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DIE GESCHÄFTSFÜHRUNG DER AKADEMIE
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THE GROWTH AND THE FOOD AND WATER CONSUMPTION OF THE RESTING OR EXERCISING ALBINO RAT ON DIETS CONTAINING VARIOUS AMOUNTS OF FAT AND A REDUCED QUANTITY OF VITAMIN B_1.

(WITH 2 FIGURES AND 3 TABLES IN THE TEXT).

BY : A. B. L. BEZNÁK.
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It appears definitely to be proved that almost normal growth takes place on a diet free of the vitamins B, if the amount of carbo-hydrate is diminished in favour of the fat. (1, 2, 2a, 3, 4, 5, 6, 7.) It is also definitely proved that growth arrested by a vitamin B_1 and fat-free but carbo-hydrate-rich diet is resumed — without the addition of vitamin B_1 — if the carbo-hydrate is replaced by 30—40% fat. (8, 9.) When on a diet free of fat, the polyneuritis is cured and growth is restored by the addition of vitamin B_1 (but no fat), a synthesis of fat from carbo-hydrate takes place, this running parallel with the resumption of growth. (10, 11.) To ensure normal growth as well as absence of the symptoms of acrodynia, however, vitamin B_1 and an adequate amount of fat are not sufficient; riboflavin (vitamine B_2) and especially pyridoxin (vitamin B_6) are also necessary. (12, 13, 9, 14, 15, 10, 16.)

As regards the rôle of choline in growth it is now known that on a vitamin B_1-free diet containing varying amounts of fat, the ingestion of choline insures fairly good growth during the first 10 days but in course of the late period on this diet, it cannot prevent a loss of weight. (19.) When the vitamins B_1, B_2 and B_6 are supplied in sufficient amounts, even on a fat-free diet, choline appears to be dispensable. (10, 16, 20, 21, 22.)

On a mixed diet containing — besides the known vitamins and salts — protein and carbo-hydrate, the fatty acid composition of the fat consumed seems to be the determining factor of growth. The dietary fat may be rich in the essential unsaturated fatty acids, yet to good growth these must be balanced with the saturated short-chain C_{10}—C_{16} fatty

acids. On a diet containing much unsaturated non-essential fatty acid, linolic acid is indispensable to good growth (in spite of the presence of the vitamins B) yet this is dispensable (if the supply of the vitamins B is adequate) on a fat-free but carbo-hydrate rich diet. If the natural fat — in relation to the other fatty acids — contains too much saturated long-chain, or too much non-essential fatty acid, with several double-bonds, its growth-promoting effect decreases. (23.)

Thus, in order to ensure good growth, the multiplexly correlated dietary constituents should be polylaterally balanced, but this is not all. The extent of the metabolism of the animal also plays an important rôle in its growth. It has been observed that forced exercise invariably causes either a fall in the body-weight, or a retardation of growth. (23, 24, 25.) The reciprocal value of this decrease is a very sensitive index of the dietary value of a fat. (23.) While this loss in body-weight and retardation of growth has invariably been observed during a period of *forced* exercise, *Donaldson* has proved that *spontaneous* exercise is favourable to the growth of the albino rats. (26, 27.)

Regarding the effect of the metabolism on the need of the organism of vitamin B_1, it is known from the experiments of *Cowgill* and his collaborators that increase of the metabolism raises the amount of vitamin B_1 necessary to prevent anorexia, the first symptom of B_1 avitaminosis. It makes no difference whether the metabolism is increased by thyroid-feeding or by exercise. (31, 30, 29, 28.)

The relationship between food consumption and growth has repeatedly been subjected to investigation. *Hopkins* (31a) observed that growth runs parallel to the calorie (food) intake. We, in collaboration with *Gáspár* (32), found in statistically controlled experiments that the correlation between food consumption and velocity of growth is not great (coefficient of correlation = r = only 0,55). On the other hand the correlation between „efficiency of growth" and growth was found to be r = 0,90, $\mu = \pm$ 0,034. We defined efficiency of growth as: growth x 100/food consumption. Thus we confirmed the conclusion of *Palmer* and *Kennedy* (33) that individual variations in the growth of animals are due primarily to the individual variations in their avidity to use part of the food for growth, and secondarily to differences in appetite.

From all these it was concluded that different amounts of the same fat in the presence of the same amounts of all vitamins B, may have different effects on the growth of the albino rat during rest and during forced exercise. To test this hypothesis we arranged experiments which, while proving it to be correct, led to an interesting discovery. It was seen that on a diet upon which growth stopped during rest at an early age,

in consequence of its low fat and vitamin B_1 content, growth was again resumed when forced exercise was begun. It is to be noted that this occurred without an increase of the fat content of the diet. Moreover the growth so resumed continued at a good space on the same diet, also after the cessation of this exercise. With the growth of the animals, we recorded also their food and water consumption, thus we have been able to make some interesting observations regarding the effect of the fat-content and exercise on the efficiency of growth.

METHODS.

In these experiments 48 male albino rats of Wistar origin were used. They were closely inbred litter mates divided into four groups, 12 rats in each. The animals lived in individual screen-bottomed cages,

TABLE I.

The composition of the food consumed by the four spring groups.

	Group I. 3 % fat.	Group II. 8 % fat.	Group III. 16 % fat.	Group IV. 32 % fat.
Sunflower oil	3,0%	8.0%	16,0%	32,0%
Starch	53,0%	48,0%	40,0%	24,0%
Casein	25,0%	25,0%	25,0%	25,0%
Spinach	10,0%	10,0%	10,0%	10,0%
Yeast	5,0%	5,0%	5,0%	5,0%
Salt-mixture	4,0%	4,0%	4,0%	4,0%
g calories/kg	3650	3930	4370	5250
Total fat content Including the 0·5% casein-fat *(Soxhlet)*	3,5%	8,5%	16,5%	32,5%

making refection impossible, and were placed in a basement room climatically conditioned, room temperature 18 C°, absolute humidity 11,9 g/m³, relative humidity 89%, dim daylight. This spring series of experiments lasted from the 13th II. till the 13th VI. 1943. — The average of their body-weights together with its standard deviation was determined from the first day, with few exceptions, almost daily. The significance between the differences of the averages of the four groups was also calculated. These results are all represented graphically in Figure 1.

Each group of rats inhabited a separate cage. Their food and tap-water consumption was measured daily, the remains of a known surplus of food or water being reweighed on the following day.

The food consumed by the four groups was qualitatively identical, but the relative amount of the different constituents varied as shown in Table I. Some of the properties of the sunflowerseed-oil are tabulated in Table II. Highly purified wheatstarch was used. The casein (a gastric

TABLE II.

Some of the properties of sunflower oil used in this work

Physical properties		*Chemical composition*	
Melting point	—	Palmitic acid	3,5%
Saponification n	193,2	Stearic acid	2,9%
Br-J adsorbtion n	131,9	Arachic acid	0,6%
Reichert-Meissl n	0,33	Lignoceric acid	0,4%
Emulsification n	1,0	Oleic acid	36,0%
Free acids n	1,0	Linolic acid	58,0%
Calories	5717		

The analyses have been carried out in the Chemical and Food-Research Laboratories of the City of Budapest Dir. Dr. B. Hunkár.

Data from Juckenack A. E., Bomes B. B., Meyer B., Grossfeld J.: Hdb. der Lebensmittelchemie Bd. 4. 1939.

rennin product) had not been previously defatted. The spinach was dehydrated fresh leaves, the yeast dried irradiated brewer-yeast. Mc Collum 185 salt mixture was used.

The exercise consisted of running in a large revolving drum. The velocity of the run was 1 km per hour. The daily period of exercise was gradually raised from one hour on the first day to five hours on the 13th day and then was kept on this level till the end of the exercise period, 28 days altogether.

EXPERIMENTAL RESULTS.

In Figure 1. the growth curves of the averages of the bodyweights of the four groups together with the averages of the food and water consumption per day per animal are plotted against time, in days.

The entire experimental period falls into three divisions: 1. pre-exercise inactivity period, i. e. normal resting life of the caged albino rat; 2. the exercise period; 3. the post-exercise inactivity period, i. e. return to the normal resting cage life.

In all three divisions there is a great difference between the growth of the 3% group and the other groups consuming diets containing 8-, 16- and 32% fat. *During the pre-exercise inactivity period* the growth of the 3% group begins to decrease after the 8.h day and from the 17th day their growth is arrested for the remaining 24 days. The curve is characteristic of a diet deficient in some essential constituent. The other three groups, on the other hand, grow about equally well, although the 8% animals lag behind slightly.

During the exercise period the difference between the 3% and the other groups becomes fundamental. The 3% group from the second day of the exercise begins to *grow*, whereas all the other groups stop growing and begin to *lose weight*. Growth during the period of exercise — in all

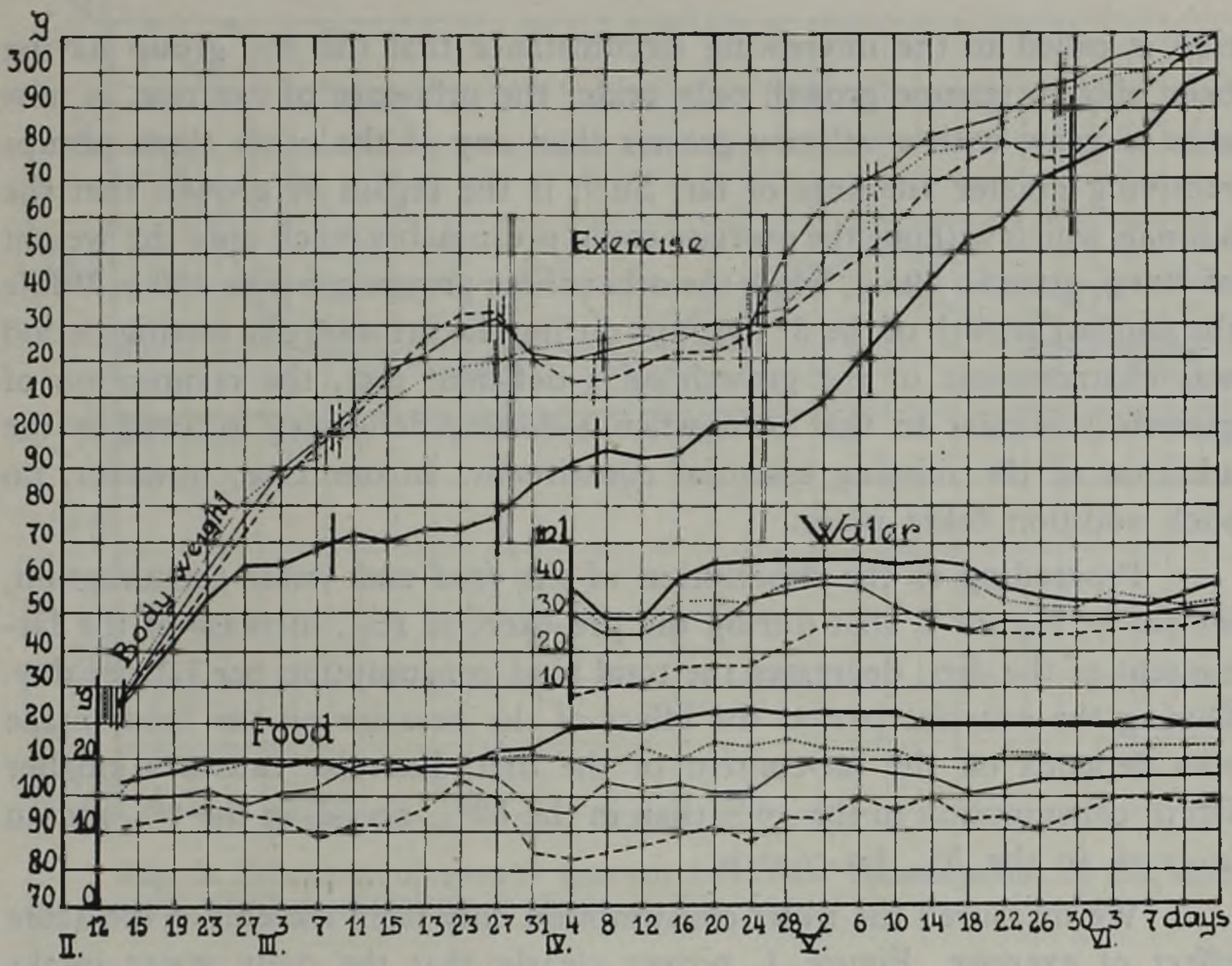

Fig. 1. Growth, water and food consumption of groups of albino rats on diets of different sunflower-seed oil content, previous to, during and after exercise. —— 3%, ... 8%, —— 16%, ------- 32% sunflower oil. The short vertical lines across the growth curve of each group correspond to twice the standard deviation of the mean.

groups —- falls into two divisions: the first is the subdivision of the acute exercise, the second is the subdivision of the compensatory adaptation. In the 3% group, during the acute effect of exercise, growth starts with a velocity as good as the best of normal growth, then during the compensatory period it slackens down but continues. In the other three groups, eating the diet of high fat content, there is a sharp loss of weight during the acute effect of exercise. The loss is inversely proportional with the fat content of the diet. Indeed in the 8% group instead of loss there is a slight gain of body-weight. During the compensatory period growth is slowly resumed. The higher the fat-content of the diet the slower the recovery of growth. It is remarkable that in the case of the 8% group where, during the acute effect of the exercise, instead of loss of weight a slight increase took place, this is followed by a small retardation as in the case of the 3% animals.

During the post-activity resting period, at the cessation of the exercise, growth is resumed with a great velocity in all the four groups. Atten-

tion is called to the interesting circumstance that the 3% group having been able to resume growth only under the influence of exercise, is now able to grow with a velocity greater than any of the other three groups receiving greater amounts of fat. Such is the regain of growth that the animals which without the exercise could presumably reach only the weight of 200 g, grow to 295 g, while the other three groups grow to 320 g. While the stunted growth of the 3% groups during the pre-exercise resting period was characteristic of the growth on a deficient diet, the resumption of growth is similar to that seen when a dietary deficiency is cured by the addition of the missing essential constituent. In our case, however, no such addition takes place.

Proceeding to the description of the *food* and water consumption, we see in Figure 1. that during the pre-exercise rest, increase of the fat-content of the diet, decreases the total food consumption per rat per day. During the exercise period the effect of the exercise on the food intake also depends on the fat-content of the diet. Exercise causes a smaller fall of consumption in the 16% than in the 32%, no fall in the 8% and an increase in the 3% fat-content.

We measured the *water* consumption only from the end of the acute effect of exercise. Figure 1. proves clearly that the daily water intake is inversely proportional to the fat-content of the diet as is — as we have seen — the daily total food consumption. During the last part of the adaptation period of the exercise, the water intake increases. This increase, like that of the food intake, is inversely proportional to the fat-content of the diet. It sets in after a certain delay. This retardation is also proportional to the fat content. In the case of the 32% diet, the delay is so long that the increase in the water intake takes place during the post-activity resting period.

Figure 2. indicates the variations of the efficiency of growth. During the pre-activity resting period it is evenly proportional to the fat-content of the diet. During the exercising period it is increased in the low fat and decreased in the high fat animals. After the exercise it increases in all groups, afterwards it returns to its lower pre-activity resting value.

