibiting the reaction in question or whether it is a change in the corticoids which must be regarded as the principal factor responsible for the inhibition.

Table II shows an increase of the eosinophil count at the usual 4 hours after the first drawing of blood in all control groups. This phenomenon did not occur in the intact animals or those which had been subjected to adrenalectomy and subsequent treatment. Considering that diurnal variations in the

eosinophil count [9*a*] did not reach 20 to 30 per cent within 4 hours and that such variations were not demonstrable in our experiments, we suggest that the observed phenomenon was not a direct result of the normal biological rhythm. The increase in the eosinophil count observed in operated or cortisone-treated animals without acoustic stimulation must have been due to the above-mentioned hormonal disturbance.

It has repeatedly been suggested that susceptibility to audiogenic seizure may be connected with general neural excitability [27]. It is, therefore, quite possible that the excitability of the central nervous system might be correlated also with the number of circulating eosinophils. The significance of that effect may have important bearings on the problems discussed.

LITERATURE

1. Bach, I., Szmuk, I., Gyulai, E., Virányi, A.: Orv. Hetil. (Budapest) **92,** 1117 (1951).
2. Biró, J., Szokolai, V., Kovách, A.: Orv. Hetil. (Budapest) **100,** 1042 (1959).
3. Biró, J., Szokolai, V., Kovách, A. G. B.: Acta endocr. (Kbh.) **31,** 542 (1959).
4. Carter, F.: Proc. Soc. exp. Biol. (N. Y.) **86,** 660 (1954).
5. Castor, C. W., Baker, B. L., Ingle, D. J., Li, C. H.: Proc. Soc. exp. Biol. (N. Y.) **76,** 353 (1951).
6. Dury, A.: Amer. J. Physiol. **160,** 75 (1950).
7. Dury, A.: Amer. J. Physiol. **163,** 96 (1950).
8. Forgács, P., Hajdú, L.: Acta med. hung. **5,** 327 (1954).
9. Gädeke, R., Betke, K.: Z. ges. exp. Med. **121,** 181 (1953).

9*a* Halberg, F.: Z. Vitamin-, Hormon- u. Fermentforsch. **10,** 225 (1959).

10. Halberg, F., Visscher, M. B., Flink, E. B., Berge, K., Bock, F. J.: Lancet **71,** 312 (1951).
11. Halmi, N. S.: cit. Porter, R. W. — Recent. Progr. Hormone Res. **10,** 1 (1954).
12. Heni, F., Mast, H.: Z. ges. exp. Med. **117,** 282 (1951).
13. Henry, W. L. jr., Oliner, L., Ramey, E. R.: Amer. J. Physiol. **174,** 455 (1953).
14. Hohlweg, W., Laschet, U.: Acta endocr. (Kbh.) **32,** 437 (1959).
15. Ingle, D. J.: Amer. J. Physiol. **124,** 369 (1938).
16. Jakobson, T.: Acta endocr. (Kbh.) **27,** 432 (1958).
17. Jakobson, T., Hortling, H.: Acta endocr. (Kbh.) **15,** 265 (1954).
18. Jakobson, T., Hortling, H.: Acta endocr. (Kbh.) **15,** 368 (1954).
19. Kovács, K., Horváth, I., Dávid, M.: Kísérl. Orvostud. (Budapest) **11,** 473 (1959).
20. Lewis, R. A., Rosemberg, E., Wilkins, L.: Endocrinology **47,** 414 (1950).
21. Mathieu de Fossey, B., Deltour, G. A.: Ann. Endocr. (Paris) **11,** 341 (1950).
22. Rosemberg, E., Lewis, R. A.: J. appl. Physiol. **3,** 164 (1950).
23. Schweizer, M.: Endocrinology **53,** 293 (1953).
24. Schweizer, M.: Endocrinology **56,** 693 (1955).
25. Simms, E., Pfeiffenberger, M., Heinbecker, P.: Endocrinology **49,** 45 (1951).
26. Simpson, L., Dennison, M., Korenchevski, V.: J. Path. Bact. **39,** 569, (1934).
27. Snee, T. J., Terrance, C. F., Crowley, M. E.: J. Psychol. **13,** 227 (1942).
28. Soulairac, A., Desclaux, P., Soulairac, M. L., Teysseyre, J.: J. Physiol. (Paris) **45,** 527 (1953).
29. Spirtos, B. N.: Proc. Soc. exp. Biol. (N. Y.) **84,** 673 (1953).
30. Woodbury, D. M.: Recent Progr. Hormone Res. **10,** 65 (1954).
31. Woodbury, D. M.: Pharmacol. Rev. **10,** 275 (1958).

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HORMONAL "FEED-BACK" REGULATION OF PITUITARY-ADRENOCORTICAL ACTIVITY

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Experiments on rats and cats have revealed that intracerebral administration of 10 and 50 μg cortisone acetate depressed pituitary-adrenocortical activity, when the drug was introduced into the caudal hypothalamus or mesencephalic reticular formation. When injected into other neural structures, cortisone was ineffective.

After injection into the hypothalamus, cortisone decreased ACTH secretion in direct proportion to the dose used, meanwhile from the mesencephalic reticular formation, a diminution only to the normal, resting level was elicited. The changes in the pituitary-adrenocortical activity were examined by establishing the corticoid level of adrenal venous blood. In addition, it has been observed in further experiments that aldosterone secretion was inhibited by hypertonic saline injected into the reticular formation, but not by cortisone or hypertonic dextrose.

The conclusion has been drawn that an increase in the corticoid content of the peripheral blood is capable of inhibiting the ACTH secretion of the anterior pituitary. This inhibitory effect is mediated through the central nervous system, the brain stem in particular.

The hypothesis that the corticosteroid content of the peripheral blood plays a regularly role in the activity of the pituitary-adrenocortical system has not yet been confirmed. INGLE, HIGGINS and KENDALL [8, 9] were the first to observe that administration of adrenocortical extracts led to atrophy of the gland. Some ten years later, SAYERS [12, 13] proved with functional methods that corticoid administration counteracts the stress-induced increase in the secretion of pituitary adrenocorticotrophic hormone. These data have led to the conclusion that a diminution of the blood corticoid concentration stimulates ACTH secretion, while an elevated blood corticoid level inhibits it. Though this feed-back principle doubtlessly plays a decisive part under certain pathological conditions or after removal of the adrenals, its role in physiological events is far from being clear. The well-known fact that the stress-induced increase of ACTH secretion starts within a few seconds excludes the possibility of a feed-back mechanism of trophic hormone secretion (LOVE [10]; GRAY and MUNSON [7]).

The present paper reports on experiments in which the effect on the pituitary-adrenocortical activity of intracerebrally administered corticoid and saline has been investigated.

Methods

The experiments were performed on 86 albino rats of the same breed and of both sexes, ranging in weight from 150 g to 240 g. In addition, 76 cats of both sexes, weighing 1.8 to 2.5 kg each, were employed. Intracerebral corticoid and saline administrations were made by agar-agar implantation in the rat, while in the cats by means of a chronically inserted microcannula.

In the case of the agar-agar implantation, a glass capillary of 0.3 mm inner diameter was used, which had been filled with agar-agar solution of a temperature of 55° to 56° C. The implantate contained 10 resp. 50 μg cortisone acetate in a volume not exceeding 0.005 ml. After solidification of the agar-agar, the implantate was pushed out of the tube by means of a metal wire 0.2 mm in diameter. The implantate was inserted by means of a stereotaxic apparatus adapted to rats.

In the cat, a stainless hypodermic cannula 0.5 mm in diameter, holding a volume of less than 0.01 ml was introduced into the brain and fixed to the skull by means of a plexiglass holder. For introducing solutions an apparatus allowing injection of as little as 0.001 ml was used. The volume of injected corticoid or saline solution was between 0.01 an 0.05 ml, usually 0.03 ml. The resorption half time of 50 μg cortisone acetate in agar-agar implantate was found to range from 16 to 18 hours. In the case of injection with a syringe, the penetration of the fluid into the brain was controlled by injection of India ink. Gross control examinations showed that 0.03 ml fluid had diffused over an area 2 to 3 mm in diameter.

After experimentation the brain of the animals was removed, fixed in formol and embedded in paraffin. The sections were stained with cresyl violet and the localization of implantate and cannula was determined.

The functional activity of the pituitary-adrenocortical system was studied by analysing the blood of the adrenal vein. Blood was collected under pentobarbital anaesthesia from heparinized rats for 60 minutes, and from the cats for 120 minutes. Extraction of the corticoids and their separation by paper chromatography were performed according to the methods described in previous papers (ENDRŐCZI, BATA and MARTIN [3]; ENDRŐCZI and LISSÁK [4]; ENDRŐCZI and YANG [5]). Quantitative determinations were made by the microtetrazolium reduction or the alkaline fluorescence tests or in a Beckman DU spectrophotometer at 240 mμ. For aldosterone determination, the blood extracts were chromatographed first in formamide-benzene, then in benzene-methanol-water (14 : 6 : 5) and the hormone was assayed by microtetrazolium reduction and spectrophotometry, using ethanol as the solvent.

Results

Since substances injected into the brain or administered by agar-agar implantation are not localized to one single point, the effects had to be considered as exerted over more extensive areas. Another problem in evaluation was that of the time factor. In the rat, the resorption of cortisone acetate from agar-agar takes place with a half time of 16 to 18 hours. As a consequence, the animals had to be killed 24 hours after implantation. This short postoperative period did not permit to avoid the influence on the pituitary-adrenocortical activity of the operation itself. For this reason, animals implanted with agar-agar not containing the active principle served as controls. As additional proof of the specificity of the effects observed, agar-agar containing cortisone was implanted directly under the cerebral cortex.

The results of the rat experiments are shown in Fig. 1. The diencephalon and the mesencephalon were divided into 4 areas including the (i) preoptic region; (ii) paraventricular and ventromedial nuclei, as well as tuber cinereum; (iii) reticular formation of the mesencephalon, extending ventrally to the level of the posterior hypothalamus; (iv) thalamus. This subdivision shows that the

evaluation of our findings in these experiments was not based on localization in individual nuclei or groups of nuclei, but rather on that in more complex anatomical units.

Fig. 1 demonstrates that the corticosterone content in the blood of the adrenal vein was markedly elevated in the control animals killed 18 to 24 hours after the operation, as compared to normal rats not subjected to operation The above-mentioned operated control rats were implanted with agar-agar into the

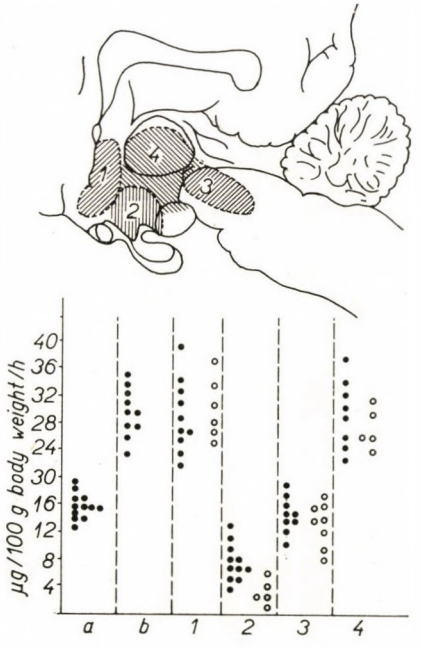

Fig. 1. Effect of intracerebrally implanted cortisone acetate on the corticosteroid content of the adrenal venous blood

a = normal, non-operated animals,
b = agar-agar implantate without cortisone acetate, resp. with 50 µg cortisone
Effect of 10 and 50 µg cortisone acetate implanted into *1* anterior hypothalamus (10 µg = ○ 50 µg = •), *2* caudal hypothalamus, *3* reticular formation, *4* thalamus

diencephalon and mesencephalon. In other control rats agar-agar containing 50µg cortisone acetate was implanted under the cerebral cortex. Throughout the rat experiments cortisone acetate was used at two dose levels, 10 µg and 50 µg. The data in Fig. 1 show that implantation into the ventral hypothalamus failed to decrease ACTH secretion. The values in these animals lay within the ranges observed in control, sham-operated rats. The same was true for implantation in the thalamus, even with 50 µg of cortisone. On the other hand, implantation of cortisone in the ventromedial nucleus, in the paraventricular nucleus, in the tuber cinereum and in the dorsomedial nucleus resulted in a

marked decrease of the corticosteroid content of the adrenal venous blood. This diminution was moderate after implantation of 10 μg cortisone acetate, and very marked after 50 μg. At the latter dose level, the corticoid content sometimes fell under the limit of determination. Similar, though slighter inhibition of adrenocortical activity occurred in animals with implantate in the mesencephalon. The effect of 10 or 50 μg cortisone was not as striking as with implantates in the caudal hypothalamus. A comparison of the effect of mesen-

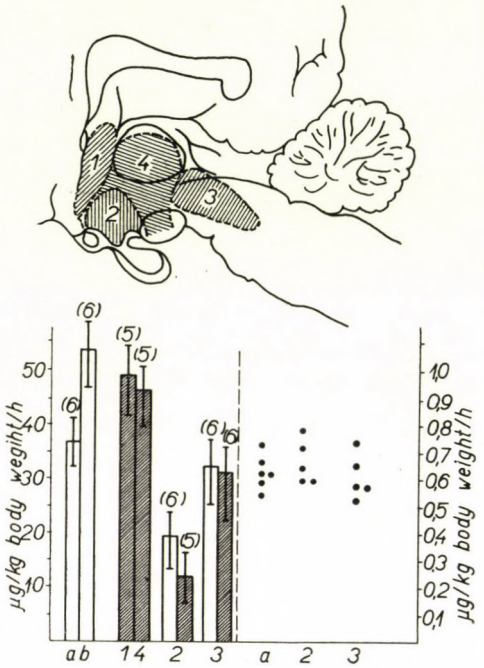

Fig. 2. Effect of cortisone acetate on the corticosteroid and aldosterone content of the adrenal venous blood in the cat. Corticosteroid values are shown by columns, and aldosterone secretion by the dotted figure on the right

Left: *a)* normal animals; *b)* cannulated controls treated with physiological saline. Columns *1* to *4* show the effect of 10 resp. 50 μg cortisone acetate administered for 7 days. White column = 10 μg; shaded column = 50 μg. Lines on left columns show extreme values

Right: aldosterone secretion. *1* = cannulated control animals treated with saline; *2* = 50 μg cortisone acetate injected into the caudal hypothalamus; *3* = 50 μg cortisone acetate injected into the mesencephalic reticular formation. In parenthesis: number of animals in individual groups

cephalic with other implantates revealed that cortisone acetate decreased the operation-induced ACTH mobilization to the normal level only; a diminution under the resting secretion was not produced by 50 μg. This observation, together with the findings on cats (see below) indicates that the depression of ACTH secretion from the mesencephalon and from the caudal hypothalamus are elicited by different mechanisms.

In the cat experiments, 10 or 50 μg cortisone acetate in physiological saline was injected daily from the 2nd or 3rd postoperative day for a period of 7 to 8 days. 2 hours after the last treatment the brain of the cats was worked up as described under "Methods".

Fig. 2 shows that, irrespective of the localisation of the electrodes, the insertion of the cannula, its presence in the cerebral tissue and the daily administration of physiological saline already resulted in increased corticoid secretion. Accordingly, all experimental values had to be compared with those found in cats cannulated but not treated. It was observed that 50 μg of cortisone acetate administered into the thalamus or ventral hypothalamus was not capable of producing depression of adrenocortical secretion even when given for 7 days. On the other hand, injection of only 10 μg of cortisone into the tuber cinereum markedly decreased ACTH secretion. A 50 μg dose of cortisone caused a further diminution. Injection of cortisone into the mesencephalic region resulted in a behaviour similar to that observed in rats. In these cats, the cannula lay mostly in the ventral reticular formation: in some cases it reached the dorsal margin of the posterior hypothalamus. In these animals daily administration of 10 μg cortisone acetate sufficed to decrease the corticosteroid content of adrenal venous blood to or even under the level found in normal, non-operated cats. A 50 μg dose of cortisone produced, however, no further decrease. Fig. 2 reveals, further, that in the secretion of aldosterone no change was brought about from those areas which in response to 50 μg cortisone acetate produced marked diminution of the corticosteroid secretion. It is to be noted that the values in Fig. 1 and Fig. 2 show the summarized data of steroids separated by paper chromatography. Thus, in the rat, they correspond to the total amount of corticosterone and of another Δ^4-3-ketocorticoid so far unknown in structure, while in the cat, to the sum of hydrocortisone and corticosterone. In view of the small amounts present, the quantitative changes in the 11-dehydrocorticosterone and Δ^4-androsterone-3,17-dione content were generally neglected in these experiments.

Next, the influence of hypertonic sodium chloride on the hormone secretion of the adrenal cortex was investigated, with special reference to the aldosterone output. For this purpose 0.03 and 0.05 ml 5 per cent saline was injected into various parts of the brain, twice or, in some cases, three times daily for a period of 6 to 7 days. No change occurred in aldosterone secretion unless the hypertonic saline was injected into the mesencephalic reticular formation. Even 0.05 ml of hypertonic saline, a volume impregnating a region of 3 to 4 mm in diameter, was ineffective when injected into the diencephalon. Fig. 3 shows, further, that 12.5 per cent glucose injected into the mesencephalic reticular formation was similarly unable to alter the aldosterone secretion. This finding indicates that the effect of hypertonic saline is due only partly to changes in the osmotic state.

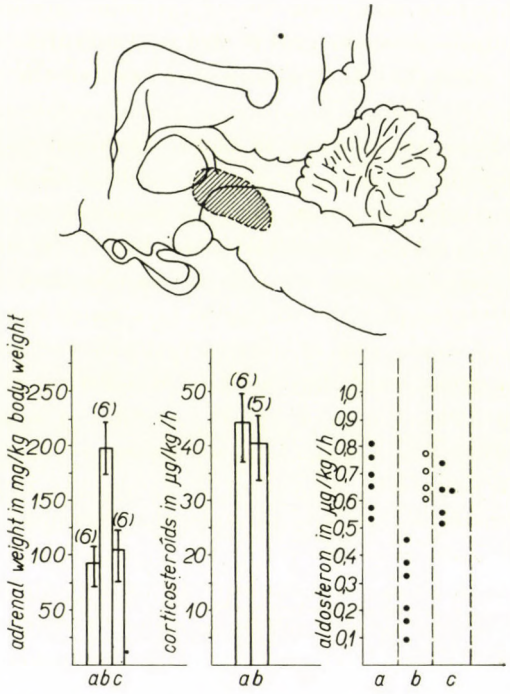

Fig. 3. Effect of hypertonic NaCl on aldosterone and glucocorticoid secretion as well as on adrenal weight

Left: adrenal weight. *a* = normal animals treated with physiological saline; *b* = treatment with 5 per cent NaCl; *c* = treatment with hypertonic dextrose.
Middle: glucocorticoid secretion; *a* and *b* as before.
Right: aldosterone secretion. *a* = cats treated with physiological saline; *b* = hypertonic saline; ○ = glucose; • = hypertonic saline. In addition, the effect of 0.05 ml hypertonic NaCl administered in various parts of the diencephalon is shown. In parentheses: number of animals

In the animals treated with injection of hypertonic saline into the mesencephalon the adrenal weight was increased by nearly 100 per cent, a remarkable elevation during an experimental period of one week. On the other hand, adrenal weight did not change if dextrose had been injected into the mesencephalon, or hypertonic saline into the diencephalon or thalamus. The marked increase of adrenal weight was not associated with changes in the glucocorticoid secretion, which was normal throughout the experiments.

Discussion

The effects of intracerebrally administered cortisone acetate in both the cat and the rat unequivocally showed that the activity of the pituitary-adrenocortical system can be decreased or even completely inhibited from certain parts of the central nervous system, namely the caudal hypothalamus and the

mesencephalic reticular formation. The reactions elicited from both nervous structures exhibited, however, marked differences. Cortisone injected into the caudal hypothalamus inhibited ACTH secretion in direct propsortion to the amount of the drug introduced, while when injected into the mesencephalon it was not capable of decreasing the corticosteroid content of the adrenal venous blood. The present investigations have failed to offer an explanation as to the mechanism of action of cortisone acetate introduced into the hypothalamus. This steroid probably decreased the secretion of ACTH directly by acting on the anterior pituitary. The possibility of such an effect is given by the anatomical localisation. On the other hand, a direct action on the nervous structures has to be supposed in the case of the mesencephalic effect. Cortisone given into this area normalized only the operation-induced activation of the pituitary adrenocortical system, a fact indicating that this corticoid influences adrenocortical activity by interruption of one of the central nervous links of the stress mechanism. In our experiments low doses (10 μg) already sufficed to produce this inhibitory effect, and no further suppression occurred on raising the dose. This fact corroborates our above-mentioned assumption about the cortisone effect. When, however, cortisone was administered in the caudal hypothalamus, the reactivity of the adrenal cortex was depressed in direct proportion to the dose, a fact pointing to a direct inhibition of ACTH secretion. It is not clear whether this effect takes place immediately through the anterior hypothalamus, or *via* the neuhormonal chain of the hypothalamus-pituitary system.

The central role of the reticular formation in the activation of the pituitary-adrenocortical system has been pointed out in a previous paper, in which we have demonstrated that, *via* the thalamus and the posterior hypothalamus, this structure represents a direct neural activation of ACTH secretion. The last link is the neurohormonal relay between hypothalamus and anterior pituitary, the morphological substrate of which is the medial eminenta. The present investigations have shown that the reticular formation in addition to representing the central transmission of neural activation, is sensitive to hormonal factors involved into regulation. This structure is known to be electrophysiologically activated by adrenaline, CO_2 or other stressor effects, a fact indicative of the marked hormonal sensitivity of this part of the central nervous system.

The investigations of ANDERSON *et al.* [1] have pointed to the decisive part of the mesencephalon and hypothalamus in the regulation of aldosterone secretion. The experiments of these authors clearly demonstrated that the mesencephalon maintains aldosterone secretion primarily by hormonal stimuli, transection at the boundary of the mesencephalon and medulla oblongata failed to alter aldosterone secretion, while complete decerebration brought about its marked decrease. The mechanism of this regulation has not been completely elucidated, even though the investigations of RAUSCHKOLB and FARRÉLL [11]

as well as DÁVID, WEISZ and KOVÁCS [2] have pointed to the possibility that a substance (glomerulotrophine) released from the mesencephalon or the brain stem enters the blood and is of decisive importance in the maintenance of aldosterone secretion by the glomerular zone. This assumption is supported by the fact that ACTH does not participate in the regulation of aldosterone secretion. In our present studies, injection of hypertonic saline into the mesencephalic reticular formation resulted in a marked decrease of aldosterone secretion. No such effect occurred after injecting hypertonic dextrose, or on administration of hypertonic saline into other neural structures. Inhibition of aldosterone secretion was accompained by a marked increase in adrenal weight. The mechanism and significance of this effect are not clear. The observation that the increase in adrenal weight did not go parallel with the rate of glucocorticoid secretion, once more calls attention to the fact that tissular hypertrophy and synthesis of 17,21-hydroxycorticoids are regulated by two independent mechanisms, which under certain experimental conditions show an opposite behaviour. Hypertonic saline injected into the mesencephalon decreased aldosterone secretion. This observation suggests the possibility that this regulation might have a role also under physiological conditions. Further studies are, however, needed to decide the specificity of this phenomenon for sodium chloride and for the osmotic state. It should be remembered that under identical conditions injection of 2 per cent potassium chloride had no effect whatever on aldosterone secretion.

In the evaluation of the experimental findings we are faced with the question of the role of the feed-back effect of the corticoids in the regulation of pituitary-adrenocortical activity. In stress of short duration such a phenomenon is certainly not involved. The corticoids liberated after a stress-increased ACTH secretion are probably capable of delaying resp. decreasing the ACTH outflow from the pituitary gland. This can take place even during the time of the intervention, either by acting directly on the anterior hypothalamus, or indirectly, *via* the neural structures of the mesencephalon. The decreasing tendency of adrenocortical reaction after repeated stresses, *i. e.* the so-called resistance, is probably brought about by this same mechanism. The final decision of the question needs, however, further studies.

LITERATURE

1. ANDERSON, E., BATES, R. W., HAWTHORN, E., HAYMAKER, W., KNOWLTON, K., RIOCH, MCK., SPENCE, W. T., WILSON, H.: Recent Progr. Hormone Res. **13,** 21 (1957).
2. DÁVID, M., WEISZ, P., KOVÁCS, K.: Annual Meeting of the Hungarian Physiological Society, Debrecen, 1960.
3. ENDRŐCZI, E., BATA, G., MARTIN, J.: Endokrinologie **35,** 280 (1958).
4. ENDRŐCZI, E., LISSÁK, K.: Acta physiol. hung. **15,** 25 (1959).

5. ENDRŐCZI, E., YANG, T. L.: Acta physiol. hung. (In press.)
6. FARRELL, G. L., RAUSCHKOLB, E. W., FLEMING, R. B., YATSU, F. M.: Report of the 30th Meeting of the Endocrine Society, New York, 1957.
7. GRAY, W. D., MUNSON, P. L.: Endocrinology **48,** 471 (1951).
8. INGLE, D. J., HIGGINS, G. M.: Endocrinology **22,** 458 (1938).
9. INGLE, D. J., KENDALL, E. C.: Amer. J. Physiol. **122,** 585 (1938).
10. LOVE V. D.: Proc. Soc. exp. Biol. (N. Y.) **75,** 639 (1950).
11. RAUSCHKOLB, E. W., FARRÉLL, G. L.: Endocrinology **59,** 526 (1956).
12. SAYERS, G., SAYERS, M. A.: Endocrinology **40,** 265 (1947).
13. SAYERS, G., SAYERS, M. A.: Recent Progr. Hormone Res. **2,** 81 (1948).

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CONTRIBUTIONS TO THE HYPOTHALAMIC CONTROL OF PITUITARY, OVARIAN AND ADRENAL CORTICAL FUNCTION

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In rat experiments it has been observed that electrocoagulation of the anterior hypothalamic area, destroying the area between the paraventricular nucleus, optic chiasma and infundibulum and extending into the praeoptic region significantly increased uterine weight and induced constant oestrus. Pituitary-adrenocortical activity was also increased, as shown by the increased corticoid level in adrenal venous blood. The gonadal changes in response to the lesion were more marked in the adrenalectomized animals than in the controls. It has also been observed that the adrenocortical hypersecretion in response to hypothalamic lesion does not take place in ovariectomized animals.

It has been concluded that the adrenocortical reaction to the hypothalamic lesion is due to a primarily increased ovarian activity, and that adrenocortical secretion very probably lessens the gonadal changes appearing after hypothalamic lesion.

A number of reports has been published indicating the direct role of the hypothalamus in the control of gonadotrophic hormone production by the anterior pituitary, although the problems of structural localisation and of the underlying mechanism still remain unclear. Destruction of the area between the paraventricular nucleus, anterior eminentia mediana and the optic chiasma produces constant oestrus or premature puberty (DEY *et al.* [2, 3, 4]; FLERKÓ [12, 13]; DONOVAN and WERFF TEN BOSCH [5, 6, 7]; GREER [15, 16]). On the other hand, a lesion located to the more rostral area produces a state of constant anovulation (GREER [17]) while stimulation of the anterior hypothalamus is known to increase gonadotrophin secretion, elicitable also from extrahypothalamic structures (TOKAZINE and SAWYER [20]; GREEN, CLEMENTE and DEGROOT [14]; CRITCHLOW [1]). In opposition to these data HERBERT and ZUCKERMAN [19] arrived at the conclusion that surgical intervention in the central nervous system without defined structural localisation aspecifically increases gonadotrophin secretion.

In this paper an account is given of the experimental changes observed in the function of the pituitary-ovary and of the adrenal cortex following hypothalamic lesion.

Methods

A total of 98 female rats from the same stock, weighing 95 to 135 g each, was used. The hypothalamus was destroyed by means of a Horsley-Clark stereotaxic apparatus adapted to the rat, by bilateral electrocoagulation with 3 mA for 6 sec. The adrenals or the ovaries had been removed 4 to 6 days before electrocoagulation, under ether anaesthesia.

Gonadal function was examined by controlling oestrus cycles in vaginal smears, or by determining the weight of the ovary and uterus post mortem.

Adrenocortical function was assayed by determining the corticosteroid level in the venous blood of the adrenals. Adrenal venous blood from heparin-treated (100 U/100 g of body weight intravenously) animals anaesthesized with phenobarbital (5 mg/100 g of body weight intraperitoneally) was collected over 60 minutes, then the corticosteroids were extracted and separated by paper chromatography, using the methods described previously (ENDRŐCZI, BATA and MARTIN [8]; ENDRŐCZI and LISSÁK [9]). For quantitative assay the microtetrazolium reduction and alkaline fluorescence tests were employed (ENDRŐCZI and YANG [11]).

The animals were weighed at the beginning and at the end of the experimental period. The site of electrocoagulation was controlled post mortem, in sections embedded in paraffin and stained with cresyl violet.

Results

The animals were grouped in the following way.

1. Control group. No surgical intervention.

2. Ovariectomized animals, tested for adrenal cortical function.

3. Adrenalectomized animals, tested for changes in gonadal weight.

4. Animals with lesion to the anterior hypothalamus, tested for adrenal cortical hormone secretion and for gonadal activity.

5. Animals with lesion to the anterior hypothalamus, previously ovariectomized.

6. Animals with lesion to the anterior hypothalamus, previously adrenalectomized. This group was kept alive by administering 50 μg DOCA in oil and physiological saline.

The changes observed in the above groups were as follows.

Group 1. The adrenal venous blood of the controls contained two compounds, corticosterone and Δ^4-3-ketocorticoid, not reducing tetrazolium. The two compounds were present in a ratio of approximately 3 to 1. No individual relationship could be demonstrated between the ovarial and uterine weights per 100 g of body weight and the adrenal cortical hormone secretion (Fig. 1).

Group 2. Fourteen days following ovariectomy there was no significant change in the corticoid content of the adrenal venous blood, though the values were closely similar to those representing the lowest of the control ones (Fig. 2).

Group 3. The adrenalectomized rats did not differ in ovarian and uterine weight from the controls. Likewise, there was no difference or change in the course of the oestral cycle during the 16 days of observation (Fig. 3).

Group 4. The lesion to the anterior hypothalamus was rostrally and ventrally from the paraventricular nucleus, extending to the area between the optic chiasma and infundibulum and in some cases also the ventromedial nucleus. Anteriorly the lesion extended to the area of the preoptic region, to the level of the anterior commissura. Some lesions destroyed the paraventricular nucleus, but no lesion was demonstrable in the area of the infundibulum and median eminentia.

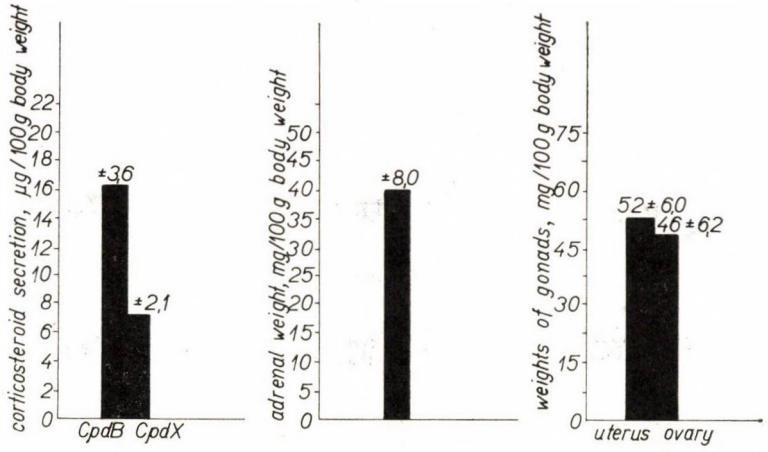

Fig. 1. Adrenal venous corticosteroid levels, adrenal weight, uterine and ovarian weights of 16 normal female rats

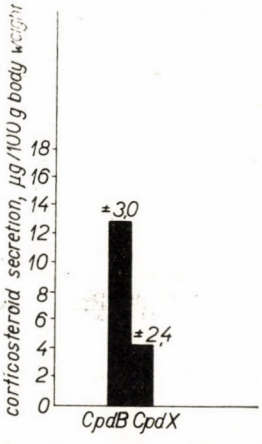

Fig. 2. Adrenal venous corticosteroid levels 14 days after ovariectomy in 12 rats

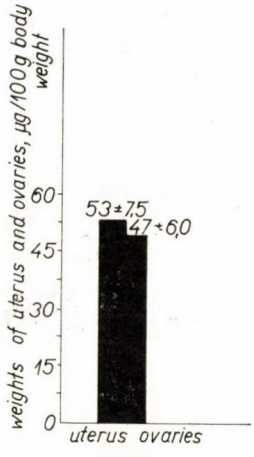

Fig. 3. Changes in uterine and ovarian weight 16 days after adrenalectomy in 10 rats

In the animals, in which bilateral electrocoagulation caused a lesion extending from over the optic chiasma to the preoptic region and caudally to the first level of the tuber cinereum, the weight of the gonads increased significantly. At the same time, these animals developed constant oestrus as early as 4 to 7 days after sustaining the lesion. The corticoid level of the adrenal venous blood (as determined on the 14th to 16th postoperative days) showed no unequivocal changes, although it was above the control level. Adrenal weight increased slightly (Fig. 4).

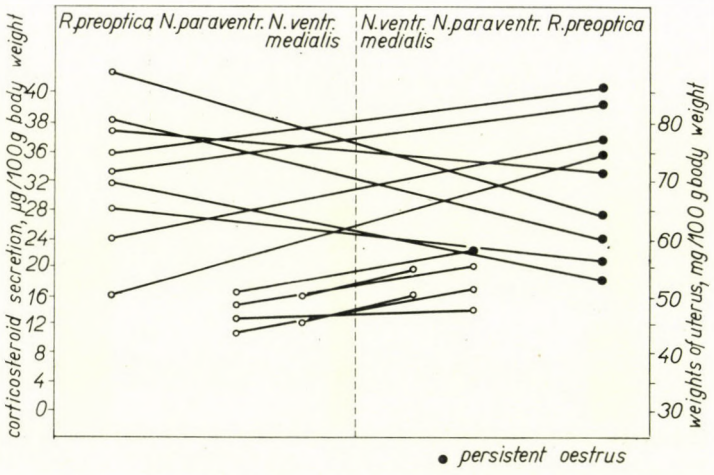

Fig. 4. Adrenal venous corticosteroid levels and changes in gonadal weight two weeks after electrocoagulation of the anterior hypothalamic area. The lines connecting the values show the reaction of the individual rats

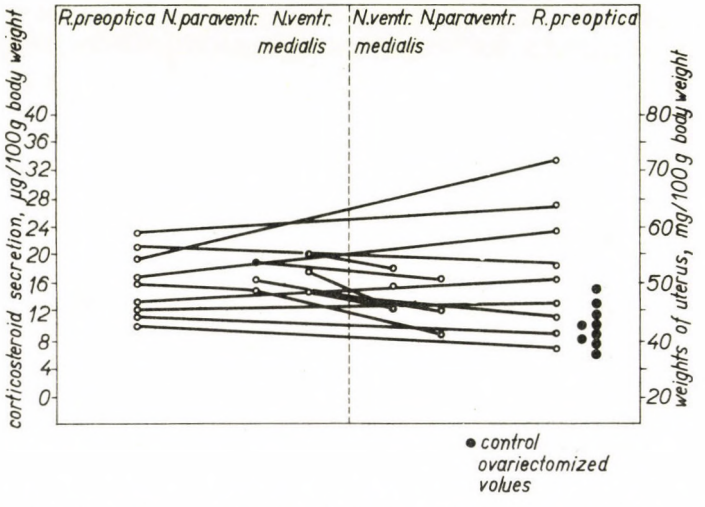

Fig. 5. Changes in adrenal venous blood and uterine weight two weeks after anterior hypothalamic lesion in 15 female rats. Electrocoagulation was performed 4 days after ovariectomy. The values for the 9 ovariectomized controls are also shown

Group 5. These animals had been ovariectomized 4 days before afflicting the lesion to the anterior hypothalamus. Subsequently, the animals studied at 14 to 16 days were tested for adrenocortical hormone secretion. As opposed to the data for group 4, the values for group 5 hardly differed from the control ones. The increase of steroid secretion characterizing the controls with lesion to the anterior hypothalamus was absent. It was noteworthy in this group that

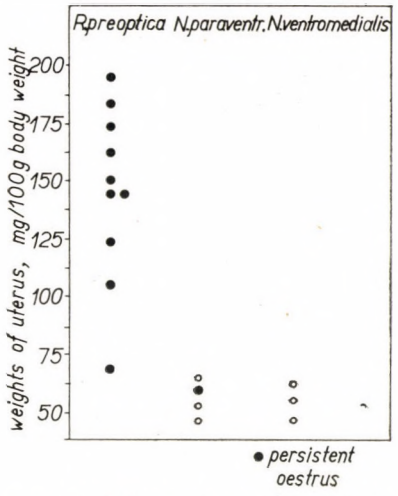

Fig. 6. Changes in uterine weight following hypothalamic lesion in 17 adrenalectomized female rats

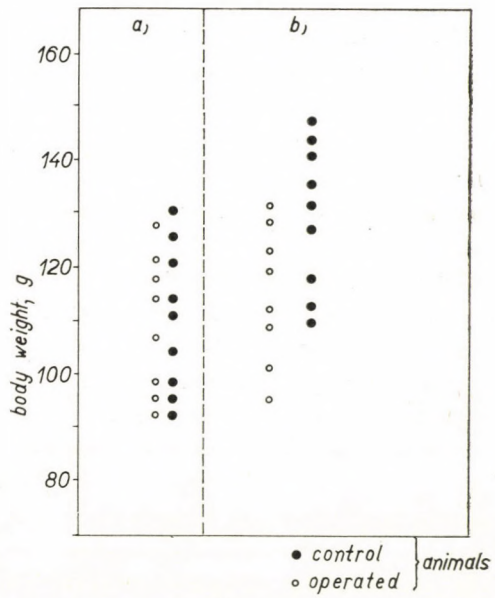

Fig. 7. Weight curves for the operated and control rats in the 16-day test period

in some animals the uterine weight was definitely higher than in the castrated control rats (Fig. 5).

Group 6. The animals of this group had undergone adrenalectomy 4 to 6 days before afflicting lesion of the anterior hypothalamus. As shown in Fig 6, the uterine weights increased much more markedly in this group than in the

controls with hypothalamic lesion. As determined by vaginal smear studies, the animals were in constant oestrus by the 6th to 7th day.

As to the site of the lesion, it is clear from the figures that electrocoagulation in the anterior hypothalamic area affected most profoundly the pituitary-ovarian function when it involved the preoptic region. No such changes resulted from lesioning the group of paraventricular nuclei or causing isolated lesion to the ventromedial nucleus.

Lesion to the preoptic region interfered with somatic development. As shown in Fig. 7, the weight gain of these animals during the test period was minimal, as compared to the weight curves for the controls.

Discussion

The present observations suggest that adrenal cortical function is interrelated with ovarian function and that these interrelations are significantly influencing the experimental results following electrocoagulation of the anterior hypothalamic area. It was remarkable that the hypothalamic lesion elicited not only a constant oestrus, but significantly increased uterine weight as well, a finding not mentioned in pertaining reports by other authors. For example, GREER [17] found no change either in the uterine or in the adrenal weight following hypothalamic lesions, although the animals showed constant oestrus.

The increase of uterine weight, a reliable indicator of gonadal activity is apparently due to an increased oestrogen output, and is in harmony with the constant oestrus.

As to the site of the lesion, the bilateral lesions extending from the level of the optic chiasma to the preoptic region anteriorly and to the first level of the tuber cinereum dorsally and caudally, were the most effective. No gonadal changes of similar nature occurred following lesion to the paraventricular nuclei alone or to the ventromedial nucleus alone.

While in the control adrenalectomized and ovariectomized rats the interaction between the two endocrine systems was not conspicuous, in the animals with lesion to the anterior hypothalamus considerable changes were noted. In the ovariectomized rat the hypothalamic lesion does not increase corticoid output. This fact indicates that the endocrine changes elicited by the lesion may be traced back to an increased gonadal activity. The increase of corticoid secretion following the administration of exogenous oestrogens has been confirmed also by us (MARTIN and ENDRŐCZI [21]). It has been observed further, that the weight of the uterus shows a more marked increase in adrenalectomized animals with hypothalamic lesion than in the controls. This inhibitory action of the adrenal cortex has been reported previously. The administration of exogenous corticoids lessens the increase of uterine weight produced by the

exogenous oestrogens and inhibits the feed-back action of oestrogens upon the gonadotropin secretion by the hypophysis (MARTIN and ENDRŐCZI [21]).

The mechanism of the changes which follow the lesion to the anterior hypothalamus remains unclear. The increased gonadotrophin secretion in response to the lesion, that may be considered a primary phenomenon, seems to be due to some non-specific tissue excitation. This is suggested also by the increased gonadotrophin secretion in response to anterior hypothalamic stimulation. On the other hand, it is difficult to interpret the view according to which the anterior hypothalamus would control the trophic hormone secretion by the anterior pituitary through inhibitory nervous structures. The gonadal atrophy following transaction of the pituitary stalk likewise points to a primary activating influence of the diencephalon. The experiments with transplanted anterior pituitary lobe yielded similar conclusions (HARRIS [18]; GREER [17]).

The pituitary-ovarial-adrenocortical connexions observable following the lesion of the anterior hypothalamus appear to play a role in physiological and pathological phenomena alike. We had shown earlier that the stimulation of the septal area causing an increased 17-ketosteroid output by the adrenal cortex had no such effect following ovariectomy (ENDRŐCZI and LISSÁK [10]). The present experiments indicate that adrenocortical secretion is capable of reducing the increased release of gonadotrophin from the anterior pituitary that results after injuring the anterior hypothalamus. This complex synergistic and antagonistic connexion between the two endocrine systems or a shift in their equilibrium might have a significant role in the genesis of certain adreno-ovarian diseases.

LITERATURE

1. CHRITCHLOW, V. B.: Anat. Rec. **127,** 283 (1957).
2. DEY, F. L., FISCHER, C., BERRY, C., RANSOM, S. W.: Amer. J. Physiol. **129,** 39 (1940).
3. DEY, F. L.: Amer. J. Anat. **69,** 61 (1941).
4. DEY, F. L.: Endocrinology **39,** 75 (1943).
5. DONOVAN, B. T., VAN DER WERFF TEN BOSCH, J. J.: Nature (Lond.) **178,** 745 (1956).
6. DONOVAN, B. T., VAN DER WERFF TEN BOSCH, J. J.: J. Physiol. (Lond.) **132,** 123 (1956).
7. DONOVAN, B. T., VAN DER WERFF TEN BOSCH, J. J.: J. Physiol. (Lond.) **147,** 78 (1959).
8. ENDRŐCZI, E., BATA, G., MARTIN, J.: Endokrinologie **35,** 280 (1958).
9. ENDRŐCZI, E., LISSÁK, K.: Acta physiol. hung. **15,** 25 (1959).
10. ENDRŐCZI, E., LISSÁK, K.: Acta physiol. hung. **17,** 39 (1960).
11. ENDRŐCZI, E., YANG, L. T.: Acta physiol. hung. **18,** 125 (1960).
12. FLERKÓ, B.: Acta morph. hung. **3,** 65 (1953).
13. FLERKÓ, B.: Acta morph. hung. **4,** 475 (1954).
14. GREEN, J. D., CLEMENTE, C. D., DE GROOT, J.: J. comp. Neurol. **108,** 505 (1957).
15. GREER, M. A.: Proc. Soc. exp. Biol. (N. Y.) **77,** 603 (1951).
16. GREER, M. A.: J. clin. Endocr. **12,** 1259 (1952).
17. GREER, M. A.: Recent Progr. Hormone Res. **13,** 67 (1957).
18. HARRIS, G. W.: Neural Control of the Pituitary Gland. Arnold, London, 1955.
19. HERBERT, J., ZUCKERMAN, S.: J. Endocr. **17,** 433 (1958).
20. TOKAZINE, T., SAWYER, C. H.: Arch. Neurol. Psychiat. (Chicago) **77,** 259 (1957).
21. MARTIN, J., ENDRŐCZI, E.: Acta physiol. hung. In press, 1961.