DISCUSSION.

The most interesting feature of our results is perhaps the dependence of the profound difference present in the growth response to exercise, on the fat-content of the diet. It has already been concluded that the organism of an animal on a fat diet is very different from one on a carbo-hydrate diet, because its response to adrenalin (42), insulin (42),

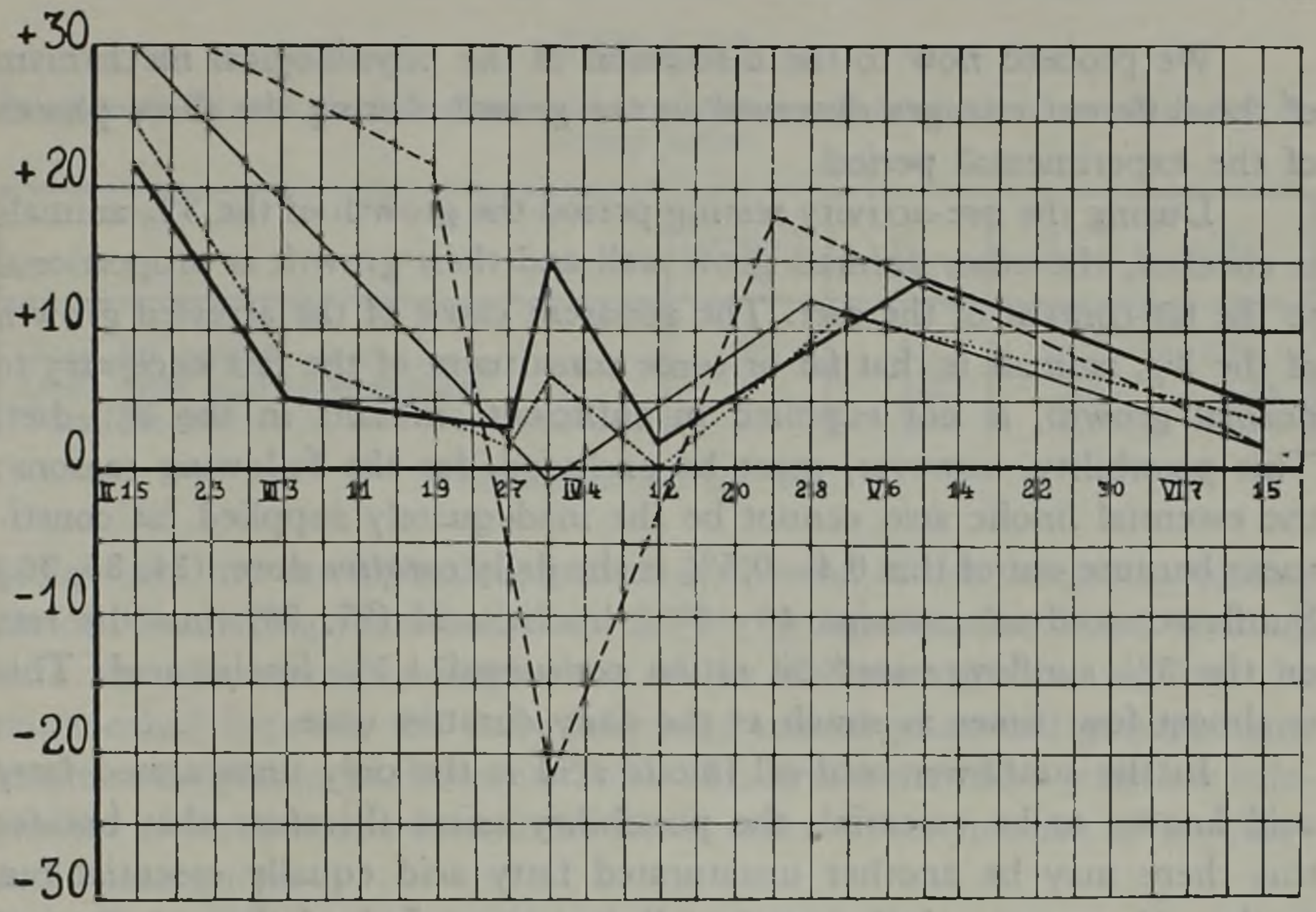

Fig. 2. Efficiency of growth (growth 100 : food consumption) of the albino rat groups. Their designations as shown in Fig. 1.

pitressin (42), lack of vitamin B_1 (3—7), and efficiency during exercise (25) is different, but the fact that at a definite fat per carbo-hydrate ratio such an organism is built as responds to the increased metabolism of the exercise with the resumption of growth, proves more strikingly than any of the others the delicate dependence of the functional structure of the body on the fat-content of the diet.

Moreover the fact that there is only one fat-content upon which growth is at its optimum both during rest as well as during exercise, settles positively the long disputed question of the existence of a „fat optimum".

As was mentioned introductorily, there is an apparent contradiction regarding the effect of exercise on growth. *Donaldson* observed an improvement, others on the other hand, a slowing of growth or loss of weight during exercise. Our experiments give a clue to the explanation of this apparent contradiction. The diets upon which exercise depressed growth were rich in fat. (1c, 23, 24, 25.) Those upon which spontaneous exercise improved growth were poor in fat for that particular strain of rats. (26, 27.) Moreover the difference between spontaneity and the forcedness of the exercise may also play a rôle. Is it that certain diet develops in the organism a hunger for exercise, which when satisfied brings forward changes in the intermediary metabolism speeding up growth?

We proceed now to the discussion of the physiological mechanism of the different changes observed in the growth during the three phases of the experimental period.

During the pre-activity resting period the growth of the 3% animals is checked, the other animals grow well and their growth is proportional to the fat-content of the diet. The apparent cause of the arrested growth of the 3% animals is that fat or some constituent of the fats necessary to normal growth, is not supplied in sufficient amounts in the 3% diet. This possibility, however, must be excluded, for the following reasons: the essential linolic acid cannot be the inadequately supplied fat constituent because out of this 0,4—0,5% is the daily *curative* dose. (34, 35, 36.) Sunflower-seed-oil contains 46—58% linolic acid (37, 38) thus the rats on the 3% sunflower-seed-oil ration consumed 1,7% linolic acid. This is almost four times as much as the daily curative dose.

In the sunflower-seed-oil linolic acid is the only unsaturated fatty acid known to be essential, the possibility arises therefore that besides this there may be another unsaturated fatty acid equally essential but in this oil present only in very small quantities. Indeed the experiments of *Mc Kenzie* et al (1c, 34) render this supposition possible. Since in the casein our diets contained 0,5% fat, it is almost certain that this butter-fat contained *Mc Kenzie's* hypothetical essential factor.

The possibility that the amount of the short-chain fatty acids was relatively small to the amount of the unsaturated fatty acids, and that caused the arrest of growth, is also rendered invalid by the circumstance that the casein-content was kept constant in all the diets and the amount of the sunflower-seed-oil was increased at the expense of the carbo-hydrate; it is therefore in the high fat diets that the ratio between the saturated and unsaturated fatty acids is unfavourable. Growth should have been arrested in the high fat animals and not in the 3% group.

It is equally impossible that a lack of choline could have been responsible for the arrest of growth in the 3% group, since undefatted casein and irradiated yeast, two powerful sources of choline, were fed in all the diets in quantities which were always sufficient to prevent the effects of the lack of choline.

Comparisons of the carbo-hydrate content of the diet with the growth, as well as another comparison between the ketogen per antiketogen ratio of *Woodyat* and growth, have definitely shown that growth is independent of both the carbo-hydrate content and the value of the ketogen per antiketogen ratio.

On the other hand considering the fat-content we find a very close relationship between the actual total daily fatty acid intake of the different

TABLE III.

Relationship between the daily total food consumption and total growth at the end of the resting period.

	Group I.	II.	III.	IV.
Total fat content	3%	8%	16%	32%
Daily fatty acid consumption	1	1,25	1,32	1,34
Total growth	1	1,26	1,39	1,46

For explanation see text.

groups and the corresponding growth at the end of the preactivity resting period. Rendering these values in the 3% group equal to 1 and dividing with this the corresponding values of the different groups, we find the relationship between the daily fatty acid consumption and growth items in the same group, as shown in Table III. While therefore *growth is independent of the actual carbo-hydrate intake it is a direct function of the fatty acid consumption.*

From the above the conclusion is obvious that the arrested growth of the 3% fatty acid animals cannot be due to the direct effect of the absence of some essential fat constituent. One indirect mechanism known to us by which an inadequate amount of fat causes arrest of growth is the inadequate supply of vitamin B_1. *Evans* et al. (1c, 4—7) showed that when the vitamin B_1 supply is small or absent, growth is proportional to the fat-content. The more recent researches have also been quoted introductorily proving that even though the essential fatty acid supply is adequate (as in our case) growth only takes place if the diet contains sufficient vitamin B_1 and B_6. In vitamin B_6 deficiency the symptoms of acrodynia are present; we have seen no signs of this. It follows that the vitamin B_6 supply must have been adequate and the vitamin B_1 was the insufficient food constituent. Is not this conclusion contradictory to the circumstance that our diets contained 5% dried u. v. irradiated brewer's yeast? This amount if the yeast is unirradiated is generally a good average maintenance dose of the vitamin B_1. On the other hand it has been shown (39a) that irradiation destroys thiamine. *The likeliest cause of the stunted growth of the 3% animals is that our diets contained a small amount of vitamin B_1, this amount while enough to ensure good growth at an abundance of fat, was insufficient to support growth at the low fat-content diet.*

We now come to the discussion of our most interesting observation: the effect of the variation of the fat-content on growth during exercise. If the fat-content is only 3%, exercise restores normal growth, if it is more than 8%, exercise causes arrest of good growth.

On the other hand, observations of *Hajdu* (25) seem to be contrary to these. This investigator found that exercise caused a loss of weight, no matter whether the diet contained 0%, 20% or 41% fat. As far as loss of weight in the case of high fat-content diet is concerned, our results confirm his. Since *Hajdu* observed a loss of weight following exercise if the diet contained only traces of fat, and we a resumption of growth if it contained 3% fat, the explanation of this contradiction appears to be that a certain small amount of fat must be present in the diet to enable exercise to act beneficially on growth.

What are the changes in the organism brought about by the variations of the fat-content of the diet causing the organism to respond in varying ways to the same exercise, i. e. to an increase of metabolism?

The arrested growth of the 3% animals during the resting period previous to exercise has had to be attributed to a deficiency of fatty acids relative to the vitamin B_1 content. Hitherto only one expedient was resorted to in curing the consequence of a food deficiency, viz. the restoration of the missing food factor. Our experiment, however, disclosed the existence of a new means, since exercise cured the growth effect of the deficiency of fatty-acids — (vitamin B_1) — without the addition of either of these two to the diet. This effect of the low fat content on growth during exercise is the more surprising because exercise and growth are both known to increase the vitamin B_1 requirement, and a further deterioration of growth was to be expected.

The cause of the different growth responses of the low fat, or high fat diets, i. e. increased metabolism, can only be a difference in the intermediary metabolism of the two kinds of organisms during exercise. The most obvious difference between the intermediary metabolism of an exercising low fat-high carbo-hydrate and that of a high fat-low carbo-hydrate animal is that in the former exercise causes an increase in the amount of the intermediary metabolites of the carbo-hydrates, while in the latter the intermetabolites of the fatty acids are increased. The low fat and low vitamin B_1 organism is therefore one whose intermediary metabolism does not produce enough of some growth essential carbo-hydrate intermediary metabolite. Exercise, increasing considerably the metabolism of the carbo-hydrates, supplies this substance. How can an increase of some carbo-hydrate intermetabolite cure the arrested growth due to the relative lack of vitamin B_1, or fat? On a vitamin B_1 deficient diet growth can be restored by the feeding either of fat or of vitamin B_1. When the avitaminosis is cured by the feeding of vitamin B_1, a synthesis of fat from carbo-hydrate takes place. The hypothesis is advanced from this that the increase of some intermetabolite of the carbo-

hydrate metabolism (possibly pyruvic acid) consequential to the exercise cures the arrested growth by being converted into fatty acids essential to growth.

Although this hypothesis is very attractive, the circumstance that hypovitaminosis B_1 cannot be cured by the forced feeding of carbo-hydrates, presents a hindrance to its acceptance. We must fall back on some correcting hypothesis and suppose that either during exercise other intermediary metabolites are produced than during rest, or that by exercise the concentration of intermediary metabolites can be increased much higher than by any forced feeding or, finally, that a combined increase of carbo-hydrate *and* protein metabolism is necessary to supply the intermediary metabolite necessary to the synthesis of the fatty acid in question.

Contrary to this hypothesis are the observations of *Hajdu* (25) who found a decrease in the fat-content of the liver and muscle at the end of a 14 days exercise period, no matter whether the diet contained 0%, 20% or 41% fat. The contradiction, however, is only apparent. Our 3% rats have grown during exercise, whereas his had been steadily losing weight. It seems natural therefore that in such animals the fat content of the liver and muscle decreased.

Why is it that on a low fat diet — according to our experiments — exercise cures avitaminosis B_1, and contrary to this, according to *Cowgill's* works, any increase of the metabolism enhances the development of the avitaminosis (28—31).

In *Cowgill's* experiments the diet was completely void of all the vitamins B, in ours all these were present, the *amount of vitamin B_1 alone being reduced*. To the fat synthesis the indispensable condition of the amelioration of the avitaminosis — the presence of some vitamin B_1, B_2 and B_6 is necessary. These were all absent in *Cowgill's* diet and the increase of the metabolism quickened the depletion of the body from its vitamins B. Finally, *Cowgill's* animals consumed 35% fat, ours only 3%. His dogs metabolised chiefly fat, our rats carbohydrates, the accumulation of whose intermediary metabolites is supposed to lead to the fat synthesis and consequential growth.

Let us consider now the loss of weight following exercise in the groups consuming 8%, 16% and 32% sunflower-seed oil. As we have seen, the loss of weight has the following peculiarities: 1. it is proportional to the fat content of the diet, 2. it begins with the commencement of the exercise, 3. it is not preceded but paralleled by a decreased food intake. It follows from this last, that the loss of weight cannot be caused by the reduction of the food intake, but by some change in the intermediary metabolism. This change apparently causes both the loss of weight as

well as the diminution of the appetite. The extent of the loss of weight is small, transient, and it is proportional to the fat content of the diet. It follows from this latter that it is caused by some intermediary metabolite of the fatty acids, whose concentration increases in consequence of the exercise in proportion to the amount of fat in the diet: possibly, the keto-acids.

The circumstance that the loss is small and transient argues for a loss of water. It is a well established fact that the human body loses water when the nutrition is changed from a carbo-hydrate diet to a fat diet, i. e. when the intermediary metabolites of the fatty acids suddenly begin to multiply. (40.) The loss is small and transient, it is 1·6% of the body weight, in the case of man. Our rats lost 0—20% depending on the fat-content. In our case this increase of these metabolites is caused not by the increased consumption of fat but the increased metabolism of fat due to the exercise. Some of the experimental results of *Hajdu* at first sight appear to be in contradiction with this supposition. This investigator found on a 41% fat diet an increase of the liver and muscle glycogen content with a corresponding increase of the water content. This observation, however, was made at the end of a 14 days' exercise period. At this stage of the exercise our rats had already recovered their body weight, whereas *Hajdu's* rats continued to lose weight.