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THE EFFECT OF CONSTANT LIVING CONDITIONS ON THE SALIVARY HYDROGEN ION CONCENTRATION IN CHILDREN

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A total of 397 tests for salivary pH have been made in 52 children, living in institutions or with their parents. The salivary pH values for the children living for longer periods in institutions were significantly higher than those for the children living with their parents. The differences in salivary pH between the single groups are attributed to dietary and environmental factors.

The extensive literature on salivary pH dates back to about 75 years ago. The first report on the subject was published by CHITTENDEN and ELY in 1883 [4], SZABÓ's [33] study "Über die chemische Reaktion des Mundspeichels", in 1900. The first electrometric salivary pH determination was made in 1905 by FOA [10]. Tests on a larger scale followed in 1914 [16, 23].

In the subsequent decades salivary pH has been studied from many aspects. Most of the investigations, preponderantly *in vitro*, were concerned with the correlation between tooth decay and salivary pH. During the past 20 years there was a tendency to claim no correlation between the two [2, 6, 15, 19, 32]. Most of the tests on which this claim was based had involved subjects not living under constant conditions and there was also a considerable divergence between the methods employed.

In our opinion, the results are significantly influenced by certain factors, such as

1. diurnal variations in salivary pH;
2. differences between the techniques employed;
3. the failure of clinical methods correctly to assess the grade of caries activity;
4. differences in the living and dietary conditions of the test subjects.

We have therefore performed our studies with especial consideration of these points.

In previous reports we have discussed in detail that the dentition of children living for a longer period and fed an approximately adequate diet in nurseries was significantly better than that of children living under inhomogeneous conditions with their parents [34, 35, 36]. It has been surmised that this

difference may be detected not only in the condition of the teeth, but in the biochemical reactions of the saliva as well.

Our experiments had the following aims. To determine the changes in salivary pH due to dietary and environmental effects; to find a difference (if any) in salivary pH between children living under constant and children under variable conditions; to analyse the correlation between the diurnal variations of salivary pH shown by the different groups.

Methods

a) Sampling of saliva. Resting saliva was collected by allowing it to flow freely at 7.30 a. m. and 2.30 p. m. Five ml were taken at a time, using 2 ml samples for pH determination and the rest in other tests. The morning sample was fasting saliva, the afternoon one was obtained 30 minutes after the midday meal. The children did not brush their teeth or rinse their mouth on the day of sample collection.

The condition of the teeth was examined by mirror and probe, in artificial light. No X-rays were used. The DMF index was applied for recording the dental status. The children having no decayed, filled or missing teeth were listed in the group "good teeth"; the rest belonged to the group of "bad teeth", depending on the DMF figure.

b) Determination of pH. The samples were immediately tested electrometrically, using glass electrodes (Universal pH Meter 22, Copenhagen).

c) Statistical analysis. Biometric evaluation of the group means was based on the "*t*" test [17], accepting a difference with $P < 0.05$ to be significant.

Number and distribution of tests (Table I)

Salivas from a total of 52 subjects (mean age 10.57 years) were tested. Twenty of them had good teeth and 32 had bad teeth.

Eleven of the test subjects lived in the State Children's Home of Szeged, 28 in the Institute for Deafmutes and 13 were children attending the Árpád Square General School.

The data for the Children's House and the deafmute children have already been reported [34, 35, 36]. In the present series the group of children attending school and living under different conditions was used as the control to the earlier two groups.

The groups were formed in the following way:

Table I

Number and distribution of tests

	Mean age years	Number of test subjects			Number of test						Total
					fasting			afternoon			
		g. t.	b. t.	total	g. t.	b. t.	total	g. t.	b. t.	total	
State Children's Home	9.27	4	7	11	18	38	56	19	45	64	120
Institute for Deafmutes	12.78	10	18	28	35	64	99	21	29	50	149
Árpád Square General School	10.15	6	7	13	34	42	76	24	28	52	128
Total	10.57	20	32	52	87	144	231	64	102	166	397

g. t. = good teeth; b. t. = bad teeth.

Group 1.: Children living for at least 2.5 years in an institution, well developed physically and mentally, fed on institute diet, brushing teeth regularly, under supervision (State Children's Home).

Group 2.: Children living in an institution for more than 2.5 years, having restricted contacts with the outer world. They are well developed physically and mentally, and are fed on a diet similar to that in group 1. They brush their teeth regularly. (Institute for Deafmutes.)

Group 3.: Children living with their parents, fed on different diets, well developed physically and mentally. Not all of them brush their teeth regularly (schoolchildren).

A total of 397 tests was made in the 52 subjects, including 231 fasting tests and 166 after the midday-meal. The average value of pH tests in one child was 7.6.

Results

I. Global results

1. The global mean pH for the 52 children tested was: fasting, morning saliva, 7.40; afternoon saliva, 7.23.

2. Dividing all the test subjects into groups of good and bad teeth, the following values were obtained.

a) In the 20 subjects with good teeth the morning salivary pH value averaged 7.48, the afternoon salivary pH 7.29.

b) In the 32 children of the bad teeth group the morning mean was pH 7.33, the afternoon one pH 7.17.

II. Results for the individual groups

Group 1. Mean salivary pH for fasting saliva 7.71, for afternoon saliva 7.46.

The means for children with good teeth were 7.80 and 7.60, respectively, and for the children with bad teeth they were 7.62 and 7.33.

Group 2. Mean morning salivary pH 7.50, afternoon salivary pH 7.29.

The means for children with good teeth were 7.47 and 7.32, respectively, and for those with bad teeth 7.54 and 7.27, respectively.

Group 3. Mean morning salivary pH 7.26, afternoon salivary pH 7.03.

The means for the children with good teeth were 7.39 and 7.11, respectively, and for those with bad teeth 7.13 and 6.95, respectively.

Discussion

1. *Salivary pH*

The pH of the saliva depends on the equilibrium between the buffer systems in the saliva. Of these, it is first of all the bicarbonate—carbonic acid system that is primarily responsible for the maintenance of the actual salivary pH [20, 21, 39]. According to the formula of HENDERSON and HASSELBACH [30],

$$pH = 6.1 + \log \frac{[HCO_3^-]}{[H_2CO_3]}$$

which essentially means that a shift in the salivary bicarbonate—carbonic acid equilibrium necessarily causes a shift in the salivary pH and vice versa.

The pH of the mixed, resting human saliva is usually reported to be around pH 7.00, with ranges of from 6.00 to 7.40 [32] and 6.40 to 7.40 [19] in groups containing subjects with good and bad teeth.

The mean salivary pH in mixed groups has been reported to be pH 6.70 [2, 6, 7, 11], around pH 7.30 [5, 14, 15, 31] and around pH 7.00 [12, 22, 25, 38].

2. *Factors influencing the pH of saliva*

POPPER and HAFFNER [26] in 1925 suggested that the salivary pH was approximately constant and might remain unchanged for long. In 1937, after detailed and careful studies, GROSSMAN and BRICKMAN [11] stated that the salivary pH was not constant, but might vary within wide ranges even in the same subject. The observations made on 5 subjects by EISENBRANDT [6] yielded the evidence that the salivary pH would change from hour to hour, from day to day. In a larger material [7] even seasonal variations were demonstrable, with the highest values occurring in autumn and the lowest values in the spring.

The lowest daily mean value was obtained immediately on awakening [13] and the highest at 5 o'clock p. m. [6]; the latter was significantly different from the fasting values.

KRASNOW [18] was the first to point out the substantial influence upon the salivary pH of the part of the day, the metabolic state of the subject, mastication, absorption of CO_2 during collection, and the length of the interval between saliva collection and pH test. According to EISENBRANDT [7], the changes in salivary pH are determined by changes in the metabolism of the subject. ANDERSON [1] suggest salivary CO_2 concentration to be responsible for the variations in salivary pH. RAPP [27] and EGGERS-LURA [8] emphasize the role of carbonic anhydrase; the resulting CO_2 loss would affect the pH of the saliva. A correlation between the diet and the alkali reserve of the saliva has been pointed out by WILLS and FORBES [40], as well as by ERICSSON [9]. SCHMIEDT-NIELSEN [28, 29] emphasized the significance of nervous stimuli, which directly influence the secretion of saliva and thus may alter salivary pH as well. The higher morning values, too, may be due to stimulation, sampling the saliva being the first and not negligible stimulus after wakening.

3. *Discussion of the results for the single groups*

The values for morning and afternoon salivary pH ranged from 6.40 to 7.50 in the group of the children living with their parents (group 3), essentially in agreement with the results published by LEICESTER [19] and SWERDLOVE [32]. However, the pH values for the children living under controlled conditions (group 1 and group 2) differed from the data in the literature. In group 2 the salivary pH values ranged from 7.10 to 8.05 and in group 1 the range was 7.20 to 8.25.

Table Ia

Details of test results

No.	Age, years	Dental state	Fasting			Afternoon		
			days	pH	mean	days	pH	mean
1.	9	good teeth	1 2 3 4 5	7.60 7.50 8.25 8.10 8.00	7.65	1 2 3 4 5	7.40 7.80 7.75 7.90 7.75	7.72
2.	8	good teeth	1 2 3 4 5	8.15 7.40 7.65 7.60 8.10	7.78	1 2 3 4 5	7.70 7.40 7.45 7.60 7.60	7.55
3.	9	good teeth	1 2 3 4	7.55 8.00 7.90 7.90	7.84	1 2 3 4	7.40 7.80 7.20 7.30	7.42
4.	8	good teeth	1 2 3 4	8.25 8.15 7.65 7.70	7.93	1 2 3 4	7.90 7.75 7.45 7.70	7.71
5.	8	bad teeth	1 2 3 4 5 6 7	8.00 7.70 8.05 8.00 8.05 7.75 8.10	7.95	1 2 3 4 5 6 7 8	7.30 7.30 7.20 7.60 7.55 7.65 7.70 7.45	7.46
6.	10	bad teeth	1 2 3 4 5	6.70 7.20 7.75 7.50 7.65	7.36	1 2 3 4 5 6	7.00 7.70 7.55 7.10 7.20 6.80	7.22
7.	10	bad teeth	1 2 3 4 5 6	7.90 7.40 8.10 7.90 7.80 7.90	7.83	1 2 3 4 5 6	7.40 7.35 7.50 7.70 7.50 7.50	7.49
8.	9	bad teeth	1 2 3 4 5	7.80 7.40 7.80 7.70 7.80	7.70	1 2 3 4 5 6	7.00 7.20 7.90 7.40 7.30 7.10	7.28

No.	Age, years	Dental state	Fasting			Afternoon		
			days	pH	mean	days	pH	mean
9.	9	bad teeth	1 2 3 4	7.60 7.90 7.60 7.50	7.65	1 2 3 4 5 6	7.40 7.50 7.45 7.40 7.20 7.35	7.38
10.	10	bad teeth	1 2 3 4 5	7.50 7.65 7.20 7.40 7.40	7.43	1 2 3 4 5 6 7	7.10 7.10 6.80 7.30 7.00 7.00 7.10	7.05
11.	12	bad teeth	1 2 3 4 5 6	7.40 7.70 7.50 7.35 6.70 7.35	7.33	1 2 3 4 5 6	7.30 7.60 7.30 7.10 7.60 7.65	7.42
12.	12	good teeth	1 2 3 4	7.50 7.40 7.70 7.45	7.51	1 2 3	7.20 7.20 7.25	7.21
13.	12	good teeth	1 2 3 4	7.10 7.10 7.15 7.25	7.15	1 2	6.90 6.90	6.90
14.	11	good teeth	1 2 3 4	7.75 7.80 7.30 7.70	7.63	1 2 3	7.70 7.40 7.90	7.66
15.	12	good teeth	1 2 3 4	7.40 7.80 7.95 7.55	7.67	1 2 3	7.30 7.70 7.40	7.46
16.	11	good teeth	1 2 3 4	7.90 7.50 7.20 7.50	7.52	1 2 3	7.25 7.20 7.50	7.31
17.	15	good teeth	1 2 3	7.45 7.15 7.40	7.33	1 2	7.20 7.20	7.20
18.	15	good teeth	1 2 3	7.10 7.10 7.30	7.16	1 2	7.10 7.00	7.05

No.	Age, years	Dental state	Fasting			Afternoon		
			days	pH	mean	days	pH	mean
19.	16	good teeth	1 2 3	7.80 7.40 7.75	7.65	1	7.70	7.70
20.	10	good teeth	1 2 3	7.70 7.95 6.90	7.51	1	7.40	7.40
21.	10	good teeth	1 2 3	7.30 7.50 7.70	7.50	1	7.60	7.60
22.	14	good teeth	1 2 3 4	7.10 7.35 7.20 7.20	7.21	1 2	6.95 7.40	7.15
23.	22	bad teeth	1 2 3 4	7.70 7.80 7.25 7.55	7.57	1 2	7.40 7.50	7.45
24.	13	bad teeth	1 2 3 4	8.00 7.70 7.40 7.20	7.57	1 2	7.90 7.85	7.87
25.	14	bad teeth	1 2 3 4	7.80 7.50 7.55 8.05	7.72	1 2	7.60 7.20	7.40
26.	12	bad teeth	1 2 3 4	7.40 7.65 7.75 7.90	7.67	1 2	7.30 7.50	7.40
27.	10	bad teeth	1 2 3 4	7.40 7.50 7.80 7.40	7.52	1 2	7.30 7.50	7.40
28.	11	bad teeth	1 2 3 4	7.90 7.10 7.70 7.70	7.60	1 2	7.50 7.25	7.37
29.	14	bad teeth	1 2 3 4	7.60 7.30 7.85 7.50	7.53	1	7.50	7.50
30.	9	bad teeth	1 2 3 4	7.80 7.40 7.30 7.40	7.47	1	7.25	7.25

No.	Age, years	Dental state	Fasting			Afternoon		
			days	pH	mean	days	pH	mean
31.	10	bad teeth	1 2 3 4	7.50 7.60 6.90 7.60	7.47	1 2	7.10 7.20	7.15
32.	9	bad teeth	1 2 3	7.10 7.80 7.40	7.43	1 2	6.80 6.95	6.87
33.	10	bad teeth	1 2 3	7.90 7.30 7.40	7.53	1	7.45	7.45
34.	12	bad teeth	1 2 3	7.35 7.50 7.65	7.50	1 2	7.10 7.45	7.27
35.	14	bad teeth	1 2 3	7.80 7.85 7.30	7.65	1 2	7.60 7.75	7.67
36.	16	bad teeth	1 2 3	7.20 7.55 7.75	7.50	1	7.45	7.45
37.	17	bad teeth	1 2 3	7.40 7.50 7.40	7.43	1	7.20	7.20
38.	13	bad teeth	1 2 3	7.20 8.05 7.90	7.71	1	7.85	7.85
39.	14	bad teeth	1 2 3	7.90 7.80 7.50	7.73	1	7.20	7.20
40.	10	good teeth	1 2 3 4 5 6	7.55 7.50 7.40 7.55 7.40 7.50	7.48	1 2 3 4	7.40 7.50 7.35 7.50	7.43
41.	10	good teeth	1 2 3 4 5 6	7.20 7.00 7.40 7.40 7.55 7.50	7.34	1 2 3 4	7.00 6.70 7.00 6.80	6.87
42.	10	good teeth	1 2 3 4 5 6	7.40 7.55 7.45 7.35 7.15 7.55	7.40	1 2 3 4	7.40 6.95 7.40 7.00	7.18

No.	Age, years	Dental state	Fasting			Afternoon		
			days	mean	pH	days	mean	pH
43.	10	good teeth	1 2 3 4 5 6	7.50 7.40 7.55 7.40 7.50 7.20	7.42	1 2 3 4	7.45 7.00 7.40 7.00	7.21
44.	10	good teeth	1 2 3 4 5	7.00 7.40 7.40 7.55 7.50	7.37	1 2 3 4	6.80 7.00 6.70 7.00	6.87
45.	10	good teeth	1 2 3 4 5	7.40 7.55 7.45 7.35 7.15	7.38	1 2 3 4	7.00 7.45 7.35 6.95	7.18
46.	10	bad teeth	1 2 3 4 5 6	7.15 7.05 7.05 6.95 7.05 7.45	7.11	1 2 3 4	7.00 7.00 7.05 6.80	6.96
47.	11	bad teeth	1 2 3 4 5 6	6.90 7.10 7.00 7.40 7.45 7.00	7.14	1 2 3 4	6.70 7.00 7.00 7.10	6.95
48.	11	bad teeth	1 2 3 4 5 6	7.00 7.50 7.00 6.80 7.10 6.75	7.02	1 2 3 4	6.90 6.95 7.00 7.05	6.97
49.	10	bad teeth	1 2 3 4 5 6	7.30 7.20 7.15 7.15 7.05 7.05	7.15	1 2 3 4	7.15 7.05 7.15 7.00	7.08
50.	10	bad teeth	1 2 3 4 5 6	6.95 7.05 7.45 6.90 7.10 7.05	7.08	1 2 3 4	6.80 6.90 6.95 6.70	6.83

No.	Age, years	Dental state	Fasting			Afternoon		
			days	pH	mean	days	pH	mean
51.	10	bad teeth	1 2 3 4 5 6	7.40 7.45 7.00 7.00 7.50 7.00	7.22	1 2 3 4	7.00 7.05 7.10 7.00	7.03
52.	10	bad teeth	1 2 3 4 5 6	6.80 7.10 6.75 7.30 7.20 7.15	7.05	1 2 3 4	6.70 6.90 6.70 6.90	6.80

As regards the fasting salivary pH it was again group 3 which was closest to data in the literature (Table II), with a mean of pH 7.26. The means were

Table II

Changes of pH in the morning and afternoon salivas

	Fasting saliva				Afternoon saliva			
	g. t.	b. t.	total		g. t.	b. t.	total	
			mean	range			mean	range
State Children's Home	7.80	7.62	7.71	±0.36	7.60	7 33	7.46	±0.26
Institute for Deafmutes	7.47	7.54	7.50	±0.33	7.32	7.27	7.29	±1.03
Árpád Square General School	7.39	7.13	7.26	±0.45	7.11	6.95	7.03	±0.14

g. t. = good teeth; b. t. = bad teeth.

higher in group 1 and group 2, pH 7.71 and pH 7.50, respectively, both significantly different from the value for group 3 (Table III, Table IV).

Table III

Comparison of the results for group 1 and 3

		Mean	Range	Degree of freedom	"t"	*P*
Fasting pH	group 1	7.71	±0.36	130	8.87	<0.01
	group 3	7.26	±0.45			
Afternoon pH	group 1	7.46	±0.26	114	7.94	<0.01
	group 3	7.03	±0.14			

Table IV

Comparison of the results for group 2 and group 3

		Mean	Range	Degree of freedom	"t"	P
Fasting pH	group 2	7.50	±0.33	173	5.36	<0.01
	group 3	7.26	±0.45			
Afternoon pH	group 2	7.29	±1.03	100	2.18	<0.05
	group 3	7.03	±0.14			

The afternoon saliva showed in every group lower pH values than those for the fasting saliva (Table I). The only significant difference was that between the mean morning and mean afternoon salivary pH values in group 1.

The lowest mean afternoon salivary pH was recorded in group 3 (pH 7.03), as compared with the pH 7.46 value for group 1. Here, too, the mean pH values for the children living in institutions were significantly higher than the mean for the children living with their parents (Table III, Table IV).

Although group 1 differed from group 2 only in that the children in the latter had a chance to take food other than that received in the institute (pocket money, parcels from home, contact with the outer world), they showed different salivary pH values. The fasting and afternoon means for group 1 were higher than those for group 2, and the difference between the morning means was significant. Salivary pH values seem to show an increasing tendency under constant conditions.

4. *Degree of caries activity and salivary pH*

Many data indicate that no correlation exists between the degree of caries activity and salivary pH [2, 6, 15, 19, 32].

The degree of caries activity or the onset of the development of disposition to caries cannot be determined by simple clinical examination [36]. For this reason we have used the terms good teeth and bad teeth instead of the terms caries-resistant and caries-active. The means for the single subgroups were different in every case. In general, the children with good teeth showed higher salivary pH values both in the morning and in the afternoon, except for the fasting values in group 2. In latter case the children with bad teeth showed a higher salivary pH mean value.

The higher mean salivary pH values of caries-resistant persons, as compared with that of caries-active ones have been pointed out by KARSHAN [15] and HUBBEL [14]. After a test period of 12 months' duration, BRAWLEY [3] expressed the view that an increase of the caries index would be accompanied

by changes in salivary pH, *i.e.* a higher caries index is characteristic of a lower pH and vice versa.

In our subjects, too, those with good teeth showed higher salivary pH means than those with bad teeth, but there was a significant difference exclusively with the afternoon values for group 1 and with the fasting values for group 3. However, no definite conclusions as to the exceptable salivary pH values of an individual may be drawn from the group mean. Subjects showing low pH values occurred also in the groups with high means and vice versa.

5. *Diurnal variations of salivary pH*

EISENBRANDT [6, 7] has called attention to the significant differences in the results of salivary pH tests made at different times during the day. For

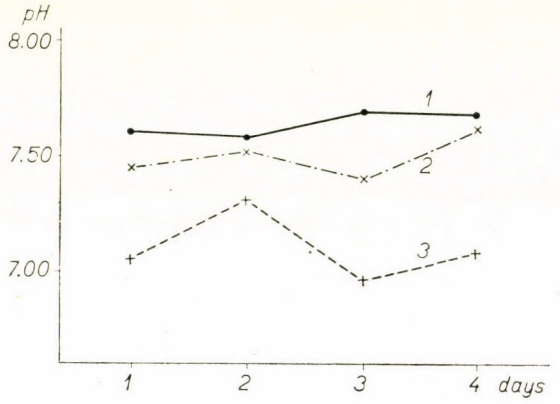

Fig. 1. Salivary pH changes in the three groups, as determined in four consecutive mornings. *1:* State Children's Home; *2:* Institute for Deafmutes; *3:* Árpád Square General School

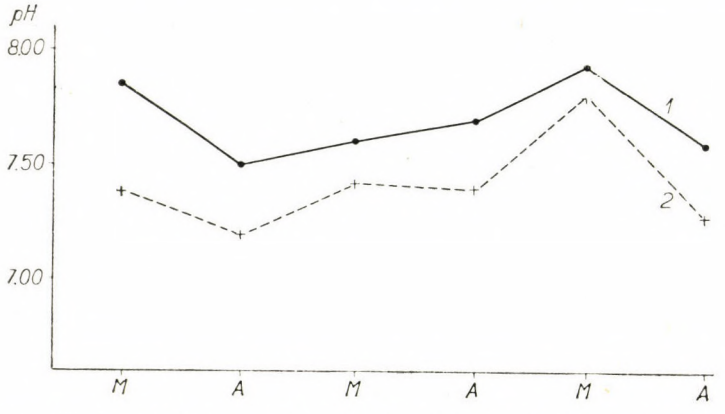

Fig. 2. Salivary pH changes in the good teeth *(1)* and bad teeth *(2)* children of group 1, as determined on 3 consecutive days (M: in the morning, A: in the afternoon)

this reason, salivary pH tests are usually done in the same period of the day, under identical experimental conditions. Even then the results are different, but they are at least comparable.

We, too, have made our tests with regard to these considerations, and found differences in the individual and group results alike. The greatest differences occurred in the group of children living with their parents and the smallest in the Children's Home. The fasting values offered the best example. From the data in Fig. 1, it is clear that the means for group 1 were slightly, those for group 3 markedly, divergent in the tests made in the morning of four consecutive days.

The diurnal salivary pH changes in the good teeth and bad teeth groups are shown in Fig. 2. In group 1 there was a definite difference in the pH curve between the children with good and those with bad teeth, with a range of 0.48 and 0.10 pH (mean, 0.24), but the same value has not been obtained twice.

6. *Salivary pH and diet*

WILLS and FORBES [40], as well as ERICSSON [9] have shown that subjects fed on different diets have different salivary alkali reserve. Those on protein and vegetable diet showed higher values than those on carbohydrate diet. TÓTH and HATTYASY [35] and ORAVECZ [24] found the diets fed at the State Children's Home in Hungary to be adequate by present standards. TÓTH [34] observed a significant reduction in caries incidence among children fed on that diet for longer periods.

The present investigations, too, indicate a correlation between the diet and the hydrogen ion concentration of the saliva.

Conclusions

No conclusions of general validity may be drawn from the evidence obtained for the small groups tested. It may still be stated that the saliva of children living in closed institutions for longer periods is significantly more alkaline and shows smaller diurnal variations *(i. e.* its pH is more constant) than the saliva of children living with their parents.

The cause of this difference is suggested to lie in differences in the dietary and living conditions.

The differences in salivary pH values between children with good teeth and those with bad teeth were usually not significant mathematically. As to an eventual correlation between caries activity and salivary pH, no answer can be given before a reliable test has been developed for the determination of the degree of caries activity or of the onset of caries disposition.

LITERATURE

1. ANDERSON, D. J.: J. dent. Res. **28,** 72 (1949).
2. BRAWLEY, R. E.: J. dent. Res. **15,** 55 (1935).
3. BRAWLEY, R. E.: J. dent. Res. **15,** 361 (1935).
4. CHITTENDEN, R. H., ELY, J. S.: Amer. chem. J. **4,** 329 (1883).
5. CUPROVA, N. D. (ЦУПРОВА, Н. Д.): Стоматология **2,** 12 (1950)
6. EISENBRANDT, L. L. :J. dent. Res. **22,** 147 (1943).
7. EISENBRANDT, L. L.: J. dent. Res. **23,** 363 (1944).
8. EGGERS-LURA, H.: Die Enzyme des Speichels und der Zähne, Carl Hauser, München. (1949). P. 44
9. ERICSSON, Y.: Acta odont. scand. **17,** 131 (1959).
10. FOA, C.: Arch. Fisiol. **3,** 369 (1905).
11. GROSSMAN, L. I., BRICKMAN, B. M.: J. dent. Res. **16,** 409 (1937).
12. HATTYASY, D.: Öst. Z. Stomat. **36,** 163 (1938).
13. HENDERSON, M., MILLET, J. A. P.: J. biol. Chem. **75,** 559 (1927).
14. HUBBEL, R. B.: Amer. J. Physiol. **105,** 436 (1933).
15. KARSHAN, M., KRASNOW, F., KREJCI, L. E.: J. dent. Res. **11,** 573 (1931).
16. KIRK, E. C., CROWELL, W. S., APPLETON, J. L. T.: J. Allied. Dental Soc. **9,** 186 (1914).
17. KOVÁCH, A. ed.: A kísérleti orvostudomány vizsgáló módszerei. Akadémiai Kiadó, Budapest, Vol. 2. (1954). P. 667—702.
18. KRASNOW, F.: Dental Cosmos **78,** 301 (1936).
19. LEICESTER, H. M.: Biochemistry of the Teeth. Mosby, St. Louis (1949) P. 262.
20. LILIENTHAL, B.: Oral Surg. **8,** 828 (1955).
21. LILIENTHAL, B.: J. dent. Res. **34,** 516 (1955).
22. LŐRINCZY, E., NÁDOR, K.: Orv. Hetil. (Budapest) **89,** 567 (1948).
23. MICHAELIS, L., PECHSTEIN, H.: Biochem. Z. **59,** 77 (1914).
24. ORAVECZ, P.: Fogorv. Szle (Budapest) **52,** 34 (1959).
25. POPOVA-MILASEVSKAYA, V. A. (ПОПОВА—МИЛАСЕВСКАЯ, В. А.): Стоматология **2,** 14 (1950).
26. POPPER, E., HAFFNER, F.: Dtsch. med. Wschr. **51,** 561 (1925).
27. RAPP, G. W.: J. Amer. dent. Ass. **33,** 191 (1946).
28. SCHMIEDT-NIELSEN, B.: Acta odont. scand. **7,** Suppl. 2 (1946).
29. SCHMIEDT-NIELSEN, B.: Acta physiol. scand. **2,** 139 (1946).
30. STRAUB, F. B.: Biochemie. Ungarische Akademie der Wissenschaften. (Budapest) 1960. P. 546.
31. SULLIVAN, J. L., STORVICK, C. A.: J. dent. Res. **29,** 173 (1950).
32. SWERDLOVE, C. K.: J. dent. Res. **21,** 73 (1942).
33. SZABÓ, J.: Ungar. med. Presse, **5,** 653 (1900).
34. TÓTH, K.: Fogorv. Szle (Budapest) **50,** 361 (1957).
35. TÓTH, K., HATTYASY, D.: Acta med. hung. **14,** 141 (1959).
36. TÓTH, K., SZABÓ, I.: Fogorv. Szle (Budapest) **52,** 141 (1959).
37. TÓTH, K., TÖRTELI, Á.: Fogorv. Szle (Budapest) **48,** 73 (1955).
38. VERING, F.: Medizinische Chemie. Maudrich, Wien, 1950. P. 388.
39. WAH LEUNG, S.: J. dent. Res. **30,** 403 (1951).
40. WILLS, J. H., FORBES, J. C.: J. dent. Res. **18,** 409 (1939).

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ORGAN BLOOD FLOW IN UNANAESTHESIZED RATS AND IN RATS ANAESTHESIZED WITH PENTOBARBITAL, URETHANE AND CHLORALOSE

By

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In rats anaesthesized with pentobarbital (40 mg/kg, intraperitoneally), urethane (1 g/kg, intraperitoneally) resp. chloralose (0.10 g/kg, iv.) we determined the cardiac output, blood pressure and by the indicator fractionation method of SAPIRSTEIN [1, 2] the organ fractions of cardiac output. From these data organ blood flow and vascular resistance were computed. In a group of unanaesthesized animals the cardiac output fractions alone were studied.

The results obtained for the animals under pentobarbital anaesthesia agreed well with those in the literature. As related to the results for the pentobarbital group, lower blood presure and cardiac resistance values were obtained in the urethane-treated group. The cardiac, carcass fractions of the cardiac output were higher, the renal and intestinal fractions were lower. Chloralose increased the blood flow in the skin, carcass and reduced the resistance of the heart and the carcass. The renal fraction of cardiac output was lower, the carcass fraction higher. In the unanaesthesized group the cardiac, renal and hepatic fractions of the cardiac output decreased, the carcass fraction increased.

On the basis of the results, of the three anaesthetics tested pentobarbital seems to be the most suitable for use in circulation experiments in rats.

In circulation studies rats are much less frequently used then the bigger mammals. Recently, SAPIRSTEIN [1, 2] has described the method of "isotope-indicator-fractionation", by which every important organ fraction of cardiac output (except for the cerebral one) can be determined in the rat, using ^{86}Rb resp. ^{42}K. When cardiac output and blood pressure are known, visceral blood flow and vascular resistance may be computed. Thus, a combined application of the above methods makes it possible to study in detail the circulation of the rat, and to use rats in investigations which up to now had to be made on bigger animals.

In the present experiments the circulatory effects of pentobarbital, urethane and chloralose were observed in the rat, to determine which of these drugs is the one most suited for use in circulation studies. In order to assess the normal values, a group of unanaesthesized animals was tested for the fractions of cardiac output.

Methods

Male rats were used. Sodium barbital was injected intraperitoneally, in a dose of 40 mg/kg, in a 1 per cent solution. The dose of urethane was 1.0 g/kg, injected intraperitoneally in the form of a 20 per cent solution. Chloralose was administered intravenously, in a dose of

0.10 g/kg in a one per cent solution. Experimentation began 20 to 40 minutes after the injection of the anaesthetics. In the group of the unanaesthesized rats ("alert") ^{86}Rb had been administered through a polyethylene cannula implanted one or two days before the experiment into the jugular vein and filled with 50 mg/ml heparin. Owing to technical reasons, only the fractions of the cardiac output were studied.

In the circulation studies we followed essentially the procedure described by SAPIRSTEIN [2]. Blood pressure was measured in the carotid artery, using a membrane manometer (type Kállay—Vorsatz.) To prevent blood clotting, heparin (2.5 mg in 0.2 ml) was injected into the tail vein before the insertion of the cannula. Cardiac output was determined by the dye dilution method [3]; 0.3 ml of a 1.5 per cent solution of Evans blue was injected into the femoral vein and at intervals of 0.66 sec blood samples were taken by means of the fractional blood collector (type Kállay—Vorsatz) from the carotid artery. The concentration of dye in 20 μl samples of blood was determined in a Beckman B spectrophotometer. Cardiac output and the fractions of cardiac output were determined in the same animal, the dye was injected 50 to 55 sec after the injection of ^{86}Rb.

The organ fractions of cardiac output (except for the cerebral one) were determined by the "indicator fractionation" method of SAPIRSTEIN [1, 2], using ^{86}Rb. $^{86}RbCl$, 5 to 10 μC in 0.5 ml of physiologic NaCl, was injected into the femoral vein. Sixty-five to 70 seconds later the animals were killed by injecting 0.5 ml of a saturated KCl solution into the tail vein. The organs were dissolved in a 20 per cent solution of KOH and the samples were tested for activity by means of a GM tube with end-window. Except for that of the brain, the organ fractions of cardiac output are given as the percentage of the activity of the ^{86}Rb, considering that during the experiment there was no difference in extraction between the organs [1, 2]. According to our unpublished control experiments, the ^{42}K or ^{86}Rb content of the total circulating blood volume does not amount to more than 5 per cent of the dose of isotope injected. We did not extrapolate to "0 venous drainage", since in 60 to 70 sec there is no significant change in the concentration of ^{86}Rb in the viscera [4]. The cerebral fraction of cardiac output [5] was not determined.

From the data for cardiac output, the organ fractions of cardiac output and organ weight we computed for the single organs the blood flow for 100 g of organ weight, and from the data for blood pressure the vascular resistance. The units of measurements are to be found at the end of Table I. As the within-sample variance proved to be practically equal (BARTLETT's test) the standard deviations were computed from the pooled sum-of-squares. The data were evaluated by STUDENT's "*t*" test. The changes were related to the pentobarbital group.

Results

In the group anaesthesized with pentobarbital sodium the mean cardiac output was 28.6 ml/100 g, blood pressure 120 mm Hg, total vascular resistance $336 \cdot 10^3$ dyne sec cm^{-5}. The cardiac output of 28.6 ml/100 g was higher than that reported for rats by SAPIRSTEIN *et al.* [2, 6, 7] (20.5 to 23.1 and 23.0 ml/ /100 g, respectively). There was practically no difference in the blood pressure values. Under urethane anaesthesia the mean cardiac output was somewhat lower, 24.4 ml/100 g (not significant), blood pressure definitely decreased (to 97 mm Hg) and as regards resistance there was no difference from the pentobarbital group. In the chloralose group blood pressure and resistance were lower and cardiac output higher than in the pentobarbital group, but the differences were not significant.

In *pentobarbital* anaesthesia the values for the fractions of cardiac output were in good agreement with those reported by SAPIRSTEIN *et al.* [1, 2, 6, 7]. There was a minor difference in the case of the kidney, in which we found a cardiac output fraction of 14.6 per cent, as compared with the 16.1 to 17.8 and 14.9 per cent described by the above authors.

Table I

The effect of anaesthetics on the circulation of the rat

	Pentobarbital 40 mg/kg i. p. (control)	Urethane 1 g/kg i. p.	Chloralose 0.10 g/kg i. v.	"Alert"	Within-sample standard deviation
Number of cases (n)	22—30	11—14	11—14	11	
		Means			
Total :					
Cardiac output	28.6	24.4	32.8	—	8.7
Blood pressure	120.0	96.7··*	109.2	—	20.0
Resistance	336.1	344.9	286.9	—	110.0
Heart :					
Blood flow	211.3	219.9	225.0	—	75.5
Resistance	50.9	34.7···	38.8··	—	11.5
Fraction	2.8	3.4··	2.6	1.9···	0.6
Kidney :					
Blood flow	415.0	328.7	417.3	—	135.1
Resistance	29.0	24.8	22.9	—	12.4
Fraction	14.6	10.8···	12.2···	9.8···	1.8
Lung :					
Blood flow	97.7	75.3	82.3	—	33.0
Resistance	124.5	110.9	117.2	—	44.7
Fraction	2.9	3.2	2.4	2.5	0.9
Liver:					
Blood flow	67.2	53.6	75.6	—	23.9
Resistance	170.4	154.1	131.1	—	62.4
Fraction	8.6	8.1	8.2	6.3··	1.8
Intestines :					
Blood flow	75.8	58.6	84.7	—	30.5
Resistance	135.8	126.9	114.5	—	51.3
Fraction	17.0	14.0··	15.1	14.8	3.2
Skin :					
Blood flow	12.0	10.1	16.6·	—	5.7
Resistance	964.6	877.5	652.0	—	394.0
Fraction	8.8	8.1	10.8·	9.7	2.4
Carcass :					
Blood flow	19.6	20.2	25.0·	—	7.1
Resistance	526.8	444.6	378.0·	—	155.1
Fraction	45.8	52.1··	49.2·	55.5···	5.5

Scales

Cardiac output	ml/min/100 g body weight
Blood pressure	mm Hg
Resistance (body)	10^3 dyne sec cm^{-5}/100 g body weight
Blood flow	ml/min/100 g organ weight
Resistance (organ)	10^3 dyne sec cm^{-5}/100 g organ weight
Fraction	blood flow of the organ expressed in percentage of cardiac output

* *Difference from the controls*

No sign:	not significant	$P > 0.05$
. :	significant	$P < 0.05$
.. :	highly significant	$P < 0.01$
... :	very highly significant	$P < 0.001$

From these data it follows that, provided there was no difference in the weight of organs, in our experiments with pentobarbital the values for blood flow of organs were slightly higher and those for vascular resistance slightly lower.

Under *urethane* anaesthesia the mean values of organ blood flow (except for those for the heart and for the skeletal muscle and bone) were somewhat, though not significantly, lower than the values found in the pentobarbital group. Vascular resistance of the heart was definitely and significantly diminished. There was no marked change in the resistance of other organs. Deviations in the visceral fractions of cardiac output were found at some sites: the cardiac and carcass (skeletal muscle, bone, endocrine organs) fractions were higher and the renal and intestinal fractions lower.

In the *chloralose* group there was no difference from the pentobarbital group as regards blood flow of organs; the blood flow of the skin increased significantly, by 38 per cent, and that of the carcass by 25 per cent. The mean resistance was somewhat lower in every organ than in the pentobarbital group, but the difference was not significant exclusively in the case of the heart and the carcass. As to the organ fractions of cardiac output, that of the kidney was lower and those of the skin and carcass were higher.

In the *unanaesthesized group* we determined the fractions of cardiac output only. Those of the heart, kidney and liver diminished and that of the carcass definitely increased.

Discussion

We have undertaken to determine which of the anaesthetics pentobarbital, urethane and chloralose would be best suited for circulation studies in the rat, relying upon the results obtained by the "indicator-fractionation", dye dilution and blood pressure measurement methods. Just one dose (that most often employed in the literature) of each anaesthetic was used. Of course, the "most suitable" anaesthetic would be the one causing the least change in the basal circulation of the "normal", unanaesthesized animal. That the question was justified is clearly shown by Table I where not less than 13 of the 48 data for the urethane and chloralose groups differed significantly from the corresponding values in the pentobarbital group.

The "normal" values on which a comparison could be based being unknown, it is difficult to find an answer to the question. Fractions of cardiac output were estimated also in non-anaesthesized animals, in order to determine the "normal" values. For technical reasons the cardiac output could not be measured in the unanaesthesized state and therefore the "normal" blood flow and resistance values for the organs are not complete. The values for the

fractions of cardiac output in unanaesthesized animals, too, should be evaluated with caution: the fact that the animals must have been excited by the presence of the investigators, is not negligible from the point of view of circulation.

According to our hypothesis, the cardiac fraction of cardiac output may supply one of the data by which the "normal" state of circulation may be set fast. On the basis of several observations [8, 6, 10] it may be surmised namely that in response to a deterioration of circulation, to a decrease in cardiac output *(e. g.* in shock, haemorrhage, dehydration) the cardiac fraction of cardiac output increases so that the heart may keep itself operating. In conditions associated with an increased cardiac output *(e. g.*arterial hypoxia), the increased work of the heart is similarly accompanied by a higher cardiac output fraction.

As other data, the renal fraction of cardiac output and renal blood flow [see 6, 8—10] may be taken into consideration. It is namely known that the diminution of cardiac output or the increase thereof (for example in stagnant and arterial hypoxia and muscular work, *etc.)* is often accompanied by a decrease of renal blood flow and of the renal fraction of cardiac output.

Of course, unusually high or low cardiac output and blood pressure values also indicate that the anaesthetic is unsuitable for use.

Evaluating our experiments according to the above points, it will be seen that the anaesthetics used did not produce major changes in cardiac output or blood pressure. Except for the small drop of blood pressure in the urethane group, the values did not differ substantially.

In the pentobarbital and chloralose groups the cardiac fraction of cardiac output showed closely similar values, identical with those reported by SAPIRSTEIN *et al.* [1, 2, 6, 7]. Urethane increased the cardiac fraction of cardiac output. It was most remarkable that the cardiac fraction of the unanaesthesized animals was much lower than that of the animals anaesthesized with any of the drugs used. According to SAPIRSTEIN [11], adrenaline increases the cardiac fraction, thus a sympathicotonia presumably caused by agitation or excitation cannot be held responsible for it. We feel tempted to suggest that the "normal" cardiac fraction is lower than what we measure under the influence of pentobarbital, urethane or chloralose. And if all three drugs cause changes, those will be the ones of choice which produce the smallest changes (pentobarbital, chloralose).

Renal blood flow was similar in the pentobarbital and chloralose groups and slightly (but not significantly) lower in the urethane group. As compared with pentobarbital, urethane and chloralose significantly reduced the renal fraction of cardiac output. The renal fraction was likewise low in the unanaesthesized group, apparently as a result of the sympathicotonia, adrenalinaemia [11] due to excitation.

The comparative analysis of the data for cardiac output, blood pressure, cardiac and renal fraction of cardiac output, and renal blood flow *suggests pentobarbital to be the most suitable for use in circulation experiments in rats.* Chloralose and urethane seem to be less suitable for such purposes.