Turning to the *discussion of the growth during the post-exercise period,* we see that in all the groups growth continues with an increased velocity. Such increase in the velocity of growth is generally seen if a period of stunted growth due to some deficiency of food is followed by the recommencement of growth consequential to the replacement of the missing food-component. In our case the cessation of exercise — and not the replacement of the deficient food-constituent — causes this increase. Perhaps the mechanism is the same in both cases.

The food and water consumption remains to be discussed. These are, during the pre-exercise resting period, both inversely proportional to the fat content of the diet, and they decrease further in consequence of exercise and this decrease is again proportional to the fat content of the diet. In the 3% fat diet during exercise instead of a decrease an increase takes place. The total calory intake of the animals is independent of the fat content and runs parallel to the total food intake. The first question to be decided is whether the satisfaction of the calory requirement is the only factor determining food intake or whether the fats have some specific bearing on the food consumption? The calory intake differs very little during the pre-activity resting period in the four groups; it is however the smallest in the case of the 3% fat content. Since per unit weight the

energy content of the food increases with its fat content, from the food containing much fat, less is necessary to satisfy the same energy requirement than from foods poorer in fat. Apparently therefore the satisfaction of the energy requirement determines primarily the amount of food consumed. The fat content has, however, also a specific complicating effect, inasmuch, as it decreases the total food intake. That this is a specific effect on the appetite — food consumption — and not an indirect one through a depression of the energy requirement, follows from the fact that the inverse correlation of the food consumption to the fat content is more close than between the variations of the fat-content and calory intake.

This ability of the fat to decrease appetite is possibly due to some of its intermediary metabolites. If the concentration of these increase in consequence of the increased amount of fat in the diet, food consumption is depressed. This supposition can explain also the further decrease of the food intake during the exercise period. This also is proportional to the fat content. Exercise causes a further increase of the intermediary metabolites of the fats which are responsible for the reduction of the food intake. Naturally the increase of the fat metabolism during exercise is proportional with the fat content and with this to the reduction of the food intake.

Let us now discuss the increase in the food intake of the 3% animals during the exercise period. We have seen that these animals recommence to grow in consequence of the exercise. In these high carbo-hydrate low fat animals the intermetabolites of the carbo-hydrate metabolism increase during exercise and not those of the fat metabolism; appetite increases concomitantly. We assumed that the accumulation of the intermediary metabolites of the fatty acids were responsible for the depression of the appetite; an increase in the appetite when these are not accumulated is therefore well in accord with this assumption. Moreover food consumption in these low fat animals increases parallel with the body-weight. It is therefore simply the consequence of the increased mass of living matter.

In discussing the changes of the *efficiency of growth* during the pre-activity period let us remember that we have found in the course of another work that the correlation between growth (velocity of growth) and efficiency of growth is very high. On the other hand there is very little correlation between growth and food consumption (unpublished). The fact that growth and food consumption are, within limits, two different processes is amply supported by our present experimental results. From these observations the question arose whether in the case of the composition of a food influencing growth this effect is due to influencing solely food consumption, or to influencing efficiency of growth or a combination

of both. Since during the pre-activity resting period, with the variations of the fat content, both growth and food consumption vary, it follows that both efficiency of growth and food consumption are dependent on the composition (fat content) of the diet. Enhancement of the fat content above a certain minimum, increases growth and decreases food consumption. Below this minimum growth stops and food consumption (catabolism) goes on in almost unchanged volume. It follows from these that for certain fatty acids, or their metabolism, both the catabolism and anabolic processes compete, and that the catabolic processes (heat requirement of the body) have priority; growth can only satisfy its need out of the surplus remaining after the satisfaction of the energy requirement.

The combined effect of exercise and variation in the fat content of the food on the efficiency of growth are also due to a double effect on growth and on food consumption. We advanced above the hypothesis that on the low fat-content diet — in consequence of the exercise — the recommencement of growth is due to an increased synthesis of some fatty acids, or their metabolites which, to a lesser degree, may be derived from some intermediary metabolites of an increased carbo-hydrate metabolism. The mechanism of the changes in the efficiency of growth during exercise is in complete harmony with this hypothesis. The changes of the efficiency of growth during the post-exercising resting period are also due to the same double effect on growth as well as on food consumption. The possible mechanism of these changes is also in harmony with the hypothesis that some intermediary metabolites of the fatty acids are involved in both growth and energy production, and that growth can only satisfy its need of this metabolite out of the surplus remaining after the catabolism. When therefore after the cessation of exercise there remains a surplus of this substance growth will be stimulated by it.

Discussion of the *water-intake* must begin with the inverse relation between the fat content of the diet and the water intake. Food (protein and inorganic salts) consumption is also inversely proportional to the fat-content. The deficiency of water, i. e. the amount of water needed for the complete metabolism of 100 calories, is greatest (350 g) if these are derived from protein. Moreover one calory of protein requires 3,0 ml of water for the excretion of urea and the sulphate formed from it. One g. of ash requires 65 ml. of water for its excretion. (41.) It is therefore likely that increase of fat in the diet decreases water intake by depressing the food, i. e. protein and salt intake, thereby lessening the metabolism of substances having the greatest deficiency of water.

The extremely interesting effect of exercise on the water consumption, viz. that the retarded increase in water intake is independent both

of growth and of food intake, but is proportional to the fat content of the diet, is possibly an effect of the prolonged exercise on the intermediary metabolism of protein and of salt. Apparently the higher the fat content the later this increase of the protein and salt metabolism begins.

SUMMARY.

1. The growth curves of groups of rats consuming diets of varying fat per carbo-hydrate content, were registered during rest, forced exercise, and after the exercise. During the pre-activity resting pericd, growth stops if the diet contains 3% fat; above this value, it is proportional to the fat-content of the diet. During the first part of the period of forced exercise, the 3% fat-animals recommence growing, while the animals consuming 8% fat continue to grow at a slower rate; animals fed on larger amount of fat lose weight. The loss of weight is proportional to the fat-content of the diet. During the second part of the forced exercise period all animals begin to grow with an increased velocity which is inversely proportional to the fat content of the diet. During the post-exercising resting period all animals grow with an even more increased speed irrespective of the fat content.

2. The stunted growth of the low fat animals is attributed to an inadequate supply of vitamin B_1, relative to the small fat content. This small amount of vitamin B_1 is the cause of an inadequate synthesis of fatty acids from carbo-hydrate. It is assumed that in the low-fat/high carbo-hydrate animals, recommencement of growth during the exercise is due to an increased synthesis of some semi-essential fatty acids, or their intermediary metabolites, consequential to the increase in the intermediary metabolism of the carbo-hydrates and proteins. The accelerated growth of the post-activity period is hypothetically attributed to the decreased metabolism of these fatty acids (or their metabolites) in consequence of the decrease of the metabolism.

3. The food and water consumption was also registered. It was found that the food consumption is inversely proportional to the fat-content. During exercise in the high fat animals it diminishes proportionally to the fat-content, whereas it increases in the low fat animals. The water consumption during the exercise period is inversely proportional to the fat-content, while it increases in the second half of the exercise period or later. This increase and the delay with which it sets in is directly proportional to the fat-content of the diet.

4. The diminution of the food intake proportional to the fat-content is partly attributed to some intermetabolite of the fatty acids. The changes

of the water consumption probably reflect those of the protein and salt metabolism.

5. The efficiency of growth (growth x 100 per food consumption) has also been registered and the results discussed. It was found that it is proportional to the fat content of the diet. Growth and food consumption are two largely independent processes. The fat content of the diet affects both. The mechanism of the changes in the efficiency of growth in consequence of the combined effect of exercise and fat content is discussed.

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BEHAVIOUR OF THE TEMPERATURE OF THYROIDECTOMIZED ANIMALS IN A HOT ENVIRONMENT.*

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Today it is generally accepted that the thyroid gland is a main factor in regulating the temperature of human and animal organisms. Numerous experiments have demonstrated that men and animals with insufficient thyroid function are more sensitive to cold — their temperature sinks more readily, since their physical and chemical thermo-regulation is defective. These functional disturbances frequently respond to thyroxin treatment. (Detailed literature on this problem is to be found in the summarizing works of *Lefevre, Gautrelet* and *Binet* (1), as well as *Thauer* (2).

Strangely little attention has been paid to the question whether thyroid takes part in the defense against warm temperature. Only in the last few years, first of all by the research work of *G. Mansfeld* (3) and his co-workers, it has been pointed out that the counter-regulation, called „second chemical thermoregulation" by *Plaut* and *Wilörand* (4) and consisting in the events occurring on the passive heating of men and animals, that is, in processes depressing metabolism below the normal level of basal metabolism, is elicited and directed by the thyroid gland. The efficacious derivative of the thyroid recognized by *G. Mansfeld* (3) which is capable of diminishing oxidizing processes was produced in crystals by *A. Mansfeld* (5), and has been termed cooling hormone, or thermothyrin A.

The question to be considered regards the temperature of animals deprived of their thyroid gland, in an environment warmer than the usual average temperature. The changes of temperature of thyroidectomized animals in a cold environment have been studied by several authors with unanimous results: The temperature of these animals is less resistant to cold; it sinks more readily and lower than that of the controls (*Abder-*

* To professor *Emil Abderhalden* on his seventieth birthday with a reverence.

halden, 6). In contradistinction to this, there are only a few observations regarding the first question and the authors do not agree, their results and conclusions being frequently contradictory. *Asher* and *Ruchti* (7) have stated, on the basis of rather brief experiments, that the temperature of thyroidectomized animals is less elevated than that of the control animals and that the elevation in a hot environment takes place less rapidly. Similar results have been obtained by *Korenchevsky* (8). Unlike these writers, *Liddel* and *Simpson* (9) have concluded from their results that thyroidectomized animals are no less sensitive to heat than to cold. Similarly, *Boldyreff* (10) observed that their temperatures in hot surroundings mounted higher than that of the controls. On evaluating *Boldyreff's* results some difficulties arise, owing to the fact that all his experimental dogs suffered from convulsions following operation, whereby it may be believed that involuntary parathyroidectomy was associated with the intended operation. In view of this the results seem rather inconclusive, considering that we know from the recent experiments of Capitolo (11) that simultaneous removal of both glands results in a quicker elevation of temperature in hot surroundings. Finally, the observations of *Lévy* (12) and those of *Lévy* and *Rotschild* (13) should be mentioned, according to which individuals suffering from hypothyreosis (the normal temperature of these persons is some decigrades lower than that of healthy individuals) display a marked hyperthermia following exertion. Thus it may be seen that the problem is not as yet settled.

The experiments reported in the following refer to the temperature of thyroidectomized animals (rabbits and guinea pigs, principally the latter) in an environment warmer than the average to which the animals are accustomed.

Experiments. The animals were put into a thermostat, the temperature of which was changed according to the various experiments (and sometimes also in the course of the same experiment) between 27 and 41 C^0. The experiments lasted for from one and a half to eleven hours.

In each experiment one pair of carefully selected animals was employed. Of animals of the same sex and nearly the same weight (the maximum weight difference admitted was $\pm$ 7%) those were chosen the temperature diagrams of which took an identical course in several preliminary experiments with passive heating. One of each pair was thyroidectomized prior to the main experiment. Figure 1. shows the temperature diagram of an „appropriate" pair in a preliminary experiment. From the horizontal axis the hours can be read; from the vertical one the degrees of body temperature in centigrades. The numbers on the upper part of the figure refer to the temperature of the thermostat at the respective times.

On evaluating the results, comparison of the temperature values alone would not be correct. It sometimes happens in the course of the same experiment that the temperature sometimes of one animal, sometimes of the other, is at one time above, at another time below that of the other animal by some decigrades. Furthermore, time is to be taken into consideration as an essential factor. To cope with these postulates it is customary to draw a diagram. Temperature diagrams, however, frequently do not lend themselves for comparison, as their course can show diversities of character. To avoid this difficulty the following procedure was used: The temperature diagrams were drawn in a co-ordinate system in which the horizontal axis displays the time in hours, the vertical one temperature in centigrades. The body temperature, taken at the time of the animals' being put into the thermostat, coincides with the 0-point of the co-ordinate system. Thus temperature curve and x-axis enclose an area which is to be regarded as the result of the two factors time and temperature, that is, the common expression of the two factors which are significant from the viewpoint of our experiments. The areas obtained by this method (in the following termed „time-temperature area"), having been made on the basis of identical principles and scale, do admit of a correct comparison despite the occasional deviations in the course of the curves. Further, they may serve as the basis for statistical error calculations, provided we are not satisfied with the comparison of the temperatures at any given time, but compare the sum of the deviations distributed through time. We aimed at the latter method.

30 preliminary experiments performed with 12 animals showed that under passive heating the thyroidectomized animal yielded a larger time-temperature area than the control animal did. The average of time-temperature deviation was + 120%, they varying between + 10% and + 427%. Table I contains the differences of the time-temperature areas expressed in percentage as taken from 30 pairs of experiments.

TABLE I.

Difference of the time-temperature areas in percentage.

83	133	73
119	83	120
62	92	89
10	477	163
18	30	79
320	183	83
120	115	139
226	112	176
77	112	76
49	44	160

Average: + 120%

Figure 2 displays a diagram closely corresponding to the average which may be regarded as typical of the majority of the experiments. (The horizontal axis shows the hours, the vertical one the elevation of body temperature in centigrades; the numbers on the upper border of the figure the temperature of the thermostat at the respective times. The areas on the right side of the Figure are time-temperature areas proportionately reduced. Temperature of the normal animal has been drawn with a continuous, that of the thyroidectomized animal with a dotted, line.)

A uniform mathematical-statistical elaboration of all results was not possible, since experimental conditions varied between wide limits as to heating temperature and time of action. Only the statistical elaboration of those groups is admitted which consist of experiments performed under identical conditions as to temperature of the thermostat and duration of heating. Table II shows the time-temperature areas (expressed in mm^2) of a series lasting for two hours at a temperature varying from 35 to 36 C^0. The formula underlying the calculations is taken from *Linder:* Statistische Methoden. Birkhäuser. Basel. 1945.

$$s^2d = \frac{1}{N_1 + N_2 - 2}\left[\sum_{i=1}^{N_1}(x_i' - \bar{x}')^2 + \sum_{i=1}^{N_1}(x_i'' - \bar{x}'')^2\right]$$

$$and\ t = \frac{\bar{x}' - \bar{x}''}{sd}\sqrt{\frac{N_1\ N_2}{N_1 + N_2}}$$

TABLE II.

Time-temperature areas (mm^2). Two hour experiments at 35° C to 36° C.

Thyrect.	Norm.
851	473
677	465
627	281
813	291
756	256
711	375

X′ = 739
X″ = 357
P = 0,017

P = 0,017, thus it is ascertained that the two experiments have led to different results.