The use of urethane seems to be not suitable, due to its hypotensive effect, and its deteriorative effect on renal blood flow. Chloralose has the disadvantage of lowering the renal fraction and of apparently increasing the blood flow in the skin, and carcass. At the same time, another point in favour of pentobarbital is its widespread use in experiments of other kinds. Ether anaesthesia seems to be disadvantageous because of its increasing the cardiac output and greatly lowering the renal fraction [7].

The carcass fraction of cardiac output was significantly lower in the animals treated with pentobarbital than in those anaesthesized with urethane or chloralose and in the non-anaesthesized group. The high fraction of the unanaesthesized animals may have been due to muscle activity. In the absence of reliable "normal" values we could not decide whether the carcass fraction was decreased by pentobarbital or increase by urethane and chloralose. Even if pentobarbital had such an action, it still remains the anaesthetic of choice in view of the advantages mentioned above.

LITERATURE

1. SAPIRSTEIN, L. A.: Circul. Res. (N. Y.) **4,** 689 (1956).
2. SAPIRSTEIN, L. A.: Amer. J. Physiol. **193,** 161 (1958).
3. HAMILTON, W. F., MOORE, J. W., KINSMAN, J. M., SPURLING, R. G.: Amer. J. Physiol. **99,** 534 (1932).
4. SAPIRSTEIN, L. A., MANDEL, M. J.: Circul. Res. (N. Y.) **7,** 545 (1959).
5. SAPIRSTEIN, L. A., HANUSEK, G. E.: Amer. J. Physiol. **193,** 272 (1958).
6. SAPIRSTEIN, L. A., SAPIRSTEIN, E. H., BREDMEYER, A.: Circul. Res. (N. Y.) **8,** 135 (1960).
7. VIDT, D. G., BREDMEYER, A., SAPIRSTEIN, E. B. A., SAPIRSTEIN, L. A.: Circul. Res. N. Y. **7,** 759 (1959).
8. GÖMÖRI, P., TAKÁCS, L.: Z. ärztl. Fortbild. **50,** 286 (1956).
9. GÖMÖRI, P., TAKÁCS, L.: Amer. Heart J. **59,** 161 (1960).
10. TAKÁCS, L., KÁLLAY, K., SKOLNIK, J.: Acta med. hung. **14,** 457 (1959).
11. SAPIRSTEIN, L. A.: Fed. Proc. **16,** 111 (1957).

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THE EFFECT OF EPINEPHRINE AND NOR-EPINEPHRINE ON PULMONARY AND SYSTEMIC CIRCULATION IN THE DOG, BEFORE AND AFTER EXTIRPATION OF THE THORACIC SPINAL CORD

By

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Removal of the thoracic spinal cord (Th I—XII.) in anaesthesized dogs did not affect vascular tone in the pulmonary circulation.

Infusion of the large doses of nor-epinephrine increased cardiac output in the intact animal; no significant vasoconstriction was observed in the systemic and pulmonary circulations.

After removal of the thoracic spinal cord, infusion of the same doses of nor-epinephrine induced peripheral vasoconstriction. There was no significant elevation in pulmonary arteriolar resistance.

Single injections of epinephrine resp. nor-epinephrine increased pulmonary pressure. Removal of the thoracic part of the spinal cord increased the sensitivity of the peripheral vessels only to epinephrine.

Humoral and neural regulation of the pulmonary circulation is not yet clearly understood. Regarding the effect of sympathomimetic drugs there is general agreement that both epinephrine and nor-epinephrine increase pulmonary arterial pressure. In part of the experiments however, left auricular resp. pulmonary capillary pressure has not been recorded, therefore the causes of the rise of pulmonary pressure cannot be deduced from these studies [1—5].

Since several authors found left auricular resp. pulmonary capillary pressure to increase after the administration of epinephrine or nor-epinephrine, the augmentation of pulmonary pressure should be considered a passive effect [6—8]. According to GILBERT [9], epinephrine resp. nor-epinephrine, by inducing spasm of the pulmonary venules, causes pulmonary hypertension. BARTORELLI [10], too, found an increase in pulmonary capillary pressure during the infusion of nor-epinephrine. He emphasized, however, that the increase of pulmonary arterial pressure precedes the increase of pulmonary capillary pressure, hence — at least in the beginning — pulmonary hypertension is brought about most probably by pulmonary arteriolar spasm. Most of the authors, in fact, observed pulmonary arteriolar vasospasm after the administration of epinephrine or nor-epinephrine [11—15].

The evaluation of the results is made especially difficult by the fact that epinephrine as well as nor-epinephrine definitely affect systemic circulation and the heart. Some authors endeavoured to overcome these difficulties by

means of an isolated lung or by artificial circulation [2, 8, 9, 12, 13]. There is no agreement regarding the differences between the effect of epinephrine and nor-epinephrine [16, 17].

Recently NAHAS [18] found a 45 per cent increase of the pulmonary pressure gradient in dogs after administration of 1 μg/kg nor-epinephrine, and increased nor-epinephrine sensitivity of the lesser circulation after severing the spinal cord at Th I. In this state after the administration of nor-epinephrine a 156 per cent increase of the pressure gradient was observed. The elevation of pressure after intravenous administration indicates that with the nervous control intact the vasoactive agents exert their effect through the vasomotor centre (the effect develops simultaneously in the systemic circulation); after dissection of the spinal cord, however, a direct effect must be assumed (with pulmonary hypertension preceding the increase in systemic pressure). These results point to the vasoconstrictor effect of epinephrine and nor-epinephrine in pulmonary circulation; this effect is definitely increased by severing the sympathetic connections. Nevertheless, blood flow (cardiac output) has not been determined in these studies and although nor-epinephrine does not generally increase cardiac output (see below), the observed pulmonary hypertension has been partly attributed to increased cardiac output.

Accordingly, we studied the reaction of the pulmonary and systemic circulation to a single dose of epinephrine and nor-epinephrine, and to an infusion of nor-epinephrine. Later these experiments were repeated after removal of the thoracic segment of the spinal cord. Cardiac output was also recorded, for evaluating vasoconstrictor or vasodilatator effects.

Methods

12 dogs of both sexes, weighing 9—18 kg each were used under chloralose anaesthesia (0.10 g/kg). Systemic blood pressure was registered from the carotid artery, pulmonary pressure by cardiac catheterisation (pulmonary art. pressure, pulmonary capillary pressure) with a four channel membrane manometer (type Vorsatz and Kállay). Cardiac output was recorded by the direct Fick method, oxygen consumption by Krogh's apparatus modified for dogs. The apparatus was connected to a trachea cannula introduced at the height of the thyroid cartilage. Arteriovenous O_2 difference was estimated by an oxymeter (type Atlas). Epinephrine (Tonogen, *Richter*) and nor-epinephrine (*Hoechst*) were used at a concentration of 10 μg/ml in physiological saline solution. Injections resp. infusions were performed through a cannula inserted into the femoral vein.

The spinal cavity was opened by means of laminectomy at the I and X dorsal vertebrae and the spinal cord was divided and removed, corresponding to the I—XII thoracic segment.

The experiment was performed as follows. After a control recording of cardiac output and blood pressure, infusion of nor-epinephrine was started and after stabilization of systemic blood pressure cardiac output and blood pressure were again determined. After cessation of the infusion and stabilization of the blood pressure a single injection of epinephrine resp. nor-epinephrine was given. The procedure was repeated after removal of the spinal cord, and in several cases a third time, after cutting the vagus nerve bilaterally.

The results were evaluated by STUDENT's "t" test.

Results

(i) *The effect of the extirpation of the thoracic spinal cord (Th I—XII) on systemic and pulmonary circulation* (Table I).

According to our observations, removal of a major part of the spinal cord is followed by a significant decrease of cardiac output and systemic blood pressure. Total peripheral resistance also decreases (the decrease is nearly significant), pointing to the well-known role of the sympathetic nervous system in the maintenance of peripheral vascular tone under physiological conditions.

The situation is quite different in the lesser circulation. Although there occurred a slight decrease in the mean pressure values after removal of the spinal cord, the change was not significant. The change in pulmonary vascular resistance was not significant either.

(ii) *The effect of nor-epinephrine infusion on systemic and pulmonary circulation* (Tables II and III).

The results obtained after nor-epinephrine infusion are summarized in the upper line of Table III. The mean rate of the nor-epinephrine infusion was 4.34 μg/kg/min. Systemic blood pressure was stabilized on the average 4.1 minutes after the infusion had been started, by which time approximately 20—30 ml fluid had been infused.

In contrast with data in the literature, a significant increase of cardiac output was observed after nor-epinephrine infusion. Subsequently there occurred a definite elevation of systemic blood pressure, but no significant change in peripheral resistance

Similar results were obtained for the lesser circulation, in that there was a significant increase in pulmonary arterial pressure, with the pulmonary capillary pressure unchanged. Elevation of the pressure gradient of the lesser circulation thus proved significant. The change of pulmonary arterioral resistance was nearly significant.

The bottom line of Table III shows the results after extirpation of the spinal cord and vagotomy. The mean rate of nor-epinephrine infusion was 3.91 μg/kg/min, stabilization of systemic blood pressure was usually reached in 4.5 minutes. The same quantity of physiological saline was administered as before the operation.

After removal of the spinal cord the nor-epinephrine infusion did not induce changes in cardiac output. Systemic blood pressure, as expected, increased more definitely, than before operation. The increase of peripheral resistance was significant.

Pulmonary arterial pressure increased significantly, definitely more than before removal of the spinal cord. Pulmonary capillary pressure remained unchanged, hence the increase of the pulmonary pressure gradient was also significant. The unchanged cardiac output and the augmented pulmonary gradient

Table I

The effect of the extirpation of the thoracic spinal cord (Th I—XII) on systemic and pulmonary circulation

	CO l/min		CAR mm Hg		TPR dynseccm^{-2}		PA mm Hg		PC mm Hg		GR mm Hg		PVR dynesecm^{-5}	
	b	a	b	a	b	a	b	a	b	a	b	a	b	a
Mean	1.57	0.94	144	64	8043	6203	16.9	13.7	9.5	7.1	8.4	7.0	423	676
Standard deviation	0.47	0.53	16	27	2890	2484	3.9	3.4	3.6	3.5	3.3	2.1	166	277
n	9		10		9		10		8		8		8	
P	<0.01		<0.001		<0.05		>0.05		~ 0.5		>0.10		>0.05	
difference per cent	-40		-55		$+23$		-19		-25		-17		$+60$	

b = before; a = after.

Table II

The effect of nor-epinephrine infusion on systemic and pulmonary circulation

No.	kg	Nor-epinephrine µg/kg/min	CO l/min		CAR mm Hg		TPR dyne·sec·cm^{-5}		PA mm Hg		PC mm Hg		GR mm Hg		PVR dyne·sec·cm^{-5}	
			c	n	c	n	c	n	c	n	c	n	c	n	c	n
1.	11	1.8	2.17		120		4 420		17		10		7		258	
		4.5	0.76	1.05	50	85	5 260	6 480	10	15	4	4	6	11	633	838
2.	9	4.4	1.94	1.82	165	220	6 800	9 670	14	25	9	13	5	12	206	527
			0.70	0.73	40	110	4 570	12 080	19	19	13	10	6	9	686	986
3.	10.5	5.2	0.83	1.49	135	210	13 012	11 280	22		16		6		578	
4.	11	5.9	0.91	0.75	145	230	12 700	24 530	12	16	9	9	3	7	243	742
		11.0		0.78	30	105		10 770	10	14		10		4		410
		8.6		0.96	35	100		8 340	10	14		11		3		250
5.	13	2.7	1.20	1.23	160	170	10 340	10 820	13	15	9	7	4	8	266	520
		3.8	0.48	0.76	25	75	4 150	7 890	12	14	7	7	5	7	835	738
		5.0		0.87		90		8 280		13		7		6		550
6.	13.5	2.6	1.98	2.37	140		5 700		18	18	8	14	10	4	400	135
7.	14	2.1	1.02	1.91	160	180	12 480	7 510	14	16	10	8	4	8	315	335
		2.5	0.74		100		11 050		14							
8.	14	2.5	1.20	1.50	160	200	10 600	10 600	19	21	9	10	10	11	660	585
		2.1	1.00	1.02	75	150	6 000	11 800								
		2.5	1.24	0.97	55	130	3 550	10 700	11	23	6	10	5	13	320	1070
		2.5	1.22	1.76	50	145	3 270	6 600	16	26	9	9	7	17	462	770
9.	11.5	5.2	2,05	2,87	130	175	5 050	5 450	20	31	6	8	14	23	545	720
		4.8	0.92	1.08	65	135	5 650	10 800	16	27	5	5	11	22	1080	1620
		2.6	1.22	1.50	30	135	1 970	7 200								
		5.2	0.85	0.67	35	95	3 300	11 400								
10.	12	8.3	1.53	1.85	130	160	6 800	6 900	17	24	7	7	10	17	522	735
		3.2	0.92	0.82	70	145	6 100	14 100	13	16	4	7	9	9	782	877
11.	13	3.4		1.15	165	205		14 200	27	28	11	12	16	16		1100
		2.3		1.28	100	175		11 200	17	23	13	12	4	11		685
		4.7	0.77	0.70	50	90	5 200	10 300	16	20	11	12	5	8	520	915
12.	13	4.7	1.06	1.18	135	210	10 200	14 200	25	24	18	14	7	10	530	680
		3.0	0.74	0.69	45	135	4 850	15 700	20	18	9	11	11	7	1190	810

c = control; n = nor-epinephrine infusion.

Table III

Statistical analysis of Table II

		CO l/min c	CO l/min nor	CAR mm Hg c	CAR mm Hg nor	TPR dyneseccm^{-5} c	TPR dyneseccm^{-5} nor	PA mm Hg c	PA mm Hg nor	PC mm Hg c	PC mm Hg nor	GR mm Hg c	GR mm Hg nor	PVR dyneseccm^{-5} c	PVR dyneseccm^{-5} nor
Before severing spinal cord	mean	1.37	1.70	148	196	9770	11 200	17.9	21.8	9.6	10.2	8.3	11.6	410	556
	standard deviation	0.47	0.61	15	23	2720	5.650	4.9	5.5	3.3	2.8	4.4	2.8	161	236
	n	10		10		9		10		10		10		10	
	P	<0.05		<0 001		>0.3		<0.02		>0.5		<0.05		>0.05	
	diff. per cent	+24		+32		+15		+22		+6		+40		+36	
After severing spinal cord	mean	0.93	0.97	55	125	4520	10 420	14.4	19.6	8.3	8.8	6.8	11.7	723	958
	standard deviation	0.24	0.34	24	29	1220	2.910	3.4	4.7	3.3	2.7	2.2	4.2	283	269
	n	12		18		12		15		13		13		9	
	P	>0.2		<0.001		<0.001		<0.001		>0.3		<0.01		<0.05	
	difference, per cent	+4		+127		+131		+35		+6		+72		+33	

c = control; nor = nor-epinephrine infusion.

Table IV

Single injections of epinephrine, single injections of nor-epinephrine

op

No.	kg	CAR mm Hg b	CAR mm Hg a	PA mm Hg b	PA mm Hg a	CAR mm Hg b	CAR mm Hg a	PA mm Hg b	PA mm Hg a	CAR mm Hg b	CAR mm Hg a	PA mm Hg b	PA mm Hg a	Dose μg
1.	11					45	75	16	11					100
						45	95	14	23					100
3.	10.5	135	222	22	24	65	205	18	28	105	215	22	24	100
4.	11	145	185	12	16	30	120	10	12	145	230	11	17	100
						35	85	10	11					100
						40	75	13	14					100
5.	13	160	205	13	14	25	85	12	12	160	190	14	16	100
						25	75	12	13					100
6.	13.5	135	265	19	20	100	225	19	18	145	265	18	24	100
7.	14	160	185	14	15	50	105			155	190	15	20	100
						55	100	14	17					100
						45	85	11	13					100
8.	14	160	220	19	22	55	120	11	22	120	190	16	20	100
						55	140	15	25					100
						35	145	16	25					100
9.	11.5	130	195	9	30	65	150	8	20	125	220	12	37	100
						30	85	9	16					100
						35	95	8	12					100
10.	12	130	195	17	26	70	120	13	19	130	180	14	26	50
						50	125	11	14					50
11.	13	155	200	27	28	85	160	16	21	160	235	21	26	50
						25	75	16	18					50
12.	13	135	190	19	24	70	130	15	19	140	195	19	26	50
						40	125	16	20					50

b = before; a = after.

indicated an increase in arteriolar resistance in the lesser circulation. This increase was not significant; percentually it corresponded to the values obtained before the operation. Accordingly, the sensitivity to nor-epinephrine of the arterioles of the lesser circulation was not augmented by the removal of the thoracic spinal cord.

(iii) *The effect of a single injection of epinephrine resp. nor-epinephrine on pulmonary circulation.* Tables IV and V show the effect of a single injection of

Table V

Mean pressure differences after single injections of

	Epinephrine				Nor-epinephrine			
	CAR		PA		CAR		PA	
	b	a	b	a	b	a	b	a
Mean	61.5	77.7	5.0	4.3	72.5	82.5	7.4	3.1
standard deviation	31.0	31.0	6.2	3.9	30.5	21.0	6.8	2.5
n	10		10		10		10	
P	<0.02		>0.7		>0.1		>0.05	

b = before; a = after.

50 and 100 μg of epinephrine resp. nor-epinephrine (6.6 μg/kg on the average). The tables demonstrate that extirpation of the spinal cord increased the epinephrine sensitivity of the systemic circulation, whereas the lesser circulation was unaffected. The sensitivity to nor-epinephrine was unchanged after removal of the spinal cord both in the systemic and the lesser circulation.

Discussion

Ad (i). NAHAS [19, 20] demonstrated an average decrease of 5.3 mm Hg in pulmonary arterial pressure after extirpation of the sympathetic nervous system from Th I to Th VIII. It is not said whether or not the changes were significant. We observed a mean decrease of 3.3 mm Hg in pulmonary arterial pressure after removal of the spinal cord from Th I to Th XII. The pressure gradient in the lesser circulation suffered a mean decrease of 1.4 mm Hg. Neither of these changes were statistically significant nor was there a significant increase in arteriolar resistance in the lesser circulation.

These observations, however, do not yet deny the role of the sympathetic nervous system in the physiological regulation of pulmonary circulation, as demonstrated by the experiments of DALY *et al.* [15]. The extirpation of a large part of the spinal cord namely interrupts part of the sympathetic innervation of the systemic vessels and there are numerous interactions between peripheral circulation and pulmonary circulation.

Ad (ii). In the case of intact innervation the large doses of nor-epinephrine used by us did not elevate arteriolar resistance in the pulmonary circulation. The increase of the pulmonary pressure gradient must have been due to an increase of cardiac output. According to several authors, nor-epinephrine does not change, or even decreases, cardiac output [1, 21—24]. The doses administered by these authors were, however, definitely smaller, than ours. They infused 0.06 to 1.0 μg/kg/min while our usual rate was 4.0 μg/kg/min, since we aimed at inducing a significant increase in systemic pressure, one amounting to 50 mm Hg. This might have been the cause of the divergence. From this it follows that this part of our experiments supplied no information concerning the vascular effect of nor-epinephrine which would have been produced with no change in the flow.

After extirpation of the spinal cord, infusion of the same large dose of nor-epinephrine failed to elevate cardiac output. This was due most probably to the decreased venous tone.

Thus, after removal of the spinal cord a "pure" vascular effect was exerted by nor-epinephrine in the lesser circulation. The response of the vessels was presumably modified by nor-epinephrine accumulated in the heart muscle or in the vessels' wall. The increase of arteriolar resistance in the lesser circulation was not significant, although the percentual increase of the pressure gradient of pulmonary circulation amounted to more than before extirpation of the spinal cord.

After termination of our experiments has been published the study of DUKE and STEDEFORD [25] demonstrating a decrease in the cat's pulmonary vascular resistance after removal of the stellate ganglion and the upper sympathetic chain. Infusion of epinephrine (appr. 1.0 μg/kg/min) did not increase the pulmonary resistance only after sympathectomy. With nor-epinephrine, only a few experiments were made, and no definite conclusions were drawn from them.

As in our experiments the infusion of nor-epinephrine increased cardiac output before removal of the spinal cord while following removal of the cord the increase failed to appear, the changes in pulmonary resistance before and after the operation cannot be compared. To clarify the problem, we are studying the effect of the usual small doses of nor-epinephrine.

Ad (iii). Pulmonary capillary pressure was not recorded after single injections of epinephrine resp. nor-epinephrine. As the infusion of nor-epinephrine did not alter pulmonary capillary pressure neither before nor after extirpation of the spinal cord, the recorded changes of pulmonary pressure were reliably representing the shifts of the pulmonary pressure gradient. Our findings indicated that extirpation of the spinal cord increased the sensitivity of the vessels of the systemic circulation only, and only to epinephrine. The sensitivity of the vessels of the lesser circulation increased neither to epinephrine,

nor to nor-epinephrine. It may therefore be assumed that the actual state of the sympathetic nervous system does not affect the reaction of the pulmonary vessels to epinephrine and nor-epinephrine.

Epinephrine and nor-epinephrine were administered in large doses in these studies, to produce definite effects in the systemic circulation and to study them in connection with the changes in the lesser circulation. Pulmonary changes did not always follow the changes of the systemic circulation. This means that the regulation of the lesser circulation differs from that of systemic circulation, in agreement with our earlier findings concerning the effect of isolated cerebral hypoxia on the lesser circulation [26, 27], and the effect of different types of shock (haemorrhage, injury, dehydration) on systemic and pulmonary circulation [28].