2. In the course of the foregoing experiments the observation was made that great changes of the surrounding temperature (e. g., from 17° room temperature to 37 C^0 thermostat) result in a rapid change in the

body temperature of both the normal and the thyroidectomized animals. In comparison the differences in the time-temperature areas are relatively small. On the other hand, a slow, gradual heating or cooling is accompanied by great differences in the time-temperature areas, because on heating the temperature of the thyroidectomized animals mounts more suddenly and to a higher level as compared with the initial value than does that of the control animals. Similarly, the phase of cooling starts later and goes on more slowly than in the controls. In further experiments the problem to be solved was whether the upper limit of the surrounding temperature just bearable without augmentation of the body temperature is less for thyroidectomized animals. Namely, as in homoiothermic animals for which there exists an irrelevant zone of surrounding temperature from viewpoint of their metabolism („température de la neutralité thermique", „Behaglichkeitszone") within the limits of which metabolism remains unaltered, there exists a similar zone for the homoiothermic animals also from viewpoint of their body temperature. The question has been raised whether or not the upper limit of this zone is lower for the thyroidectomized than for normal animals.

To answer this question the animal pairs (guinea pigs) were kept in thermostats of identical temperature for six hours to observe at what surrounding temperature the body temperature exhibits a noticeable increase. To determine just when the body temperature shows an elevation is not a simple question and cannot be done without establishing certain conventions, since little fluctuations in body temperature occur physiologically. Thus we agreed to pay no attention to elevations of temperature ensuing temporarily or remaining within 0,5 C°, whereas those exceeding 0,5 C^{0} and lasting throughout the whole experiment were regarded as a sign of disturbed thermo-regulation. The lowest temperature of the thermostat causing such a definite and long-lasting elevation of body temperature was termed the upper limit of the tolerated zone of external temperature. We are not ignorant of the fact that a thermostat environment does not correspond to a free, natural one. It suffices to mention the lack of air current, irradiation, liberty of movement and consumption of water. Consequently we do not intend to draw far-reaching conclusions from our experiments nor to consider the values obtained as absolute. The data do, however, admit of a comparison from the viewpoint of thermo-resistance under identical conditions.

The experiments have shown that, taking the stable temperature as a measure, the thermo-resistance of thyroidectomized animals is less. Figures 3, 4 and 5 give the diagrams of an animal pair kept in thermostats

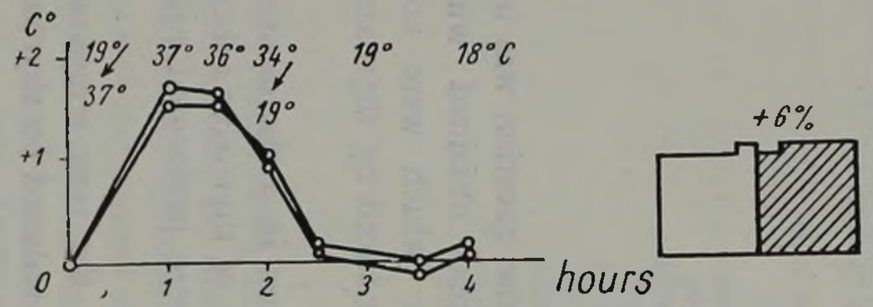

Fig. 1.

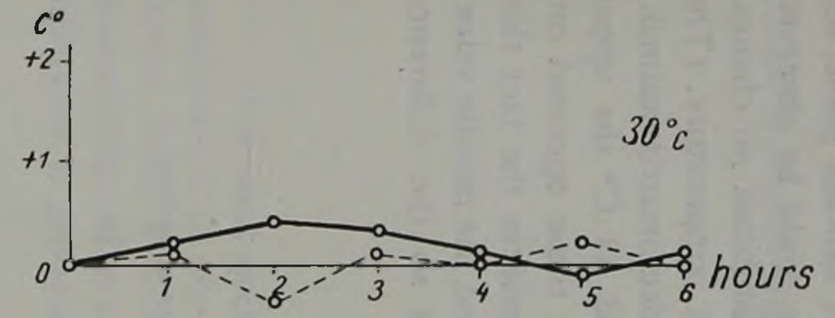

Fig. 3.

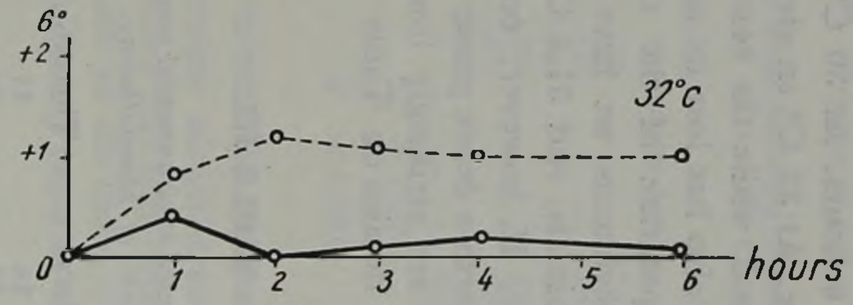

Fig. 4.

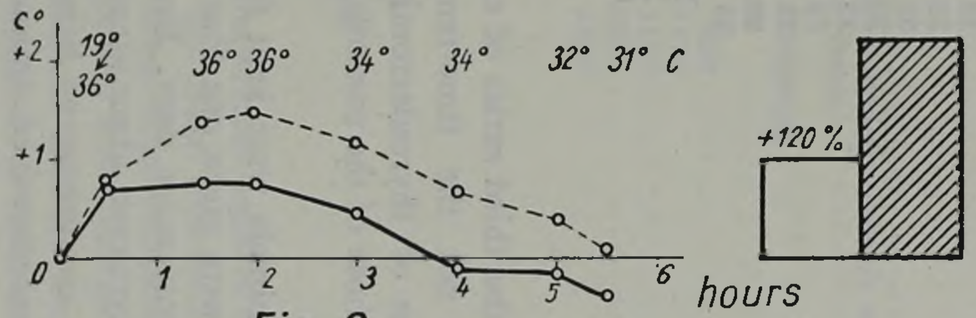

Fig. 2.

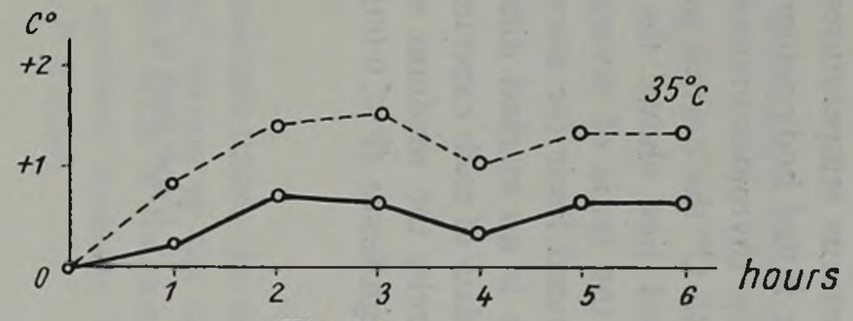

Fig.5.

of various temperatures for 6 hours. At 30 C⁰ both animals preserved their initial body temperature. At 32 C⁰ an elevation could be observed in the thyroidectomized animal, while the control exhibited no change. At 35 C⁰ the normal animal also has lost its original temperature. (The dotted line shows the temperature of the thyroidectomized animal.) On the basis of several experiments we have found 33 C⁰ the upper tolerated limit for normal animals, and 31,5 C⁰ for those operated on. This is no marked difference. If, however, one considers the fact that the data of each experiment form a dense group around the middle value, whereby the medium errors are strikingly low, and so the difference is significant ($P < 0{\cdot}001$) (v. details on Table III).

TABLE III.

Upper limit of the tolerated environmental temperature zone from viewpoint of homoiothermy.

Norm.	Thyrect.
34	31
33	30
32	31
33	32
33	32
33	32
32	31
33	32
33	32

$X' = 32{,}9$
$X'' = 31{,}5$
$P < 0{,}001$

3. In the third series of experiments the question was investigated whether or not the time-temperature areas obtained with passive hyperthermia of thyroidectomized animals (which were considerably larger than those of the controls) could be reduced by thyroxine administration.

The animals were kept for three hours in a thermostat of 34 to 35 C⁰, and were given one-fourth milligram of thyroxine subcutaneously on three consecutive days. Experiments were performed prior to and following thyroxine administration.

Other circumstances being identical, the time-temperature areas of the thyroidectomized animals were not reduced by thyroxine. They were even augmented. $P < 0{,}001$. Details in Table IV).

TABLE IV.

Changes of the time-temperature areas following thyroxine administration in thyrectomized animals. (34° C to 35° C, three hours.)

Prior to thyroxine admin.	Following
960	1350
830	1920
1040	1680
1030	2090
1570	2190
1660	1810
	2200

$X'' = 1182$
$X' = 1891$
$P < 0{,}001$

It seemed necessary to examine the control animals under the same conditions, i. e., after thyroxine administration and heating to the same extent. Table V. demonstrates that time-temperature areas of the control animals respond to thyroxine as do those of the operated animals. The difference is probably significant, because $0{,}02 < P < 0{,}05$.

TABLE V.

Changes of time-temperature areas following thyroxine administration in normal animals (34° C to 35° C, three hours).

Prior to thyroxine admin.	Following
490	820
480	1170
1030	1103
750	1260
1150	1280
710	
1030	
850	
780	
1110	

$X'' = 838$
$X' = 1127$
$0{,}02 < P < 0{,}05$

Thus it is established that the time-temperature areas of thyroidectomized animals cannot be diminished by thyroxine treatment. Contrary to our expectations, thyroxine enhances these time-temperature areas, likewise those of the control animals though to a lesser extent. Therefore we are disposed to state that thyroidectomized animals are, from the standpoint of passive hyperthermia, more sensitive to thyroxine than the nonoperated controls.

DISCUSSION.

Summarizing the data of three experimental series, the conclusion seems admissible that animals deprived of their thyroid glands are more sensitive to heat than control animals are: they exhibit larger time-temperature areas in passive hyperthermia and a lower upper limit of environmental temperature causing no alteration in body temperature than is the case with the controls. On thyroxine administration the time-temperature areas, contrary to expectation, increase to a greater degree than those of nonoperated animals; i.e., their thyroxine sensitivity is higher than that of the controls.

The fact that the basic phenomena due to thyroidectomy cannot be alleviated by, but rather become worse, subsequent to thyroxine treatment leaves no doubt as to the role of the thyroid in the protection of the organism against heating, the latter being due not to the thyroxine but to its antagonist termed thermothyrin A, produced by the thyroid in hot surroundings. The above experiments clrearly demonstrate the existence of this hormone.[1]

SUMMARY

The thermo-resistance of thyroidectomized animals is diminished as examined on the basis of normal body temperatures.

1. In passive hyperthermia they produce larger time-temperature areas than normal animals.

2. They tolerate without elevation of their body temperatures a temperature of the environment the upper limit of which lies at a lower level than is the case of the control animals.

3. The time-temperature areas of thyroidectomized animals, exceeding those of the controls cannot be diminished, they even become enhanced, by thyroxine. The enlargement of these areas is greater than in the conrol animals. That is, thyroidectomized animals are more sensitive to thyroxine from this viewpoint than normal animals are.

It is pointed out that the above phenomena are probably due to the lacking of thermothyrin A, the cooling hormone of the thyroid.

[1] The idea of replacing the lacking thyroid function by administration of thermothyrin A seems promising. This treatment could, however, not be successful except directly following thyroidectomy because, as has been shown by *Mansfeld* (3), for the effect neutralizing the action of thyroxine exercised by the two thermothyrins and for their effect in diminishing oxidations a certain supply of thyroid-colloid in the organism is indispensable. If colloid is lacking, as it is after thyroidectomy, thermothyrins stimulate oxidation, thus it is impossible that they could replace the thyroid as an impeding factor in oxidation.

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NEW DATA ON THE QUESTION OF HORMONAL THERMO-REGULATION.

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In previous works (1—3) dealing with the question of hormonal thermoregulation we reported that thyroidectomized animals are more sensitive to changes in temperature. The body temperature of such rabbits and guinea pigs mounts more rapidly and higher in hot surroundings than does that of normal animals. We measured the degree of hyperthermia with „time-temperature areas". I call time-temperature area the area enclosed in a co-ordinate system by the hyperthermic body temperature curve and the horizontal axis, measured planimetrically and expressed in mm^2, its size depending on the degree of elevation of the body temperature and the duration of the period of hyperthermia. The absolute values thus gained can be used advantageously for comparison. The time-temperature area of thyroidectomized animals measured in passive hyperthermia was essentially and significantly greater than that of the control animals in the same circumstances.

From further experiments it appeared also that this phenomenon consequent to extirpation of the thyroid gland could not be controlled by administrating thyroxine. Thus we cannot replace with thyroxine the activity of the thyroid gland. We therefore explained this observation as a consequence of the lack of thermothyrin *A* (cooling hormone). It is known that Mansfeld (4) demonstrated a thyrogen hormone in the thyroid gland and blood of animals under passive hyperthermia which the gland, during passive hyperthermia, pours out into the blood stream and which depresses metabolism. — Our experiments cited above indicate the significance of the humoral factor in „second chemical thermo-regulation". (5.)

According to the unanimous data of *Kennedy* (6), *Hartzell* (7), *Astwood* (8—9), *Mackenzie* and *Mackenzie* (10), and later many other authors, with thiouracil derivatives we arrive in a domain of substances which provide means of interfering with special cell function of the

thyroid. When given these chemicals the animals show symptoms of hypothyroidism and characteristic histological changes in the thyroid gland can be recognized. Experiments to clear up the mechanism carried out by *Franklin, Lenner* and *Chaikoff* (11—13), *Astwood* and *Bissel* (14) and *Rawson* and co-workers (15) established that thiouracil derivatives inhibit the synthesis of thryoxine in the thyroid gland. As the role played by the thyroid gland in second chemical thermo-regulation — in diminishing the oxidative processes — does not depend on the secretion of thyroxine, but of thermothyrine, it seemed worth while to investigate what effect the administration of thiouracil derivatives would have on the animals' sensitivity to temperature changes.

The experimental animals were selected by preliminary hyperthermic experiments among guinea pigs of one sex and of equal weight. We chose such pairs of animals, or groups of 3 animals, whose members under equal hyperthermic conditions showed equal changes in speed and extent of temperature elevation. That is, those which in several preliminary experiments produced time-temperature areas not essentially varying from one another. One of the animals of a pair was then fed for 3 weeks daily 0,07 gr/kg methylthiouracil.[1] The other served as control. At

TABLE I.

Time-temperature areas	No. 1 guinea pig (methylthiouracil)	No. 2 guinea pig (methylthiouracil)	No. 3 guinea pig (control)
	890	1290	810
	1240	1030	740
	1030	1190	770
	1220	870	850
	1190	630	670
	1000	850	700
	1350	1470	840
		1160	910
	1520	1340	860
	1050	1250	910
Average	1140		810

[1] The methylthiouracil was contributed by the Egger Pharmaceutical Manufacturing Company, Budapest, to which I take this occasion of acknowledging my gratitude.

the end of the 3 weeks hyperthermic experiments were carried out on the animals by putting them in a thermostat at 34—35 C⁰ for 2½hours. The increase in body temperature was measured and the time-temperature area. From the data, shown in *Tables I* and *II*, it appears that the time-

TABLE II.