LITERATURE

1. Goldenberg, M., Pines, K. L., Baldwin, E. F., Greene, D. G., Roh, C. E.: Amer. J. Med. **5,** 792 (1948).
2. Tyrer, J. H.: Quart. J. exp. Physiol. **38,** 169 (1953).
3. Forman, S., May, L. G., Bennett, A., Kobayashi, M., Gregory, R.: Proc. Soc. exp. biol. (N. Y.) **83,** 847 (1953).
4. Kleinerman, L., Marcus, N., Georgescu, M.: Rev. Fiziol. norm. patol. **2,** 44 (1955).
5. Herzenberg, H., Adams, F. H., Lind, J.: Cardiologia (Basel) **35,** 47 (1959).
6. Hamilton, W. F., Woodbury, R. A., Vogt, E.: Amer. J. Physiol. **125,** 130 (1939).
7. Fowler, N. O., Westcott, R. N., Scott, R. C., McGuire, J.: J. clin. Invest. **30,** 517 (1951).
8. Borst, H. G., Berglund, E., McGregor, M.: J. clin. Invest. **36,** 669 (1957).
9. Gilbert, R. P., Hinshaw, L. B., Kuida, H., Vissher, M. B.: Amer. J. Physiol. **194,** 165 (1958).
10. Bartorelli, C., Folli, G., Rumolo, R., Agostoni, E.: Cuore e Circol. **38,** 65 (1954).
11. Witham, A. C., Fleming, J. W.: J. clin. Invest. **30,** 707 (1951).
12. Rose, J. C., Freis, E. D., Hufnagel, Ch. A., Massulo, E. A.: Amer. J. Physiol. **182,** 197 (1955).
13. Patel, D. J., Burton, A. C.: Circul. Res. **5,** 620 (1957).
14. Patel, D. J., Lange, R. L., Hecht, H. H.: Circulation (N. Y.) **18,** 19 (1958).
15. de Burgh Daly, M., Luck, C. P.: J. Physiol. (Lond.) **145,** 108 (1959).
16. de Largy, C., Greenfield, A. D. M., McCorry, R. L., Whelan, R. F.: Clin. Sci. **9,** 71 (1950).
17. Zanetti, M. E., Opdyke, D. F.: J. Pharmacol. exp. Therap. **109,** 107 (1953).
18. Nahas, G. G., McDonald, J.: Amer. J. Phsiol. **196,** 1045 (1959).
19. Nahas, G. G., Mather, G. W., Kitchell, R. L.: Fed. Proc. **12,** 102 (1953).
20. Nahas, G. G., Mather, G. W., Wargo, J. D. M., Adams, W. L.: Amer. J. Physiol. **177,** 13 (1954).
21. Gilmore, J. P., Smythen, C. M., Handford, S. W.: J. clin, Invest. **33,** 884 (1954).
22. Barcroft, H., Starr, I.: Clin. Sci. **10,** 295 (1951).
23. Collier, H. D., Meyers, F. H., Schmidt, G. H.: Amer. J. Physiol. **189,** 224 (1957).
24. Tuckman, J., Finnerty, F. A.: Circul. Res. **7,** 988 (1959).
25. Duke, H. N., Stedeford, R. D.: Circul. Res. **8,** 640 (1960).
26. Gömöri, P., Takács, L., Kállay, K.: MTA V. Oszt. Közl. (Budapest) **8,** 269 (1957).
27. Gömöri, P., Takács, L., Kállay, K.: Acta med. hung. **16,** 75 (1960).
28. Kállay, K., Takács, L.: In press.

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A NEW TYPE OF VASOMOTOR REFLEX ELICITABLE FROM THE CORONARY SINUS

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A cannula with an inflatable balloon on it was introduced into the coronary sinus. Blood outflow from the sinus through the cannula was not interfered with even when the balloon was inflated.

Stretching the wall of the sinus by the balloon caused blood pressure to fall. The depressor response was abolished by vagotomy. The duration of the hypotensive response was very long, blood pressure remaining low as long as the balloon was inflated, in contrast with the carotid sinus reflex, in which blood pressure returns soon to its initial level in spite of the continued pressure exerted on the sinus wall. From this it follows that the reflex plays a role not in the maintenance of a given level of blood pressure, but in a lasting adjustment of blood pressure to a new level.

The adjustment of blood pressure to a new level is realized through a shift in the pressor-depressor balance in the depressor direction. During coronary sinus reflex hypotension, the carotid sinus hypertensive reflex cannot be elicited, whereas the carotid sinus hypotensive reflex can.

The reflex exerts a generalized effect and modifies circulatory equilibrium as a whole owing to the fact that its afferent fibers form connections in the reticular formation whence inhibition spreads diffusely to the centers taking part in the regulation of blood pressure.

Fundamental knowledge concerning the reflex regulation of blood pressure is based primarily on studies of the stretch receptors in the carotid sinus. Stimulation of these receptors may be carried out directly without creating changes in the systemic circulation. The possibility of "direct" stimulation made it possible to obtain detailed information on the function of these receptors. Stimulation of the other stretch receptor zones (for instance that of the auricular receptors) is usually carried out by producing changes in the systemic circulation. Thus, when describing the responses elicitable from various areas we cannot go further than to state that the response is a depressor or pressor one. Owing to the methodical difficulties involved in gaining access to these areas, it has been impossible to tell in what further respect these reflexes differ from the carotid sinus reflex. Thus, the question is: if two reflexes act alike in that they are both pressor or depressor in nature, would this determine their place in the regulation of blood pressure, or are there further qualitative differences that may justify us to speak about different types of reflexes even within the pressor and depressor categories?

In the investigations described below we have studied the coronary sinus, a receptor area not known before. Since it is possible to stimulate the receptors

in this area without producing changes in systemic circulation, the nature of the reflex response originating here could be readily compared with those elicited from other zones, notably the carotid sinus. Moreover, we could study the interaction between the responses elicited from the two receptor zones without the stimulus employed having by itself any direct effect on the other receptor zone.

Methods

Thirty-five dogs of either sex were used. The experiments were carried out under chloralose, morphine-chloralose, or chloralose-urethane narcosis. The dose of the anesthetic was individualized, the elicitability of the response depending not on the dose of the anesthetic, but on the depth of narcosis (0.05 g/kg to 0.1 g/kg of chloralose). Anesthetics were injected very slowly into the saphenous vein until the animal was narcotized, but the corneal reflex was still present. It was found that general anesthesia must be induced with the utmost precision, because in deeper narcosis, when the corneal reflex was diminished or absent, the reflex response could not be elicited.

The chest was opened by making incisions in the fourth costal interspace on both sides and transecting the sternum. Artificial respiration was started and a small incision was then made in the pericardium. A Morawitz cannula was inserted into the coronary sinus through the right auricular appendage. On the tip of the cannula there was an inflatable balloon, by means of which the tension of the sinus wall could be altered. Blood from the sinus was returned to the femoral vein through a polyethylene tube. Thus, sinus blood outflow was ensured even when the balloon on the cannula was inflated.

The carotid sinus hypertensive reflex (C.S.H.R.) was elicited by clamping one or both common carotids. The carotid sinus depressor reflex (C.S.D.R.) was elicited in some cases by pulling the carotid by means of a thread previously placed under one common carotid; the thread passed over a pulley and had a weight on it, ensuring a constant tension on the artery. In other cases a carotid sac was created in which pressure was altered by injecting different volumes of physiologic salt solution. Carotid sinus pressure was recorded by means of a mercury manometer on kymograph. Blood pressure was recorded in the usual way from the femoral artery using a mercury manometer.

In a few experiments we have studied the effect of the coronary sinus reflex on the corneal and patella reflexes. Sensitivity of the corneal reflex was registered by touching the cornea at one point at equal time intervals and equal pressure. The measure of the elicitability of the corneal reflex was determined by the number of touches required for eliciting one contraction.

In studying the knee jerk the tendon of the unilateral rectus femoris muscle was prepared and the contractions of the muscle were recorded on kymograph by the usual method. In such cases we either recorded the spontaneous reflex activity of the animal under chloralose, or applied rhythmic stimulation with rectangular impulses to the central stump of the sciatic nerve.

Atropine sulphate, and other drugs, or blood was injected into the saphenous vein.

The receptors of the coronary sinus were demonstrated histologically by the silver impregnation method of GROS-SCHULTZE [25].

Results

Stimulation of the coronary sinus receptors and the afferent pathway

Systemic blood pressure dropped in response to pressure exerted on the wall of the sinus by inflating the balloon in the coronary sinus (Fig. 1*A*). The depressor response was influenced by the depth of anesthesia and by the initial blood pressure level. When anesthesia was so deep that the formerly responsive corneal reflex became diminished or could not be elicited at all,

inflation of the balloon caused only a slight, or no fall of blood pressure. Likewise, when the initial blood pressure was low, inflation of the balloon hardly had any depressor effect.

The question arose whether the phenomenon observed was due to a true reflex response or merely to hemodynamic changes.

The most convincing evidence indicating that this phenomenon is based on a purely reflex mechanism is the fact that it could not be elicited after vagotomy. Figure 1*C* shows that after vagotomy inflating the balloon produces

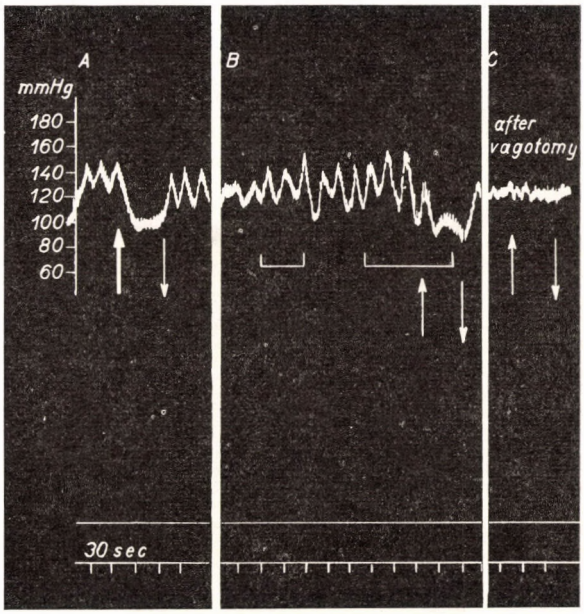

Fig. 1 A) Blood pressure response to inflation of the balloon in the coronary sinus
B) Inflation of the ballon during the carotid sinus hypertensive reflex causes a fall in blood pressure similar to that shown under *A)*
C) Inflation of the balloon causes no fall in blood pressure after vagotomy. Time signal: 30 sec. ↑ balloon inflated ↓ balloon deflated. |_____| clamping of the bilateral common carotids

no depressor response indicating that the pathway of the reflex runs in the vagus. Since atropinization does not block the reflex, it can be inferred that the *afferent* fibers are involved in the depressor response and not the *efferent* fibers.

Not only vagotomy, but also painting the environment of the sinus with cocaine abolished the depressor response; this is additional evidence indicating that the elicitability of the reflex is bound to the intactness of certain nervous structures.

Effect of coronary sinus receptors on respiration

As the experiments were carried out on dogs with an open chest, the respiratory response could be studied only superficially. After inflating the balloon in the sinus, the respiratory movements sometimes seen during artificial respiration (chest movements, gasping) were not observed. From these we conclude that the reflex also has an inhibitory action on the respiratory center.

Comparison of the coronary sinus and carotid stretch receptors

The coronary sinus reflex differs in many outstanding features from the carotid sinus depressor reflex. The most obvious difference is in the duration of the depressor response. In the case of the coronary sinus reflex, blood pressure remains depressed as long as the balloon is inflated (Fig. 1*A*, Fig. 2). In the

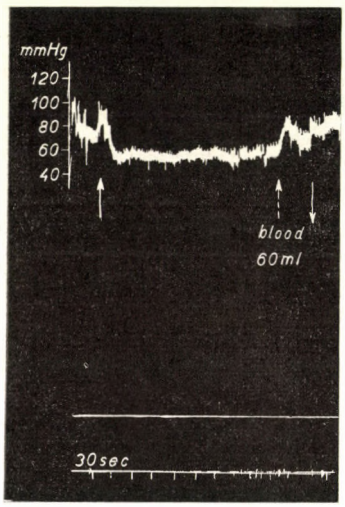

Fig. 2. The balloon in the sinus is kept inflated for sixteen minutes during which blood pressure remains at the new lower level. The arrow with the broken shaft indicates the intravenous administration of 60 ml blood, which caused only a short depressor response; in about three minutes blood pressure is back again at the resting level. Signs as in Figure 1

experiment shown in Figure 2 the balloon was inflated for 16 minutes and, as can be seen, the blood pressure was maintained at a constant low level for the period of increased sinus tension. In many experiments the balloon was kept inflated for one to two hours during which time the blood pressure remained at the new lowered level. This may indicate that the receptors involved in this reflex apparently adapt very slowly or not at all. To facilitate comparison,

we present the response typical to stimulation of the carotid sinus receptors (Fig. 3). As can be seen, the depressor response to the increase of pressure in the carotid sinus sac is quite different in nature. The blood pressure returns to the initial level in spite of continuous stretch of the walls. Thus, the decisive difference between the two reflexes may be outlined as follows: *While the carotid sinus reflex preserves a given blood pressure equilibrium for a shorter*

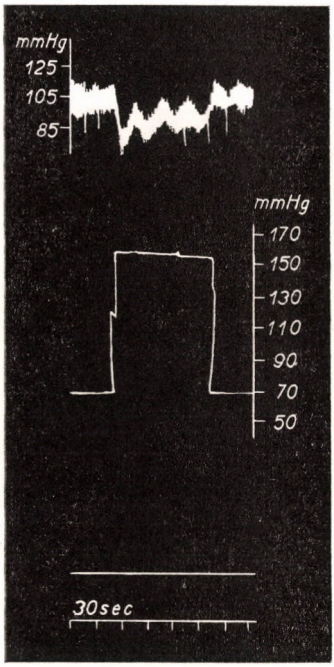

Fig. 3. Blood response characteristic of carotid sinus receptor function. In the left carotid sac blood pressure rises from 70 mm Hg to 150 mm Hg. Although pressure in the sac remains high, blood pressure gradually returns to the control level

or longer period, the long duration of the depressor response to the coronary sinus reflex suggests that here we are dealing not with the preservation of any given equilibrium, but with the adjustment and maintenance of a new blood pressure level.

Effect of the coronary sinus reflex on the elicitability of the C.S.H.R.

Adjustment of the blood pressure to a lower level means of course that the reflexes controlling equilibrium are influenced. We may say that the reflex responsible for adjusting the level influences the other reflexes involved in the maintenance of that level. In the case of hypotensive stabilization of the

blood pressure this means a suppression of hypertensive tendencies, as a result of which blood pressure equilibrium shifts in the depressor direction. As Figure 4 indicates, this is what actually happens, for the C.S.H.R. cannot be elicited as long as the balloon in the coronary sinus is inflated. However, when it is deflated, elicitability of C.S.H.R. returns gradually. Moreover, even in cases where the initial blood pressure level is low and inflation of the balloon in the coronary sinus will cause no fall in blood pressure, C.S.H.R. cannot be elicited. This suggests that the inhibitory action of the coronary sinus reflex on the pressor tendencies cannot be ascribed to the fall in blood

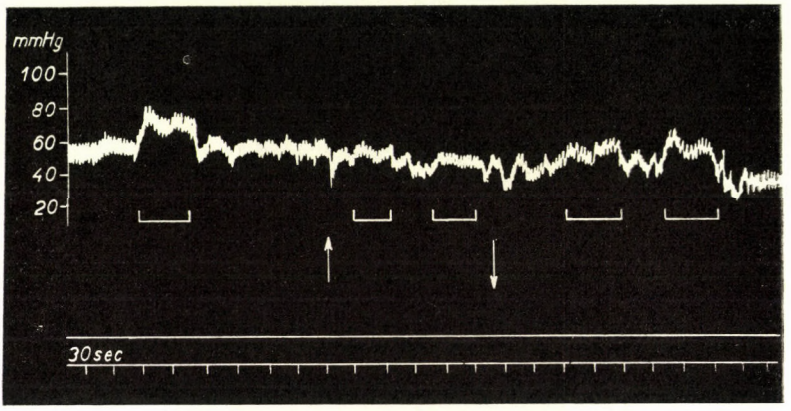

Fig. 4. Effect of inflation of the balloon in the sinus in an animal with low blood pressure; after bleeding the initial blood level is 60 mm Hg. Inflation of the balloon causes no fall of blood pressure but it prevents the development of the carotid sinus hypertensive reflex. After deflation of the balloon the elicitability of C. S. H. R. returns gradually. Signs as in Figure 1

pressure, but to an inhibitory action on C.S.H.R. which occurs in the vasomotor center. As Figure 1*B* shows, the depressor effect develops even when C.S.H.R. has been elicited and, even under such conditions, is similar to the hypotensive response shown in Figure 1*A*.

Effect of the coronary sinus reflex on other pressor tendencies

The coronary sinus reflex blocks not only the pressor response to clamping the common carotids, but also that due to other effects such as the intravenous administration of blood. As Figure 2 shows, as long as the balloon is inflated the injection of as much as 60 ml of blood causes no lasting shift from the new state of equilibrium; blood pressure soon returns to its stabilized new level and rises to the initial level only after the balloon has been deflated.

Effect of the coronary sinus reflex on the carotid sinus depressor reflex

It is obvious that a lasting stabilization of blood pressure at a new lower level is possible only when the reflexes involved in the maintenance of equilibrium remain intact. What we deal with is merely a shift in the equilibrium of the vasomotor center, where pressor effects are more or less suppressed, while the sensitivity of depressor reflexes are maintained. It is shown in Figure 5

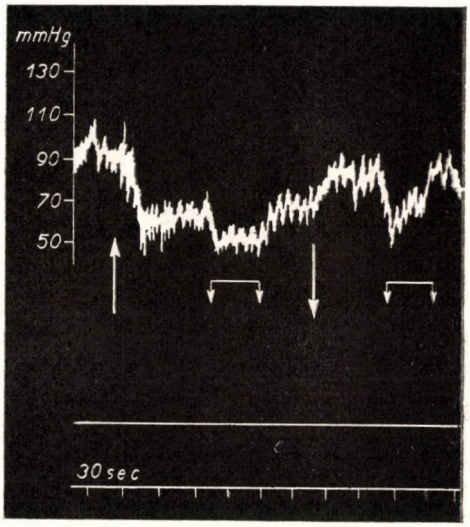

Fig. 5. Effect of stimulation of coronary sinus receptors on the depressor response to pulling the carotid nerve. The right common carotid is pulled by a thread passing over a pulley, with a weight at its end. The magnitude of the depressor response is the same, irrespective of the balloon being inflated or not. ↓ ↓ pulling the right common carotid. Other signs as in Figure 1

that as long as the balloon in inflated the depressor response to stretching the carotid is of the same magnitude as that resulting from stretching alone. Moreover, as long as the balloon is inflated, the depressor response lasts longer than in the case of stretching alone. Thus, stimulation of the coronary sinus receptors seems to increase the duration of the depressor response to stretching the carotid.

Effect of the coronary sinus reflex on the corneal reflex and knee jerk

The fact that the reflex can shift the balance of circulation as a whole suggests that the afferent impulse may extend to many central sites. We have therefore undertaken to study the influence of the reflex on two such anatomically distinct reflex centers as those of the corneal reflex and knee jerk. As it can be seen in Figure 6, eliciting the coronary sinus reflex in the animal under

chloralose the spontaneous reflex activity is extinguished and muscle tonus decreases markedly. At the same time, the elicitability of the corneal reflex is diminished. Figure 6*B* illustrates that an increased inflation of the balloon results in a more marked depressor response and in a stronger inhibition of reflex centers. Such an extensive spread of inhibition suggests that the afferent

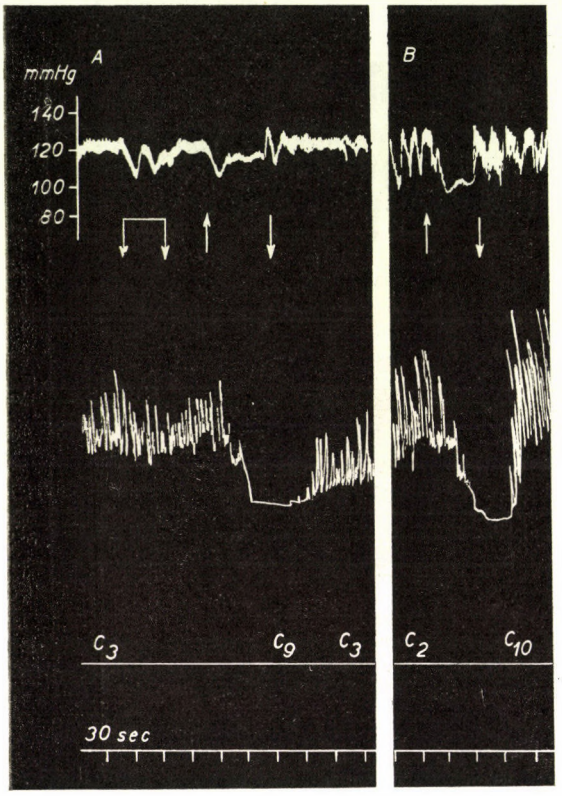

Fig. 6. Effect of carotid nerve and coronary sinus receptor excitation on the corneal reflex and knee jerk of the animal under chloralose. Above: blood pressure, center: spontaneous reflex activity traced from the tendon of the rectus femoris muscle. Figures on the upper baseline indicate elicitability of corneal reflex. Signs as in Figure 1 and Figure 5. Explanation in text

vagus fibers involved in the reflex have synapses in the reticular formation, whence inhibition spreads all over the vasomotor center. From this it follows that the coronary sinus reflex differs in many features from the C.S.D.R.; namely, that the fibers of the two carotid nerves are connected directly and only unilaterally to the right or left cell groups of the vasomotor center. Correspondingly, as it is seen in Figure 7, the depressor response to the stimulation of the unilateral carotid nerve cannot block the pressor response to clamping the contralateral common carotid. Thus, the C.S.H.R. can be

elicited even in the presence of the depressor response to stretching the contralateral carotid. However, as illustrated in Figure 4, stimulation of the coronary sinus receptors can inhibit, even without a fall in blood pressure, the hypertensive response to clamping the bilateral common carotids. As Figure 6*A* shows, while stimulation of the receptors of the coronary sinus strongly inhibits both the corneal and patella reflexes, stimulation of the carotid nerve has no influence whatever on the two reflexes even though there was a comparable

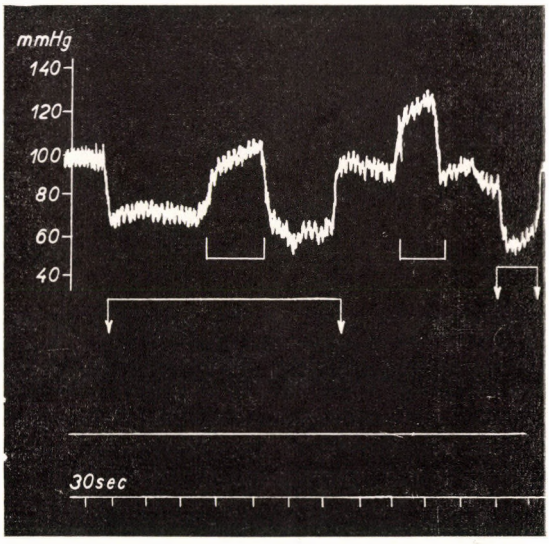

Fig. 7. Effect of pulling the right common carotid on the hypertensive response to clamping the left carotid. Blood pressure rises even during pulling. Signs as in Figure 1 and Figure 5

fall of blood pressure. This indicates that the fibers of the latter nerve *are not so* intimately connected with the reticular formation as are the afferents of the coronary sinus reflex, in accordance with the fact that the fibers of the carotid nerve are connected directly to the unilateral or contralateral cell groups in the vasomotor center.