Time-temperature areaes	No. 4 guinea pig (methylthiouracil)	No. 5 guinea pig (control)
	1350	850
	1360	1090
	1520	940
	1440	1100
	830	570
	1050	520
Average	1260	845

temperature areas of the animals given methylthiouracil were greater that those of the controls. Statistical calculations prove the significance of the difference. Thus the animals given methylthiouracil were less resistant to changes of temperature than the controls. This result corresponds essentially with the observations we made on thyroidectomized animals. If we take into consideration the quantative relation as well we find that the difference between the time-temperature areas of thyroidectomized and normal guinea pigs, measured during passive hyperthermia, is greater than that between those given methylthiouracil and their controls. This is not surprising, thyroidectomy being the more radical interference. But the difference between the thyroidectomized animals and those given methylthiouracil is only quantative, not qualitative. In both of them resistance to temperature changes diminishes. Our experiments mentioned above show that this phenomenon is not a consequence of thyroxine deficiency. We must therefore suppose that *the methylthiouracil does not only impede the synthesis of thyroxine in the thyroid gland, but that it also inhibits the production of thermothyrin;* that is, it in general impairs the secretion of the thyroid gland.

To make certain that the experimental animals really were affected by the methylthiouracil, a histological investigation of the thyroid gland seemed necessary. This Dr. L. Dános (Pathologic Anatomy, Univer-

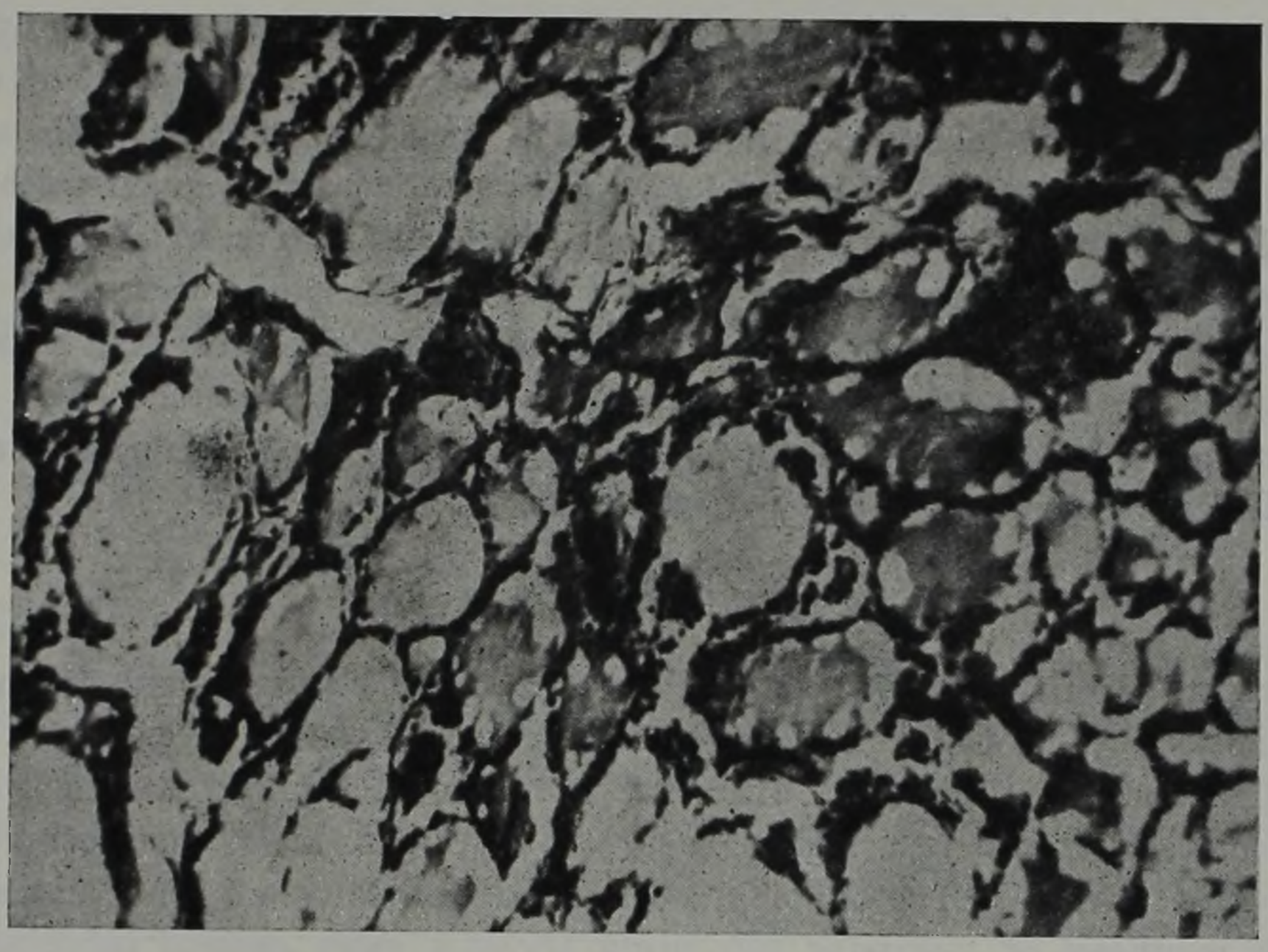

FIG. I.

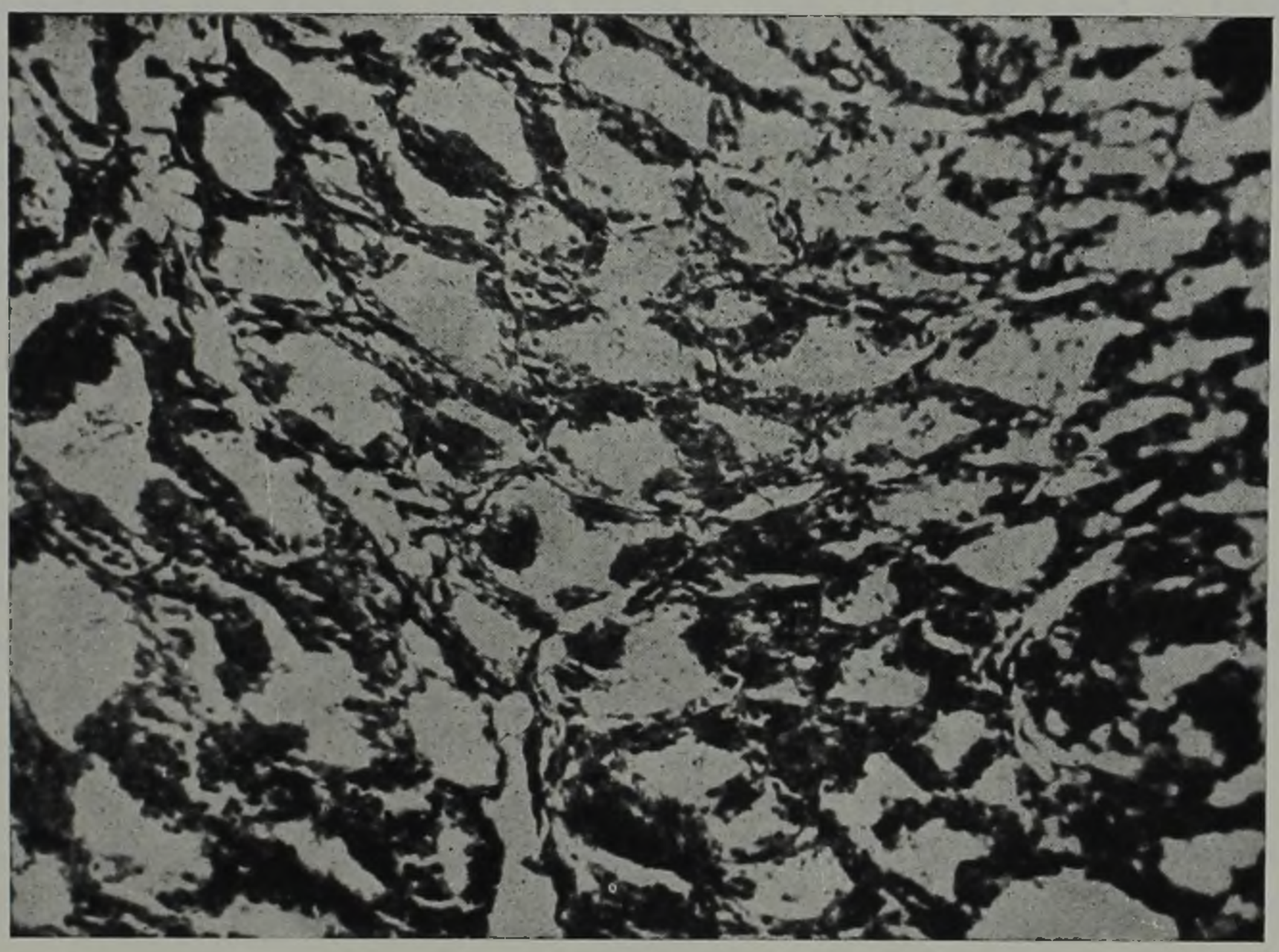

FIG. II.

sity of Pécs) had the kindness to carry out. From his microphotographs it can be seen that the histological picture of the thyroid gland of the control animals shows the normal image with flat epithelial cells and an abundance of colloid. (Fig. 1.) Whereas in the animals given methylthiouracil there can be seen a change similar to that seen in Graves' disease, which has been repeatedly and unanimously described by anglosaxon authors as characteristic of thiouracil derivatives: high hyperplastic epithelial cells and an almost entire lack of colloid. (Fig. 2.) According to all this, it is not to be doubted that methylthiouracil was efficacious in the treatment of the animals.

SUMMARIZING,

we can say that the thermal tolerance of animals treated with methylthiouracil diminishes. This circumstance corresponds in principle to what we had previously observed in thyroidectomized animals. As thermal tolerance is a symptom not of thyroxine but of thermothyrine A deficiency, it seems probable that thiouracil derivatives not only suspend the synthesis of thyroxine but bring about a more general inhibition, insofar as they impede the production of thermothyrine.

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THE PRODUCTS OF THE SPLITTING OF *ATP* BY MYOSIN.*

BY I. BANGA AND GY. JOSEPOVITS

FROM THE INST. OF BIOCHEMISTRY OF THE UNIVERSITY OF BUDAPEST

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If pure myosin is allowed to act upon ATP, one P is split off per molecule of ATP and the reaction stops. If the reaction mixture is analysed it is found to contain two substances, the one of which is ADP_1 (the same as *Lohmann*-s[1] ADP) and the other a dinucleotide-pentaphosphate (DNP). If at this stage a protein is added, which has been described before as „ADP-isomerase"[2] and will be called henceforth „protein II" the reaction proceeds, P and N split off until the nucleotide is degraded to inosinic acid. If pure crystalline myosin is allowed to act on ATP, protein II being present right from the beginning of the reaction, ATP is quickly dephosphorylated and desaminated to inosinic acid. It lies at hand to believe that also in this case the reaction proceeded over the same way as above, ADP_1 and DNP being formed first. This, however, is not the case. The experiment shows that ADP_1 and DNP are attacked by myosin + protein II but very slowly, the latter with a considerable latent period. If, however, protein II is present from the beginning, myosin splits ATP into inosinic acid rather quickly. This shows that ADP_1 and DNP cannot be formed. These results can be explained in two ways: either the primary splitting products are different according to whether protein II is present or not, a very reactive labile substance being formed in presence of protein II. which substance is split further rapidly, while in absence of protein II, the more stable ADP_1 and DNP are formed, or else we could suppose that the primary product of splitting is the same in both cases but in absence of protein II this is transformed into ADP_1 and DNP not being split further. ADP_1 and DNP in this case, are stabilisation-products.

In this paper the analyses of ADP_1 and DNP are given, while in the subsequent paper the enzymatic reaction of ADP_1 and DNP with myosin and actomyosin in the presence or absence of protein II will be discussed.

* This research was sponsored by the *Jos. Macy Jr. Foundation,* New York.

EXPERIMENTAL PART
THE ISOLATION OF DINUCLEOTIDE-PENTAPHOSPHATE (DNP) AND OF ADENOSINE-DIPHOSPHATE (ADP_1)

In 300 ml total volume 600 mg of crystallised myosin, 2,25 g of ATP as *K*-salt (starting from 2,63 g acid *Ca*-salt of ATP $C_{10}H_{14}O_{13}N_5P_3Ca\ 2H_2O$ dissolved in 200 ml H_2O adding the calculated quantity of *K*-oxalate, neutralized with *KOH* and separated from the *Ca*-oxalate by centrifugalization) and 0,1 *M KCl* were added. The reaction mixture was incubated at 38° C for 10 min. under constant shaking. At the end of the 10 min. the reaction was stopped by adding 30 ml of 10% trichloroacetic acid. During this time 33% of labile phosphate of ATP has been split off.

The inorganic phosphate was precipitated in the form of a $MgNH_4PO_4$ compound by adding 3 g of $MgCl_2$ and NH_4OH to the filtrate. Two g.—*s* of $BaCl_2$ are added whereby the dinucleotide was precipitated in the from of a water insoluble *Ba*-salt. This neutral *Ba*-salt was transformed into an acid *Ba*-salt by dissolving it in *N/10 HCl* and was precipitated by adding alcohol. The precipitate was washed in alcohol until the alcohol contained no *Cl*. The dinucleotide *Ba*-salt was then dried first in an exsiccator and then at 105° C. Yield = 1,41 g acid *Ba*-salt of the dinucleotidepentaphosphate.

The water soluble *Ba*-salt of ADP_1 was precipitated by adding equal part of alcohol. After centrifugalizing it was washed with alcohol and dried in an exsiccator. Yield 1,01 g of neutr. *Ba*-salt of ADP_1. *Preparation of the solutions for the analysis: Solution of ATP.* 200 mg of the acid *Ca*-salt of *ATP* was dissolved in 15 ml of water. 1 ml of the solution contained 2,06 mg of total P. It was in concerning of *ATP* 0,0218 M and of P 0,0654 M. The relation between labile and stable P was 2 : 1. *Solution of DNP:* 200 mg of the acid *Ba*-salt of *DNP* was suspended in 5,8 ml water and 9,6 ml of N/10 H_2SO_4. Total volume was 15,4 ml. After centrifugalizing off the $BaSO_4$, the solution does not give any precipitate with one drop of N/10 H_2SO_4 or with one drop of N/10 $Ba(OH)_2$. One ml of the solution contained 1,44 mg of total P. The relation between labile and stable P is 2,3 : 1. *Solution of* ADP_1*:* 200 mg of the neutr. *Ba*-salt was suspended in 10,5 ml water and 6 ml of N/10 H_2SO_4. Total volume was 16,5 ml. After centrifugalizing off the $BaSO_4$ the solution does not give any precipitate with one drop of N/10 H_2SO_4 nor with one drop of N/10 $Ba(OH)_2$. One ml contained 0,98 mg of total P. The relation between labile and stable P was 1 : 1.

The determination of the total P and of the total N: An aliquot part of the solutions described above was decomposed by boiling with conc sulphuric acid and H_2O_2. The *N* was determined by the micro-Kjeldah

and the *P* according to Fiske-Subbarow. Table I. gives the values received and the ratio of *N/P*.

TABLE I.

Solution employed	N mg found in 1 ml	P mg found in 1 ml	N/P
ATP adenosine triphosph.	1,52	2,06	0,74
DNP dinucleot. pentaphosph.	1,35	1,44	0,93
ADP_1 adenosine diphosph.	1,10	0,98	1,12

The theoretical *N/P* value in *ATP* 0,75, in *DNP* 0,90, in *ADP* 1,13. As the results show the *N/P* value in the water insoluble *Ba*-salt (0·93) is near to the theoretical *N/P* value (0,90) of a dinucleotide-pentaphosphate. The water soluble *Ba*-salt gives the theoretical value for *ADP*.

Determination of amino-N. The NH_2—*N* was determined by the method of Van—Slyke, results being compared with results obtained on *ATP*. The solution was being shaken with concentrated acetic acid and $NaNO_2$ for different periods and the quantity of free amino-*N* found was expressed in per cent. The amino-*N* was taken as being equal to total *N/5*. The results are given in Table II.

TABLE II.

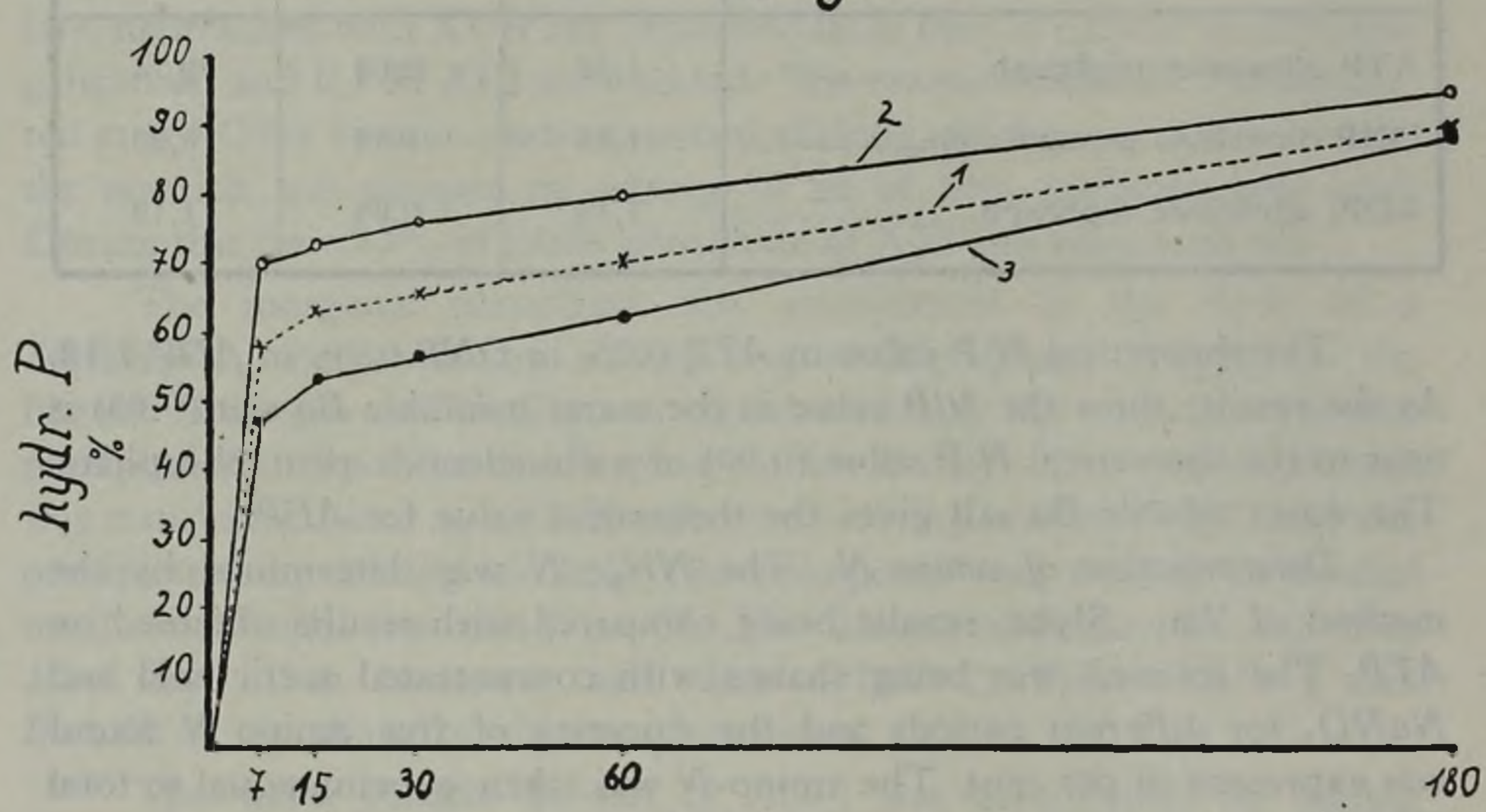
	Calcul. amino-N. mg/ml	Amino-N found mg			Amino-N split off %		
Minutes		10	20	40	10	20	40
ATP	0,304	0,120	0,340	0,340	39	112	112
DNP	0,270	0,168	0,168	0,168	62	62	62
ADP_1	0,220	0,080	0,170	0,200	37	76	90

As Table II. shows the total amino-*N* of *ATP* splits off in 20 minutes. In the supposed *DNP* (dinucleotide-pentaphosphate) only half of the quantity of amino-*N* split off in the first 10 minutes, and even after 40 minutes there is no more liberation of *N* at all. The amino-*N* of ADP_1 split off slowly during a period of 40 minutes.

Determination of the acidic hydrolysis of P. The solutions were hydrolysed in *N HCl* in a water-bath at 100° C for 7, 15, 30, 60 and 180 minutes

and the quantity of P split off was determined. Fig. I. shows the hydrolysed quantity of the *P* of *ATP*, of *DNP* and of ADP_1 expressed as % of the total *P*.

Fig. I.



Curve 1. Splitting of P from ATP in N HCl 100° C.
Curve 2. Splitting of P „ Dinucleotide in N HCl 100° C.
Curve 3. Splitting of P „ ADP_1 in N HCl 100° C.

As Fig. I. shows 70% of the total *P* of *DNP* is split off in 7 minutes at 100° in *N HCl*. 76% split off in 30 minutes. Supposing that there are five *P*-s in the molecule of *DNP*, of which four are labile and one is stable, then the one mononucleotide of the *DNP* molecule is similar to *ATP* and the other to an *ADP* which has two labile *P*-s. The hydrolysis curve of ADP_1 is the same as Lohmans *ADP*.

The analysis of the Ag-salt of DNP. The *Ag*-salt of dinucleotide-pentaphosphate was prepared by adding $AgNO_3$ in presence of nitric acid until no precipitate was formed. The *Ag*-salt was centrifuged, and crystallised twice from hot water. The *Ag*-salt thus obtained was dried first in an exsiccator and then at 100° C. The analytical data correspond to the formula $C_{20}H_{24}O_{22}N_{10}P_5Ag_5$.

The micro-analysis of *C, H, N* was made by M. Kovács—Oskolás, whom our thanks are due. The *Ag* was titrated with NH_4SCN in the presence of ferro-ammoniumsulphate as indicator. The results are shown in Table III.

TABLE III.

	C	H	N	P	Ag
Values calculated for $C_{20}H_{24}O_{22}N_{10}P_5Ag_5$	16,55	1,65	9,66	10,69	37,17
Values found for $C_{20}H_{24}O_{22}N_{10}P_5Ag_5$	16,90 17,00	1,70 1,80	9,89 9,78	10,50 10,66	37,00 36,75

The micro-analysis of the *Ag*-salt of *DNP* proves that we deal really with a dinucleotide-pentaphosphate.

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THE ENZYMATIC REACTIONS OF DINUCLEOTIDE AND ADP_1 WITH MYOSIN.

BY I. BANGA

FROM THE INST. OF BIOCHEMISTRY OF THE UNIVERSITY OF BUDAPEST

RECEIVED FOR PUBLICATION 15. XI. 1946.

It was shown in a previous paper[1] that two compounds arise when *ATP* is split by pure myosin: a dinucleotide and ADP_1.

In this paper the enzymatic splitting of these compounds by myosin, and actomyosin will be described in the presence or absence of the protein II.

In Fig. I. curves of the splitting of *ATP* will be given which could be compared with the curves of Dinucleotidepentaphosphate *DNP* Fig. II—III. and of ADP_1 Fig. IV.

Fig. II. curve 1 shows that the *DNP* does not split on myosin or on actomyosin with measurable velocity although the ratio of labile *P* to total *P*, is very close to the same ratio in *ATP*. In the presence of myosin + protein II. at the beginning the *DNP* splits only 30% as can be seen in Fig. II. curve 2 with a latent period of about 10 minutes the splitting off of the labile *P*-s begins being nearly complete in 40 minutes.

In the presence of actomyosin + II. protein (Fig. II. curve 4) the splitting off of the labile *P*-s of the *DNP* sets in immediately. Fig. III. shows the splitting off of the labile *P*-s and of NH_2 of the *DNP* in the presence of myosin + II. protein compared with the respective values of actomyosin + II. protein. In the first ten minutes, in the presence of myosin + II. protein, about 30% of the labile *P*-s split off and about 45% of the NH_2-group. (Fig. III. curves 1,2). After this latent time of 10 minutes the splitting off of the remaining labile *P*-s and the second NH_2-group sets in. In the presence of actomyosin + II. protein (Fig. III. curve 3,4) the splitting off of the labile *P*-s and of the NH_2-group sets in immediately.

The splitting of ADP_1 differs not only from that of *ATP*, but from that of the *DNP* as well. In case of ADP_1 it is the half of its total *P*-s i. e.

one of the two *P*-groups is acid labile. This readily hydrolysable *P* does not split at all on myosin as can be seen on Fig. IV.; nor does it split on actomyosin. In the presence of myosin + II. protein half of the readily hydrolysable *P* (very slow reaction) is split off. In the presence of actomyosin + II. protein on the other hand the reaction is very fast. There is no desamidation taking place, neither on myosin nor on actomyosin. In the presence of myosin + II. protein a desamidation of 40% takes place in 40 min. as shown in Table I. and in the presence of actomyosin + II. protein the values do not differ essentially (50% desamidation). While all of the hydrolysable *P*-groups of ADP_1 split off in the presence of actomyosin + II. protein only half of the NH_2 is split off. On the other hand with the method of Van Slyke[1] the entire NH_2 of ADP_1 can be determined which proves that it is free. These observation can only be accounted for if I suppose that during the enzymatic reaction ADP_1 is transformed into a dimeric modification where one *of the two ADP-s* is Lohmann's *ADP* and the other corresponds to Barrenscheen's. *(ADP_2)* According to Barrenscheen the hydrolysable *P*-groups of *ATP* are linked to NH_2-group.

The results do not show definitely what changes take place in the configuration in the course of the enzymatic reaction.

It might be supposed that in the muscle, where actomyosin as well as II. protein is present, *DNP* does not arise at all and that the ADP_1 undergoes further reactions. In order to clear up the mechanism of the splitting of *ADP* in the presence of myosin as well as of actomyosin + II. protein, further investigations are necessary. The changes of pH during the enzymatic reaction were measured; the changes observed coincide with the calculated values.

EXPERIMENTAL PART

Crystallised myosin: twice crystallised myosin, prepared after the method of *A. Szent-Györgyi.*[3]

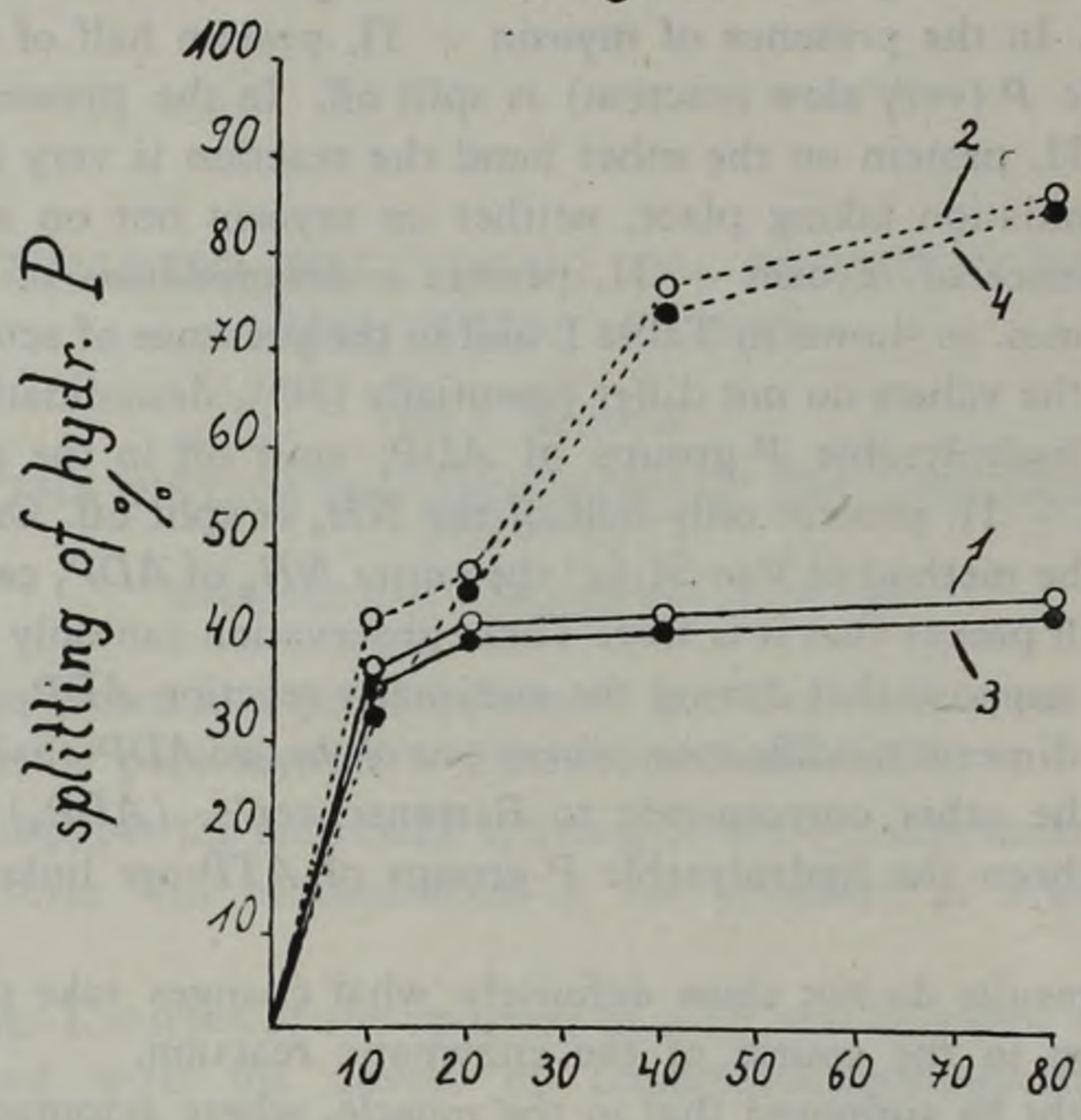
The preparation of the II. protein: the II. protein is the same as the *ADP*-isomerase[2] or as the soluble protein which was described by *K. Laki*[4] as being necessary for the splitting off of the second *P* from *ATP*.