Histological demonstration of the receptors

Detailed histological studies of the nerve elements in the coronary sinus are in progress. Here we wish to deal with them briefly. In the plane and cross sections of the coronary sinus many nerve elements are demonstrable by the silver impregnation method. Among many nerve elements, major nerve trunks are also visible near the coronary sinus. In the sinus proper the nerve

elements occur mainly in the adventitia in the form of nerve trunks and fine fibers. Single fibers may be followed through their ramifications until they end as receptors, showing brush-like branching. In the photomicrograph presented in Figure 8 shows the brush-like receptor ramifications of a fine nerve fiber.

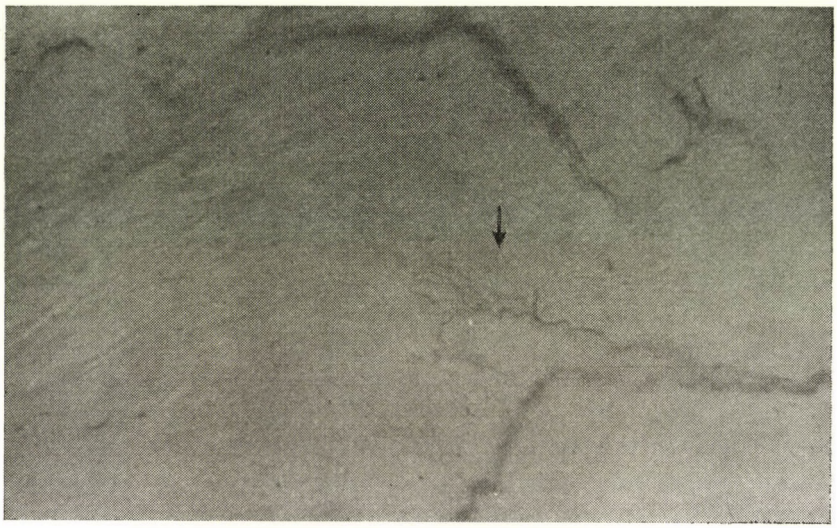

Fig. 8. Arrow pointing at brush-like receptor ramifications in the adventitia of the coronary sinus. Gros-Schultze silver impregnation

Discussion

The nervous factors taking part in the maintenance of a given level of blood pressure are well-known from the investigations of CYON [1], HERING [2, 3], HEYMANS [4] and others. A typical example of these so-called buffer reflexes is the carotid sinus reflex, the receptors of which are stimulated when blood pressure rises; as a result of their excitation, blood pressure returns to its original level. In other words, these reflexes are active only in the maintenance of a given level of blood pressure in restoring a shift from an equilibrium, but play little if any role in creating a new state of equilibrium in a permanent adjustment of the blood pressure to a new level. It is known that the hypertension following the transection of both carotid or depressor nerves is not always permanent [5] and means only that a normal factor hindering the increase of the sympathetic tone is absent; when sympathetic tone decreases, blood pressure returns to normal [6]. However, we have no knowledge about the existence of such receptor zones, the role of which would be distinct from that of these buffer reflexes in that they would not maintain a given blood

pressure, but would adjust the blood pressure to a new state of equilibrium at a new level. In addition to the carotid sinus receptor zone there are many other stretch receptor zones throughout the body. The function and physiological role of these are little known, partly because they are difficult to gain access to, and partly because their stimulation can be effected only by general hemodynamic changes which results in the simultaneous stimulation of other receptor zones. All that we know about them is that their stimulation results in a rise or fall of systemic blood pressure. We cannot say whether they play a role in the maintenance of the blood pressure equilibrium or in bringing about a shift in that equilibrium.

This applies first of all to the receptor zones in the systemic arterial areas. Vasomotor reflexes can be elicited in practically any systemic arterial area, for example, in the area of the thoracic aorta [7], in various abdominal vessels [8, 9, 10, 11], hepatic artery [12], in the extremities [13, 14, 15], and even in the carotid proper [16]. In most cases we do not know the site of the receptor, and information on the importance of these reflexes in the regulation of the blood pressure effects is by far not so complete as is for the carotid sinus receptor zones; they seem to serve local reactions and are therefore not capable of altering the level of systemic blood pressure.

Such studies on the receptors in the auricle and in the great veins [17, 18] are *a priori* impossible, because they can be stimulated exclusively by causing extreme changes in systemic circulation. More recently, the site of the receptors in those areas have been carefully analyzed [19]. We face even greater difficulties in the case of ventricular receptors, the existence and presence of which have only recently been ascertained [20, 21].

Pressor receptor zones in the pulmonary arteries have been described by many workes [22, 23]; the function of these seems to be identical with that of the carotid sinus reflex. No such reflex has thus far been described which would function to adjust blood pressure to a new level. Theoretically, such a reflex might be demonstrated on grounds of the following criteria: (1) Adjustment of blood pressure to a new level must be elicited from the receptors of the reflex and not by a stimulation of afferent fibers, (2) stimulation of the receptor zone must by itself not produce, or be associated with, changes in systemic hemodynamics, (3) the reflex should be elicitable simultaneously with the carotid sinus reflex so that the differences existing between the level-adjusting and the level-maintaining reflexes may become manifest with in one experiment, and (4) as the adjustment to a level means a change in the balance between the reflexes involved in the equilibrium, that is, a change in the sensitivity to them, it is desirable to investigate how the two kinds of reflexes act on each other. All these criteria are given in the case of stimulating the coronary sinus receptors. As the experiments prove, stimulation of these receptors without altering the outflow of blood from the sinus by means of an inflatable

balloon on a cannula will cause a reflex decline in blood pressure which is absent after vagotomy.

The coronary sinus reflex is similar to the carotid sinus reflex in that stimulation of either one results in a fall of blood pressure. There is, however, a substantial difference in the duration of the reaction. In the case of the carotid sinus reflex, blood pressure soon returns to its initial level although stretching of the sinus is maintained, whereas in the case of the coronary sinus reflex the depressor response lasts as long as stretching is maintained. In many experiments the balloon was kept inflated several hours and the new hypotensive level was maintained throughout that period. This circumstance by itself indicates that in the case of the coronary sinus reflex we deal not with a buffer system involved in the maintenance of blood pressure homeostasis at a given level, but with a reflex that provides for blood pressure homeostasis at a new level. It would not be appropriate to consider this reflex as a depressor one, because this term is reserved for the buffer systems acting against elevations of blood pressure. The term coronary sinus stabilizator reflex (C.S.S.R.) seems to be more appropriate, because it infers that the reflex plays a role in changing the level of blood pressure and in stabilizing equilibrium at that new level. Adjustment to and stabilization of the new hypotensive level of blood pressure, of course, means an adjustment to a new level of equilibrium, a shift of the pressor–depressor balance in the depressor direction. This is manifested in an inhibition of pressor tendencies, while depressor tendencies remain unaltered. In fact, even if eliciting the C.S.S.R. is not followed by a drop of blood pressure, the C.S.H.R. cannot be elicited, while the C.S.D.R. can.

The ability of the reflex to cause lasting changes in blood pressure equilibrium cannot be based on the presence of special non-adapting or slowly adapting receptors alone. To achieve a lasting shift in balance to block the action of buffer reflexes striving to eliminate the newly adjusted hypotensive level require special central nervous connections that make control of these reflexes possible. Our experiments have demonstrated the diffuse connections existing between these reflex pathways and the formation reticularis. Connection with the reticular formation is indicated by the fact that eliciting the C.S.S.R. may block so distant reflex centers as those of the corneal reflex and knee jerk. Of course, not only C.S.S.R. but also C.S.D.R. has connections with higher nervous centers, notably with the reticular formation [24, 25]. This is undersandable, because ultimately the vasomotor center is also part of the reticular formation, although a specialized one. However, these connections are not so extensive and diffuse as in the case of C.S.S.R. As Figure 6 shows, the same fall in blood pressure elicited by the coronary sinus receptors is associated with a strong inhibition of the knee jerk and corneal reflexes, whereas in the case of C.S.D.R. there is no inhibition. By increasing the intensity of stimulation, blockade of the above two reflex centers by the

C.S.D.R. can be achieved. Thus, there is a quantitative difference between the two reflexes. This difference may be explained by the fact that while the fibers of the bilateral carotid nerve are directly connected to the right and left side cells of the vasomotor center, from which they send relatively few collaterals to higher nerve centers or to other parts of the reticular formation, the afferents of the C.S.S.R. *form connections directly in the reticular formation, whence inhibitions spreads diffusely, flooding the vasomotor center as a whole.* If this were not so, the C.S.H.R. could not be blocked by the coronary sinus reflex. The unilateral vasomotor center representation of the carotids is indicated by the fact that the effect of clamping the unilateral carotid cannot be blocked by stimulating the contralateral carotid nerve.

Thus, the criteria of hypotensive stabilization seem to be, on the one hand, that the reflexes have special, slowly or non-adapting receptors and on the other hand, that the afferents have special central nervous connections. As a third criterion, it may be assumed that in the reactions of this type the "C" afferents of specially slow conductivity take part. DOUGLAS and RITCHIE [26 27, 28] have found lasting blood pressure response to the stimulation with high parameters of the vagus and depressor nerves. This fact alone is no proof that these fibers are in fact stabilizator ones. Most of them are likely to be ones of buffer nature and differ in their effect from that of "A" fibers in that they carry out the buffering of slower, more lasting changes. Stimulation of the receptors permits us to decide whether we deal with this, or with a true adjustment of level. At any rate, these investigations call attention once again to "C" fibers. Such a role of "C" fibers is suggested by the observations we have made in lightly anesthetized, waking dogs. When we elicited C.S.S.R. in such dogs, the animals which had been exhibiting increased reflex activity and respiratory movements quieted down. The fact that a similar effect was obtained after bilateral vagotomy and stimulating the unilateral central vagal stump with parameters corresponding to the threshold of "C" fibers, also may indicate that the C.S.S.R. is transmitted by "C" fibers. We think that the "C" afferents are not necessarily stabilizator fibers, though it is likely that the stabilizator fibers are "C" fibers. Their role in the regulation of blood pressure is determined by the receptors belonging to them and by their central nervous connections. It is probable that various intermediate forms exist between the buffer nerves eliminating lasting effects and the true level-adjusting stabilizator systems. The latter, however, can be distinguished from the buffer nerves of slower action, inasmuch as their role is to adjust blood pressure to a new level, just by controlling the function of the former nerves involved in the maintenance of equilibrium. If a buffer nerve has an ever so long-lasting action, this action will be restricted to a maintenance of an equilibrium determined for it by the primary, level-adjusting reflexes.

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LITERATURE

1. CYON, E., LUDWIG, C.: Arbeiten a. d. Physiologischen Anatomie zu Leipzig 1866, 128.
2. HERING, H. E.: Münch. med. Wschr. **71,** 701 (1924).
3. HERING, H. E.: Münch. med. Wschr. **72,** 1265 (1924).
4. HEYMANS, C., BOUCKAERT, J. J., REIGNIERS, P.: Le sinus carotidien et la zone homologue cardio-aortic. Doin, Paris 1933.
5. KOCH: 46. Kongr. Dtsch. Ges. f. inn. Med. cit. 6.
6. HEYMANS, C., BOUCKAERT, J. J.: C.R. Soc. Biol. (Paris) **117,** 252 (1934).
7. GRUZHIT, C. C., MOE, G. K.: Amer. J. Physiol. **171,** 730 (1953).
8. GAMMON, G. D., BRONK, D. W.: Amer. J. Physiol. **114,** 77 (1935).
9. HEYMANS, C., BOUCKAERT, J. J., FARBER, S., HSU, F. Y.: C.R. Soc. Biol. (Paris) **122,** 115 (1936).
10. HEYMANS, C., BOUCKAERT, J. J., WIERZUCHOWSKI, M.: C.R. Soc. Biol. (Paris) **123,** 286 (1936).
11. HEYMANS, C., BOUCKAERT, J. J.: C. R. Soc. Biol. (Paris) **123,** 986 (1936).
12. WAELE, H. DE, VAN DER VELDE, J.: C. R. Soc. Biol. (Paris) **113,** 85 (1933).
13. FERNANDEZ MOLINA, A., ACHARD, O., WISS, O. A. M.: Helv. physiol. pharmacol. Acta **11,** 1 (1953).
14. FOLKOW, B.: Acta physiol. scand. **17,** 289 (1949).
15. COMROE, J. H., JR., SCHMIDT, C. F.: Amer. J. Physiol. **138,** 536 (1943).
16. BOSS, J., GREEN, J. H.: Circul. Res. **4,** 12 (1956).
17. BAINBRIDGE, F. A.: J. Physiol. (Lond.) **50,** 65 (1915).
18. MCDOWALL, R. J. S.: The Control of the Circulation of the Blood. Dawson & Son, London, 1938.
19. COLERIDGE, J. C. G., HEMINGWAY, A., HOLMES, B. L., LINDEN, R. J.: J. Physiol. (Lond.) **136,** 174 (1957).
20. AVIADO, D. M., JR , SCHMIDT, C F.: Amer. J. Physiol. **196,** 726 (1959).
21. DOUTHEIL, U., KRAMER, K.: Pflüg. Arch. ges. Physiol. **196,** 726 (1959).
22. CHURCHILL, E. D., COPE, O.: J. exp. Med. **49,** 531 (1929).
23. SCHWIEGK, H.: Pflüg. Arch. ges. Physiol. **236,** 206 (1935).
24. GELLHORN, E., YESINICK, L., KESSLER, M., HEILMANN, H.: Amer. J. Physiol. **137,** 369 (1942).
25. SCHULTZE, F. J., HENATSCH, H. D., BUSCH, G.: Pflüg. Arch. ges. Physiol. **269,** 248 (1959).
26. DOUGLAS, W. W., SCHAUMAN, W.: J. Physiol. (Lond.) **132,** 173 (1956).
27. DOUGLAS, W. W., RITCHIE, J. M., SCHAUMAN, W.: J. Physiol. (Lond.) **132,** 187 (1956).
28. DOUGLAS, W. W., RITCHIE, J. M.: J. Physiol. (Lond.) **134,** 167 (1956).

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TOLERANCE TO TREMORINE

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Tremorine has been shown to cause unusually rapid habituation in the mouse. Tolerance comprises all of the three main central effects of the drug, *viz.*, tremor-producing action, analgesic effect and anaesthesia-prolonging action. Sensitivity to tremorine returns after discontinuing of drug administration for 1 to 2 weeks. The property of tremorine to be a drug of tolerance has to be kept in mind, when used in routine pharmacological screening.

Tremorine (1,4-dipyrrolidino-2-butyne) is a drug characterized by the ability to produce in laboratory animals sustained tremor strikingly resembling human parkinsonism [1, 2]. The tremorine test has been introduced into the routine screening methods of pharmacology by EVERETT *et al.* [1, 2], and is now increasingly used in the search for new anti-parkinsonism substances, since the tremor induced by this drug is selectively antagonized by agents effective in the treatment of human parkinsonism [1, 3, 4, 5, 6, 7, 8]. Apart from the tremor-producing action, tremorine exhibits marked analgesic and anaesthesia-prolonging effects [7]. The analgesic action has also proved useful in testing of anti-parkinsonian drugs [8].

In our mouse experiments [9], the analgesic activity of tremorine exceeded that of morphine, while its anaesthesia-prolonging effect was about equal in strength with that of chlorpromazine. These effects, too, were completely counteracted by antiparkinsonian drugs, a fact indicating that not only the tremor-producing action, but also the antagonism against the analgesic or anaesthesia-prolonging effects can be used for testing new anti-parkinsonian substances [9], a possibility expressed also by CHEN [8].

In the course of the above experiments, the observation has been made that the effects of tremorine are gradually diminishing after repeated treatment with the drug. The present paper deals with a more detailed analysis of this finding.

Methods

White mice weighing 17—20 g each were used. Two sets of experiment were carried out as to the addictive properties of tremorine. First, the tolerance to the analgesic action was investigated. For this purpose, 40 mice were treated with 6 mg/kg tremorine subcutaneously at alternative days. This dose produces nearly complete analgesia, without causing visible tremor

or any marked prolongation of anaesthesia. The animals were treated for 16 days and the analgesic action was continuously measured.

In other experiments, 50 mice were treated with 18 mg/kg tremorine administered intraperitoneally or subcutaneously. Tremorine at this dose level elicits marked tremor, complete analgesia and very marked prolongation of ether anaesthesia. Treatments were performed at two-days intervals for 10 days. Administration of the drug was then discontinued and the return of sensitivity checked after a period of 1 to 2 weeks.

The tremor of the animals was judged by gross observation, considering a mouse "positive", when showing definite tremor also at rest. In some cases, quantitative evaluation of the tremor was carried out by the apparatus described by AHMED and TAYLOR [6].

The ether anaesthesia-prolonging effect was estimated in the manner previously described [14].

The analgesic action was determined in two ways: (i) by making use of the hot plate method described by WOLFE and MCDONALD [10], as modified by HERR and PÓRSZÁSZ [11]; (ii) by employing the procedure of HAFFNER [13], with the modifications given by BIANCHI and FRANCESCHINI [12].

Results

Tolerance to the analgesic action of low doses (6 mg/kg subcutaneously) of tremorine is demonstrated in Fig. 1. As seen, tremorine at this dose level

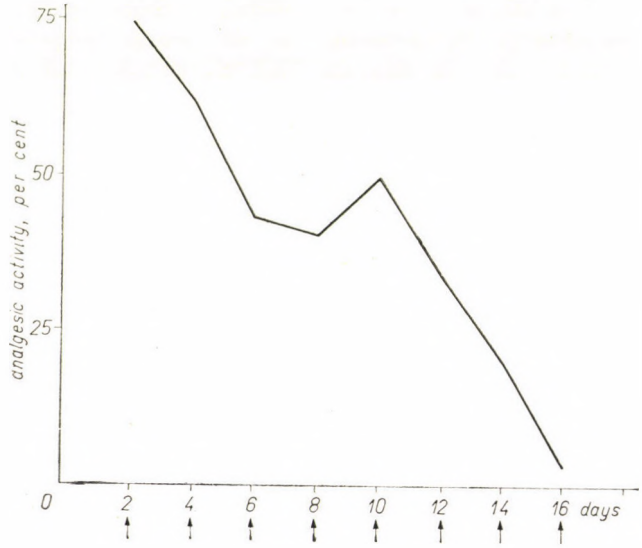

Fig. 1. Decrease of the analgesic activity of tremorine after repeated administration of 6 mg/kg intraperitoneally. † = 6 mg/kg tremorine

causes analgesia above 75 per cent, when given on the first occasion. Thereafter, the analgesic activity gradually decreases, completely to disappear after 16 days, in other words after 8 doses administered at two-days intervals. Fig. 1 shows the results obtained by the hot plate method: the results yielded by HAFFNER's method were in agreement with these.

Fig. 2 shows the results of continuous treatment with 18 mg/kg tremorine. As said above, this dose induced not only analgesia, but also a marked prolongation of anaesthesia, in addition to tremor, in all animals. This was, however, true only for the first and, sometimes, for the second treatment. Thereafter, sensitivity to tremorine rapidly decreased, and the fourth or fifth administration had no effect whatever. All of the three effects investigated were equally involved in the addition. After administering tremorine on five occasions, neither tremor, nor analgesia were induced, nor was anaesthesia prolonged.