Actin: prepared after the method of *F. B. Straub.*[5]

Fig. I. shows the splitting off of the readily hydrolysable *P* from *ATP:* 1. in the presence of myosin, 2. in the presence of myosin + II. protein, 3. in the presence of actomyosin, 4. in the presence of actomyosin + II. protein.

Note: The splitting off of the first and the second *P* was taken as 100% and thus the splitting off of the first *P* was 50%.

Fig. I.



Curve 1. Splitting of ATP on cryst. myosin.
Curve 2. Splitting of ATP on cryst. myosin + II. protein.
Curve 3. Splitting of ATP on actomyosin.
Curve 4. Splitting of ATP on actomyosin + II. protein.

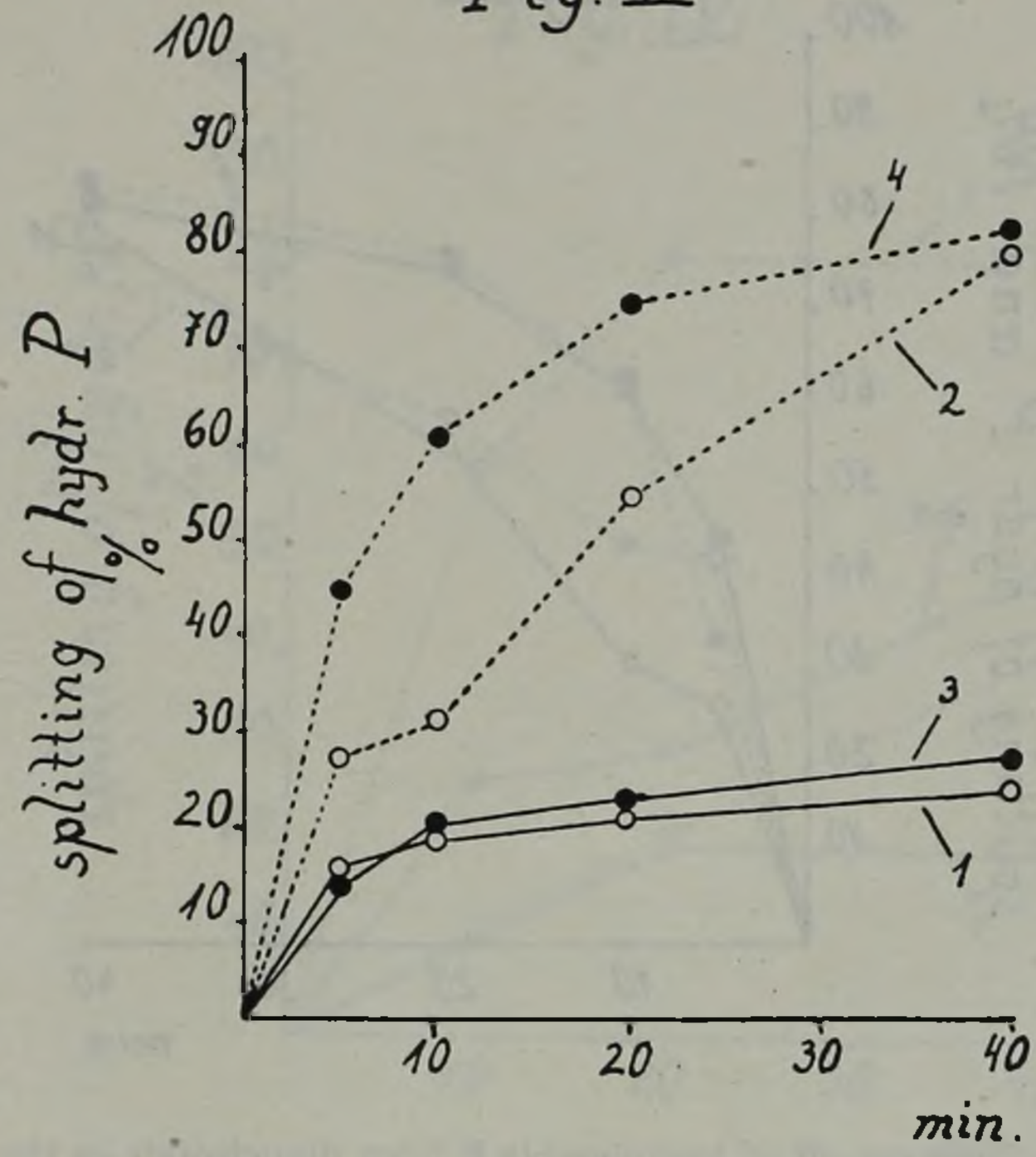
The reaction mixture in Fig. I. contained in 5 ml. total volume 5 mg of cryst. myosin, 15 mg *ATP* as *K*-salt and 2 ml veronal-acetate buffer of pH 7. The quantity of the II. protein was 100γ in 5 ml, that of actin 0,85 mg. The mixture was shaken at 38° C. After having been precipitated with 1 ml of 10% trichloroacetic acid the inorganic *P* was measured after the method of Fiske—Subbarow.

Fig. II. shows the time-curves of the readily hydrolysable *P*-s of the *DNP :* 1. in the presence of myosin, 2. in the presence of myosin + II. protein, 3. in the presence of actomyosin, 4. in the presence of actomyosin + II. protein.

The reaction mixture in the case of Fig. II. is the same as in Fig. I. with the exception that 15 mg of *DNP* (*K*-salt) has been employed instead of *ATP*.

Fig. III. shows the time-curves of the splitting off of the readily hydrolysable *P*-s and of that of the NH_2-group of *DNP* : 1—2 in the

Fig. II.



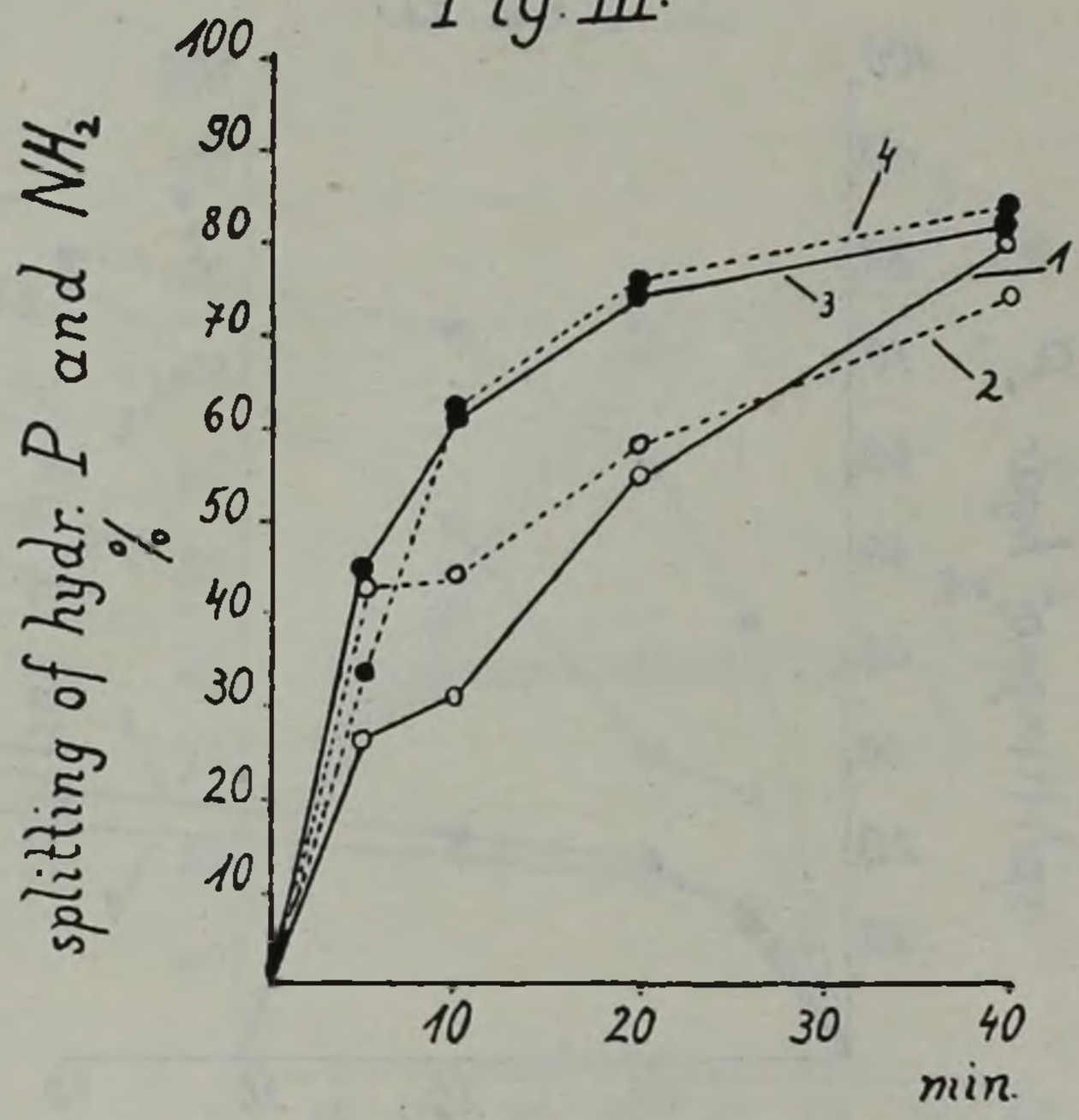
Curve 1. Splitting of dinukleotide on myosin.
Curve 2. Splitting of dinukleotide on myosin + II. protein.
Curve 3. Splitting of dinukleotide on actomyosin.
Curve 4. Splitting of dinukleotide on actomyosin + II. protein.

presence of myosin + II. protein, 3—4 in the presence of actomyosin + II. protein. The reaction mixture in the case of Fig. III. is the same as with Fig. II. but here, in addition to the splitting off of *P*, the splitting off of NH_2 has also been determined in an aliquot part of the trichloroacetic acid filtrate. The liberation of NH_3 was determined by micro-Kjeldhal. The apparatus used in the destillation was that of Wagner—Parnas. The NH_2-group was calculated as 20% of the total *N* and the liberated NH_3 was compared with that value.

Fig. IV. shows the splitting off of the readily hydrolysable *P* from ADP_1: 1. in the presence of myosin, 2. in the presence of myosin + II. protein, 3. in the presence of actomyosin, 4. in the presence of actomyosin + II. protein.

The reaction mixture contained in Fig. IV. in 5 ml of total volume 5 mg of cryst. myosin 15 mg of ADP_1 (*K*-salt) 100γ of II. protein + 0,85 mg of actin; the pH was 7.

Fig. III.



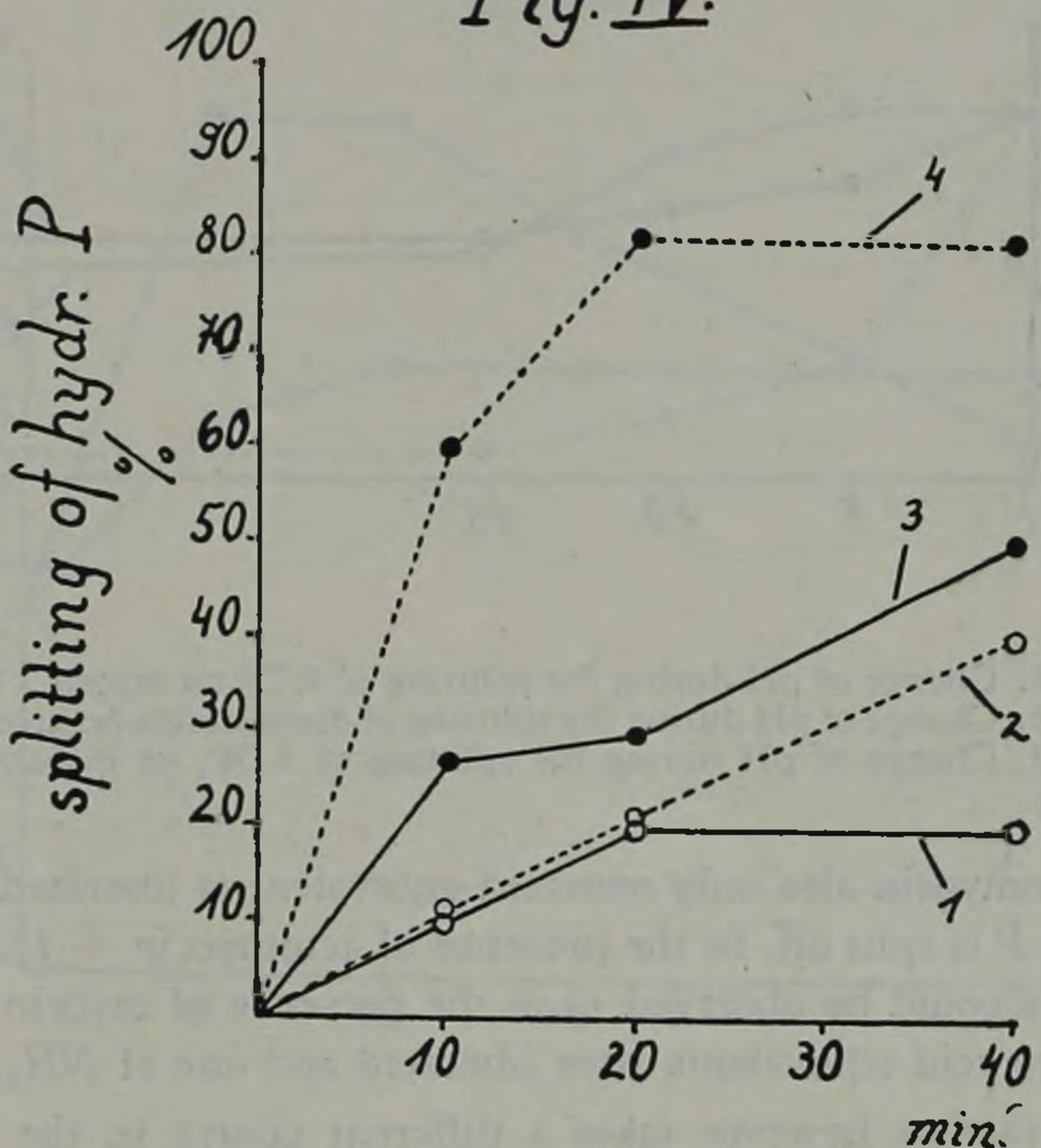
Curve 1. Splitting off of hydrolysable P from dinucleotide in the presence of myosin + II. protein.
Curve 2. Splitting of NH_2 from dinucleotide in presence of myosin + II. protein.
Curve 3. Splitting off of hydrolysable P from dinucleotide in the presence of actomyosin + II. protein.
Curve 4. Splitting off of NH_2 from dinucleotide in the presence of actomyosin + II. protein.

TABLE I.