Next, we investigated the duration of habituation. After the development of complete tolerance, tremorine administration was discontinued. Part of

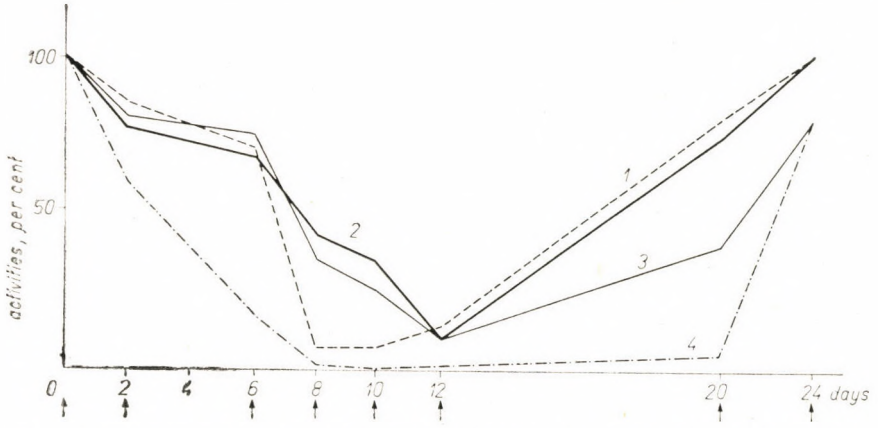

Fig. 2. Tolerance to tremorine after repeated administration of 18 mg/kg intraperitoneally. ↑ = 18 mg/kg tremorine. *1*: analgesic activity, HAFFNER's method; *2*: analgesic activity, hot plate method *3*: tremor-producing action *4*: prolongation of ether anaesthesia

the animals was then investigated after a rest of 8 days. As seen from Fig. 2, sensitivity to tremorine then began to return: the drug was about half as effective as in normal, untreated mice. The rest of the animals was reinvestigated 12 days after discontinuing tremorine administration. Sensitivity at this time was practically identical with that of untreated control mice, a fact indicating that the tolerance was already over.

Discussion

After the introduction of the use of tremorine as a test in the search for antiparkinssonian drugs [1, 2] were demonstrated its analgesic [7, 8, 9] and anaesthesia-prolonging effects [7, 9] which have also proved suitable for the same purpose [8, 9]. To these properties of tremorine now a new one has been added, namely its causing tolerance in animal experiments. The development of tolerance is surprisingly rapid. Three or four treatments already

suffice to cause complete insensitivity to the drug, especially after administering high doses. The development of tolerance does not depend on the route of administration (intraperitoneal, resp. subcutaneous). It should be noted, however, that about 30 per cent of the animals did not tolerate a 18 mg/kg dose of tremorine for more than 6 to 8 days. The duration of tolerance is short. The original sensitivity was partially restored after a rest of one week and wholly after an interval of two weeks. There was, however, a tendency for a more rapid re-addiction.

The property of tremorine to cause rapid addiction in the mouse must not be left out of consideration when the drug is used in routine pharmacological screening.

LITERATURE

1. EVERETT, G. M.: Nature (Lond.) **177,** 1238 (1956).
2. EVERETT, G. M., BLOCKUS, L. E., SHEPPERD, I. M.: Science **124,** 79 (1956).
3. FARQUHARSON, M. E., JOHNSTON, R. G.: Brit. J. Pharmacol. **14,** 559 (1959).
4. P'AN, S. Y., CARIOTO, R., TIMMENS, E., GARDOCKI, J. F.: Arch. int. Pharmacodyn. **120,** 222 (1959).
5. PREZIOSI, P., DE VLEESCHHOUWER, G. R.: Arch. int. Pharmacodyn. **120,** 108 (1959).
6. AHMED, A., TAYLOR, N. R. W.: Brit. J. Pharmacol. **14,** 350 (1959).
7. LENKE, D.: Arch. exp. Path. Pharmak. **234,** 35 (1958).
8. CHEN, G.: J. Pharmacol. exp. Ther. **124,** 73 (1958).
9. VÁRSZEGI, M., DECSI, L., MÉHES, Gy.: Acta physiol. hung. Suppl. **18,** 89 (1961).
10. WOLFE, G., MCDONALD, A. D.: J. Pharmacol. exp. Ther. **80,** 300 (1944).
11. PÓRSZÁSZ, J., HERR, F.: Kísérl. Orvostud. (Budapest) **2,** 245 (1950).
12. BIANCHI, C., FRANCESCHINI, J.: Brit. J. Pharmacol. **9,** 280 (1954).
13. HAFFNER, F.: Dtsch. med. Wschr. **55,** 731 (1929).
14. DECSI, L.: Arzneimitt.-Forsch. **8,** 348 (1958).

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Recensio

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"Die pharmakologische Beeinflussung der Kapillarresistenz und ihrer Regulationsmechanismen"

(Capillary Resistance: Its Response to Drugs and Its Control) Akadémiai Kiadó (The Publishing House of the Hungarian Academy of Sciences) Budapest, 1960

The monograph gives a survey of the author's investigations followed for about 10 years partly in cooperation with other workers at the same University from where had originated the reports on rutin, on the inhibitory action of flavonoids on permeability (RUSZNYÁK, SZENT-GYÖRGYI, ARMENTANO, BÉRES, BENTSÁTH).

In the general part of the monograph the concepts of capillary resistance, capillary permeability and capillary fragility are classified and differentiated. The methods for studying capillary resistance, the mechanism of the formation of petechiae, the changes in capillary resistance (diurnal, seasonal, age-bound), and its normal values in males and females are described. On the basis of the pertaining literature and the results of experiments of his own, the effects of physical factors, such as thermal effects and the actions of ultraviolet and X-ray irradiation are discussed. The author has succeeded in showing that flavonoids and the indenochrome derivatives offer protection against the untoward effects of radiation. The problem of the correlation between capillary resistance and blood circulation is also touched upon.

In the main part of the monograph (chapter II) the effect of various drugs on capillary resistance is discussed. It is pointed out that by the use of new methods it has been confirmed that calcium increases capillary resistance and that capillary resistance decreases in response to histamine. Skin irritants are diminishing capillary resistance (i) reflectorily; (ii) by causing the release of histamine and other H-substances; and (iii) through a direct vascular action. In contrast with this, the antihistamine drugs (Antergan, Neoantergan, Synopen, Benadryl, Antistine) increase capillary resistance. Must authors claim the same for epinephrine, too. A similar action of ascorbic acid has been described by some authors, while others found vitamin C ineffective in this respect. Capillary resistance increased in response to the oral administration of thiamine, while it suffered a transitory decrease after the administration of nicotinic acid. Capillary resistance is increased also by paraaminobenzoic acid, vitamin E and vitamin K.

Of the favonoids, citrine restored to normal the pathologically-diminished capillary resistance in cases of vascular purpura and also in experiments. Similar observations have been made with rutin, and it is only with the experimental scurvy of guinea pigs that conflicting evidence has been reported. The low capillary resistance was increased also by hesperidine, as well as by quercetine. Sophoricoside, isolated from Japanese acacia, was found to be even more potent.

The indenochrome derivatives inhibit the histamine-induced chemosis in the conjunctiva of the guinea pig. Haematoxylin affords protection against the bronchospasm elicited by histamine and restores the capillary resistance lowered by the Sherman—La Mer—Campbell diet. Capillary resistance was increased also by haematein, brasilin and brasileine.

Among the catechols, d-epicatechol was the most effective in increasing capillary resistance. Of the anthocyanides, leucocyanidine increased by 30 to 100 per cent capillary resistance, and also the cumarin derivatives increase it.

It is shown that anticoagulants (heparin, glutathione, acetopurpurin, Chicago-blue, Germanin, Liquoid, lanthanum chloride) reduce capillary resistance, irrespective of their being natural or synthetic products, organic or inorganic substances.

The author and his co-workers have succeeded in blocking the action of these anticoagulant and capillary resistance-reducing agents. For example, the action of Liquoid could be inhibited by pretreatment with haematein and that of heparin by pretreatment with protamine sulphate or toluidine blue. The same has been achieved by treating the animals with rutin for some days before

administering heparin. In contrast with this, $CaCl_2$, administered intraperitoneally in doses of 25 mg/100 g of body weight, did not suffice to block the capillary resistance lowering action of heparin. On the other hand, aminopyrine and phenylbutazone inhibited development of the heparin effect. Phenylbutazone was inhibitory only exceptionally. The reduction of capillary resistance by Germanin was inhibited by toluidine blue.

In chapter III the effects of DOCA, ACTH and cortisone on capillary resistance are analysed, in connection with the research done by EICHHOLTZ, NITSCH, ROBSON and DUTHIE, KRAMÁR and others. All authors agree in that capillary resistance is enhanced by cortisone and this action may be suspended by the administration of somatotrophic hormone. The author and DUX showed the part in the control of capillary resistance of the ACTH-heparin and the cortisone-heparin systems. These results are in agreement with the view of KRAMÁR, according to whom cortisone plays the principal rôle in the regulation of capillary resistance. The author and his associates can claim for themselves the credit for proving that beside cortisone heparin, too, plays a significant rôle in these mechanisms.

With its bibliography embracing 225 references, the monograph is a valuable account of a systematic research representing the continuation and expansion of investigations in which Hungarian workers have been the pioneers. The monograph will be useful to all those engaged in studies concerning capillary resistance.

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РЕЗЮМЕ

ПРОСТОЙ И ЧУВСТВИТЕЛЬНЫЙ МЕТОД ДЛЯ ОПРЕДЕЛЕНИЯ НЕОРГАНИЧЕСКОГО ФОСФОРА

Н. А. БИРО, А. МЮЛРАД и ДОБРОНАИ

Авторы разработали метод определения неорганического фосфора путем осаждения фосфора в виде соли стрихнина фосфорномолибденовой кислоты, растворяя промытое осаждение и измеряя поглощение света при 254 *mμ*. Наименьшее определяемое количество составляет 0,05 микрограмм в 1 мл. Аденозинфосфат, кальций, магний, трихлоруксусная кислота и хлорная кислота не препятствуют определению. Соль мышьяковой кислоты должна отсутствовать. АТФ не показывает измеряемого гидролиза, а креатинфосфорная кислота выявляет в примененных авторами экспериментальных условиях до 5%-ого гидролиза.

ФОТООКИСЛЕНИЕ МИОГЛОБИНА

М. ШАЙГО

БИОСИНТЕЗ КОРТИКОСТЕРОИДОВ В НАДПОЧЕЧНИКАХ КРОЛИКА

Д. А. ФАЗЕКАШ

Образование 11 β ОН группы кортикостероидов в надпочечниках кроликов возможно также путем восстановления 11-оксогруппы. Для этого процесса необходимо присутствие молекулярного кислорода. Передающий водород коэнзим не является ДПН. Этот процесс можно стимулировать добавлением промежуточных продуктов цикла Сент-Дьёрдьи—Кребса. Неповрежденной клеточной структуры не требуется. Биосинтез альдостерона и кортизола следует из 11-дегидрокортикостерона. Кортикостерон получается главным образом из 11-дезоксикортикостерона, но его можно получить также из 11-дегидрокортикостерона. Первая реакция происходит приблизительно в два раза интенсивнее, чем последняя. В надпочечниках кроликов 17-гидроксиляция может иметь место также после введения 21-гидроксилгруппы, но только в том случае, если соединение обладает также 11-оксогруппой.

МОДИФИЦИРОВАННОЕ ИОДОМЕТРИЧЕСКОЕ ОПРЕДЕЛЕНИЕ ПЕНИЦИЛЛИНАЗЫ

В. ЧАНЬИ

Для определения пенициллиназы принято пользоваться иодометрическим методом Перре, дающим точные величины, но непригодным для серийных измерений. Для серийных измерений этот метод стал пригодным благодаря применению вольфрамата натрия и желатины в качестве ингибитора. Приводятся результаты нескольких определений, проведенных по этой модифицированной методике.

РОЛЬ КАЛЬЦИЯ В РЕГУЛИРОВАНИИ НАКОПЛЕНИЯ КАЛИЯ В СРЕЗАХ КОРЫ ГОЛОВНОГО МОЗГА МОРСКОЙ СВИНКИ

Д. ГАРДОШ

1. Для накопления калия с максимальной скоростью в срезах коры головного мозга морской свинки необходимо присутствие ионов кальция.
2. При отсутствии ионов кальция накопление калия совершенно прекращается.
3. Ионы кальция при примененных в экспериментах автора концентрациях совершенно не влияют на углеводный обмен тканей и производство энергии тканями.
4. По мнению автора кальций является основной составной частью механизма, регулирующего транспорт катиона.

ИССЛЕДОВАНИЯ О-ГЛИЦЕРАЛЬДЕГИД-3-ФОСФАТ-ДЕГИДРОГЕНЕЗЫ. XVIII. ЛИПИДНЫЙ КОМПОНЕНТ ЭНЗИМА

Т. ДЕВЕНИ, Т. КЕЛЕТИ, БРОНИСЛАВА СОРЕНИ и М. ШАЙГО

ДЕЙСТВИЕ УДАЛЕНИЯ ЭНДОКРИННЫХ ЖЕЛЕЗ НА ВЫЗВАННУЮ ЗВУКОВЫМ РАЗДРАЖЕНИЕМ, ЭОЗИНОФИЛИЮ

Я. БИРО, В. СОКОЛАИ и Я. ФАХЕТ

У крыс с удаленными надпочечниками (разными способами сохраненных в жизни), а также у крыс с удаленной щитовидной железой и с положительной реакцией на сильный звук и у крыс с удаленным гипофизом, аудиогенная эозинофилия не имеет места. У животных с удаленной щитовидной железой, но с негативной реакцией на сильный звук, удалось вызвать аудиогенную эозинофилию. Постоянный прием кортизона препятствовал повышению числа эозинофильных клеток также у неповрежденных животных. Данные авторов говорят за то, что аудиогенная эозинофилия может развиваться, как правило, только в случае хорошо уравновешенного снабжения гормонами.

СВЯЗЫВАНИЕ КАЛЬЦИЯ ИЗОЛИРОВАННЫМИ МИОФИБРИЛЛАМИ

(Предварительное сообщение)

Н. А. БИРО и А. МЮЛРАД

ВОПРОС ГУМОРАЛЬНОЙ «FEED BACK» РЕГУЛЯЦИИ ФУНКЦИИ ГИПОФИЗ-КОРЫ НАДПОЧЕЧНИКА

Э. ЭНДРЕЦИ, К. ЛИШШАК и М. ТЕКЕРЕШ

В опытах на собаках и кошках было установлено, что интрацеребральное введение ацетата кортизона в количестве 10 или же 50 мг в каудальную часть гипоталамуса и **formatio reticularis** среднего мозга, снижает функцию гипофиз-коры надпочечника. У других нервных структур этот стероид, в примененной концентрации, не вызывал изменений. В то время как кортизон при введении в область гипоталамуса снижал секрецию АКТГ соответственно с введенным количеством, со стороны **formatio reticularis** среднего мозга удалось вызвать снижение только до величины, достигаемой в покое, что авторы определили измерением содержания кортизона в крови надпочечной вены. В дальнейших исследованиях наблюдалось, что гипертонический раствор хлористого натрия, введенный в **formatio reticularis** снижал выделение альдостерона; этого явления не наблюдалось ни при введении кортизона, ни при введении гипертонического раствора декстрозы. Результаты опытов указывают, на то, что повышение содержания кортикоидов в периферической крови может тормозить выделение гипофизом АКТГ, посредством центральной нервной системы, и посредством ствола головного мозга.

ВЛИЯНИЕ АДРЕНАЛИНА И НОРАДРЕНАЛИНА НА БОЛЬШОЙ И МАЛЫЙ КРУГИ КРОВООБРАЩЕНИЯ СОБАК ДО И ПОСЛЕ УДАЛЕНИЯ ГРУДНОГО СЕГМЕНТА СПИННОГО МОЗГА

К. КАЛЛАИ, Л. ТАКАЧ и Т. ФЕНЬВЕШИ

Удаление грудного сегмента спинного мозга (th I—XII) у наркотизированных собак не влияет на сосудистый тонус легочного кровообращения. Капельное вливание больших доз норадреналина повышает минутный объём сердца неповрежденных животных, и значительного сосудосуживающего эффекта мы не наблюдали ни в большом ни в малом кругах кровообращения.

Идентичные дозы норадреналина, после удаления сегмеита спинного мозга, вцзвали сосудосуживающий еффект на периферии, однако в малом кругу кровообращения статистически показателного повьжениа артериоларного сопротивлениа не набюдается.

Однократное введение адреналина или норадреналина повышает легочное артериалное давление. Удаление грудного сегмента спинного мозга повышает чувствительность всего лишь периферических сосудов и только к адреналину.

ВАЗОМОТОРНЫЙ РЕФЛЕКС НОВОГО ТИПА, ВЫЗЫВАЕМЫЙ ИЗ ВЕНОЗНОГО СИНУСА

М. СЕНТИВАНИ и А. ЮХАС-НАДЬ

В венозный синус вводили канюлю, снабженную вздуваемым баллоном. Отток крови из синуса через канюлю был обеспечен и в случае вздувания баллона.

Натяжение стенки синуса баллоном привело к падению кровяного давления. Это явление удалось прекратить перерезкой блуждающего нерва. Снижение кровяного давления весьма длительно, и продолжается до тех пор, пока баллон содержится во вздутом состоянии, в противоположность рефлексу пазухи сонной артерии, при котором, вопреки вызванному на стенку давлению, кровяное давление скоро достигает первоначального уровня. Из этого можно сделать вывод, что роль рефлекса заключается не в сохранении определенного уровня кровяного давления, а в прочной установке кровяного давления на новом уровне.

Установка кровяного давления на низком новом уровне осуществляется путем смещения прессорно-депрессорного равновесия в направлении депрессора: во время рефлекторной гипертензии нельзя вызвать гипертенсивного рефлекса пазухи сонной артерии, в отличие от гипотенсивного рефлекса сонной артерии, который можно вызвать.

Рефлекс генерализирован и его модифицирующее действие на равновесие всей циркуляции обусловливается тем,что афферентные волокна включаются в ретикулярное образование, причем торможение диффузно распространяется оттуда на центры, участвующие в регуляции кровяного давления.

ПРИВЫКАНИЕ К ДЕЙСТВИЮ ТРЕМОРИНА

Л. ДЕЧИ, М. ВАРСЕГИ и Д. МЕХЕШ

У мышей развивается поразительно быстрое привыкание к действию треморина. Это привыкание наблюдается в отношении всех трех действий треморина на центральную нервную систему: — при вызывающем тремор, при потенцирующем наркоз и при болеутоляющем действии его. Через 10—14 дней после прекращения введения треморина, чувствительность к нему вновь восстанавливается.

ДАННЫЕ О РЕГУЛЯЦИИ ФУНКЦИИ ГИПОФИЗА, ЯИЧНИКА И КОРЫ НАДПОЧЕЧНИКОВ СО СТОРОНЫ ГИПОТАЛАМУСА

Э. ЭНДРЕЦИ

В опытах на крысах было установлено, что проведенная в передней части гипоталамуса электрокоагуляция, разрушающая область между **nucleus paraventricularis**, **chiasma opticol** и **infundibulum**, которая распространилась также на **regio preoptica**, в значительной мере повысила вес матки, и обусловливала постоянное состояние течки. Эти повреждения повысили также функцию гипофиз-коры надпочечника, которую авторы проверяли определением содержания кортикоидов в крови надпочечной вены. Было установлено, что у животных с удаленной корой надпочечников изменения половых желез более выражены чем у контрольных животных. Далее было установлено, что у животных с удаленными яичниками повышенной секреции гормона надпочечников не наблюдается. Из результатов экспериментов можно сделать заключение, что реакция коры надпочечника, на повреждение гипоталамуса, является следствием первично повышенной функции яичников. С другой стороны кажется весьма вероятным, что секреция коры надпочечников уменьшает наступающие после повреждения гипоталамуса изменения половых желез.

ВЛИЯНИЕ ПОСТОЯННЫХ ЖИЗНЕННЫХ УСЛОВИЙ НА КОНЦЕНТРАЦИЮ ВОДОРОДНЫХ ИОНОВ СЛЮНЫ ДЕТЕЙ

И. САБО и К. ТОТ

Исследовалось pH 397 образцов слюны, взятых от 52 детей, живущих в институте, и живущих у своих родителей. Было установлено, что pH слюны детей, живущих продолжительное время в институте, сигнификантно выше, по сравнению pH слюны детей, живущих у своих родителей. В отдельных группах pH слюны детей с хорошими зубами, отличалось от pH слюны детей с плохими зубами, однако, как правило, математически несигнификантно. Авторы приписывают различие pH слюны у отдельных групп факторам питания и влиянию среды.

ИССЛЕДОВАНИЕ ОРГАНОВ КРОВООБРАЩЕНИЯ КРЫС ПРИ НАРКОЗЕ ПЕНТОБАРБИТАЛОМ, УРЕТАНОМ И ХЛОРАЛОЗОМ, И В БОДРСТВУЮЩЕМ СОСТОЯНИИ

К. КАЛЛАИ и Л. ТАКАЧ

У наркотизированных пентобарбиталом (внутрибрюшинно 40 мг/кг), уретаном (внутрибрюшинно 1 г/кг) и хлоралозом (внутривенно 0,10 г/кг) крыс, проводилось определение минутного объема, кровяного давления и методом «**indicatorfractionation**» ($Pб^{86}$) Сапирштейна (1,2) фракции минутного объема органов. По этим данным можно вычислять кровообращение органов и их сопротивление кровообращению. На ненаркотизированной группе крыс исследовались только фракции минутного объема.

Полученные при наркозе пентобарбиталом результаты,, соответствуют литературным данным. В наркозе уретаном кровяное давление и сопротивление циркуляции сердца ниже чем при наркозе пентобарбиталом. Сердечная и костно-мышечная фракции минутного объема выше, а почечная и кишечная фракции ниже. Хлоралоз повышает ток крови в кожно- и костно-мышечной системе, но снижает сопротивление сердца и костно-мышечной системы. Минутного объема почечная фракция ниже, а костно-мышечная фракции выше. В ненаркотизированной группе крыс поразительно снижение минутного объема, сердечной, почечной и печеночной фракций и повышение минутного объема костно-мышечной фракции.

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