Vessels contain:	Splitting of NH_2% in 40 min.
Myosin + ADP_1	13
Myosin + II. protein + ADP_1	38
Actomyosin + ADP_1	34
Actomyosin + II. protein + ADP_1	53

Table I. shows the desamidation of ADP_1 after an incubation time of 40 minutes. The reaction mixture employed was the same as in Fig. IV.

Fig. IV.

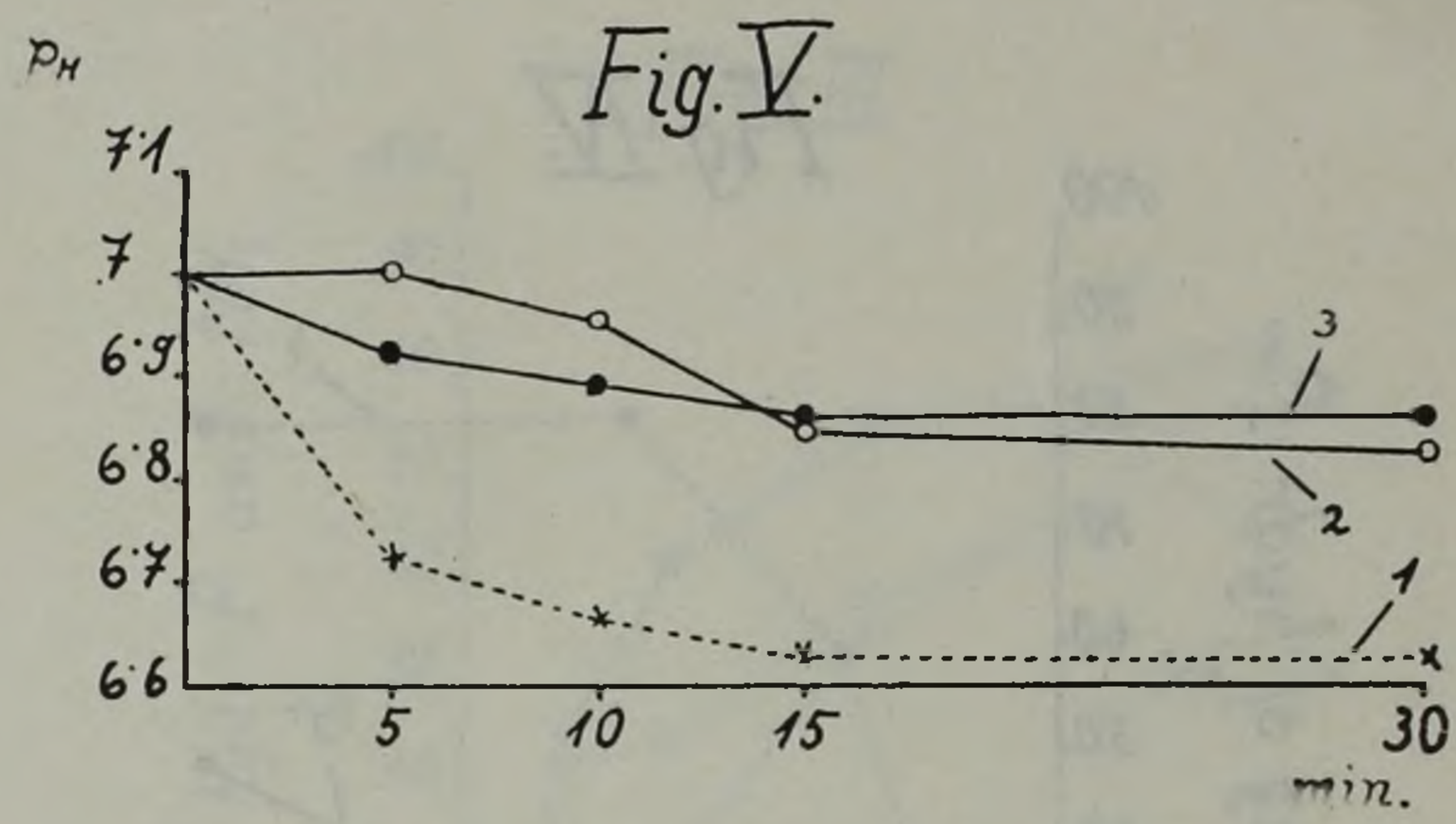


Curve 1. Splitting of ADP_1 on myosin.
Curve 2. Splitting of ADP_1 on myosin + II. protein.
Curve 3. Splitting of ADP_1 on actomyosin.
Curve 4. Splitting of ADP_1 on actomyosin + II. protein.

THE pH CHANGES IN THE ENZYMATIC SPLITTING OF ATP, DINUCLEOTIDEPENTAPHOSPHATE AND ADP_1

When *ATP* is split on crystallised myosin, the splitting off of the first *P* is accompanied by the liberation of one acid equivalent per molecule. The liberation of the acid equivalent can be determined by colorimetric measurement of the pH. The system was set up in such a way that the appearance of one acid equivalent should correspond to a change in pH 0,35—0,40. The changes in pH were determined in the case of the enzymatic splitting of *ATP*, *DNP* and ADP_1. In the case of *ATP* in the presence of myosin + II. protein, both readily hydrolysable *P*-s are split off while one M NH_3 was also split off and thus the changes in pH was the same as with myosin alone. This can be accounted for by supposing that the acid equivalent, liberated with the second *P* is neutralised by NH_3. In the pre-

Fig. V.



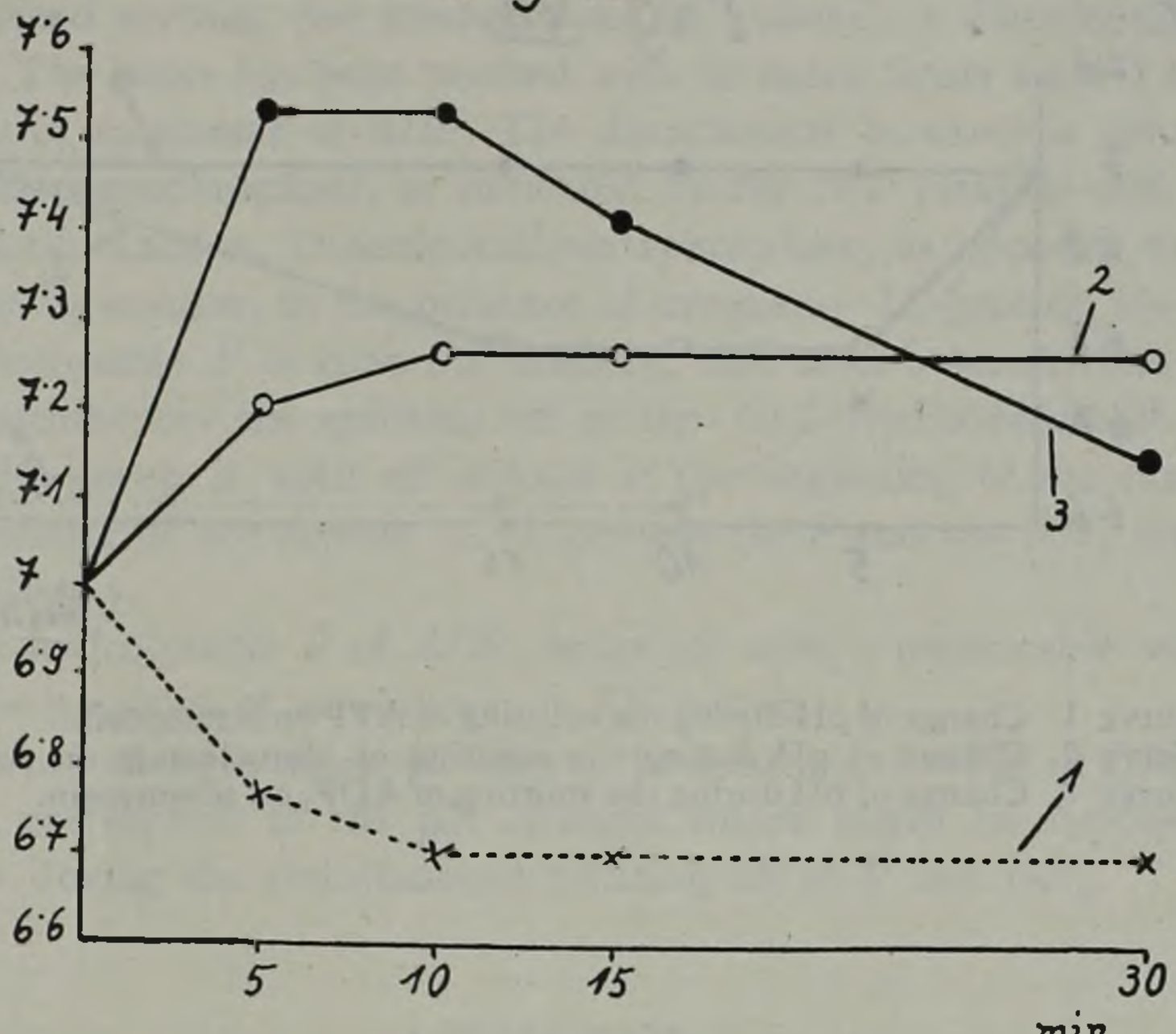
Curve 1. Change of pH during the splitting of ATP on myosin.
Curve 2. Change of pH during the splitting of dinukleotide on myosin.
Curve 3. Change of pH during the splitting of ADP_1 on myosin.

sence of actomyosin also only one acid equivalent is liberated since here, too, the first *P* is split off. In the presence of actomyosin + II. protein the same process could be observed as in the presence of myosin + II. protein, i. e. two acid equivalents were liberated and one of NH_3.

The reaction however takes a different course in the case of the *DNP*, when in the presence of myosin only 20—25% of the hydrolysable *P* is split off and a half of the acid equivalent is liberated. In the presence of myosin + II. protein there is a quick NH_3 liberation observable corresponding to the splitting off of one hydrolysable *P* i. e. of one *P* out of the three hydrolysable *P*-s. Therefore alkalisation may be predicted and does actually take place as can be seen in Fig. VI. Curve 2. since the NH_3 represents two alkali-equivalents as against the one acid equivalent that accompanied the splitting off of one *P*. In the presence of actomyosin also only one *P* was split off out of the three and therefore one acid equivalent was liberated which disappeared, however, between 15—30 minutes due to some unknown reaction (Fig. VII. curve 2). In the presence of actomyosin + II. protein a weak alkalisation could be observed which accounted for the splitting off of the three hydrolysable phosphate and the two NH_2-groups. This reaction also suggests that a *DNP* has actually been present.

Investigating the pH changes of ADP_1, during enzymatic splitting it was found that in the presence of myosin there is a slow (20%) splitting off of *P* as well as a slight decrease of the pH. In the presence of myosin + II. protein there was a great initial rise of pH which disappeared at

Fig. VI.



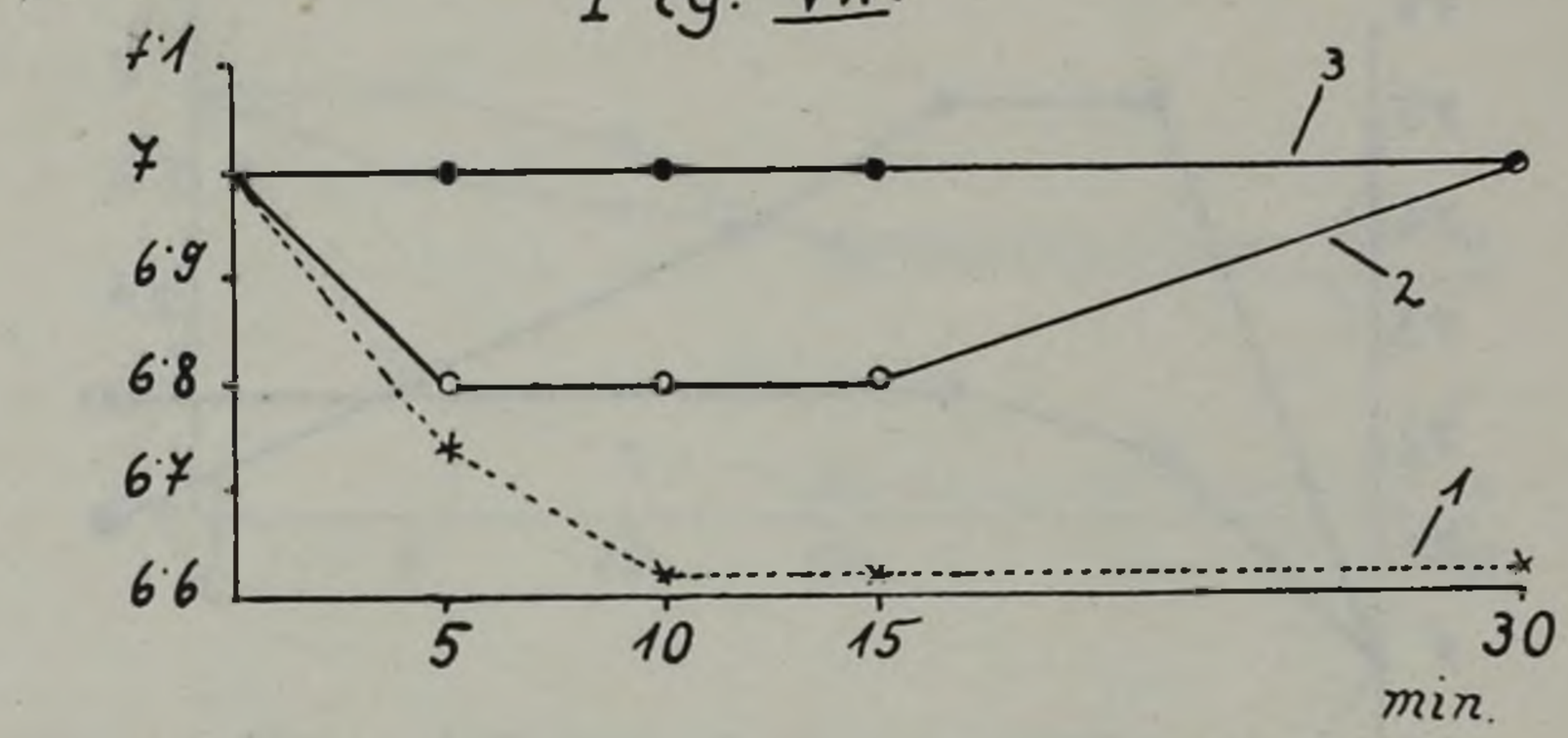
Curve 1. Change of pH during the splitting of ATP on myosin + II. protein.
Curve 2. Change of pH during the splitting of dinucleotide on myosin + II. protein.
Curve 3. Change of pH during the splitting of ADP_1 on myosin + II. protein.

about 10 minutes when the pH changed towards acidity. This could be accounted for by supposing that one half of the hydrolysable *P*-s was split off (half acid equivalent) and one M NH_3 was liberated. In the presence of actomyosin no change was observed. There was no change of pH in the presence of actomyosin + II. protein either, since all the hydrolysable *P* were split off against only a half of the NH_2-groups.

These changes of pH suggest also that ADP_1 has been isomerised into ADP_2 in the presence of myosin + II. protein. It can be surmised therefore that the *P* and the NH_2-groups are split off only from ADP_2.

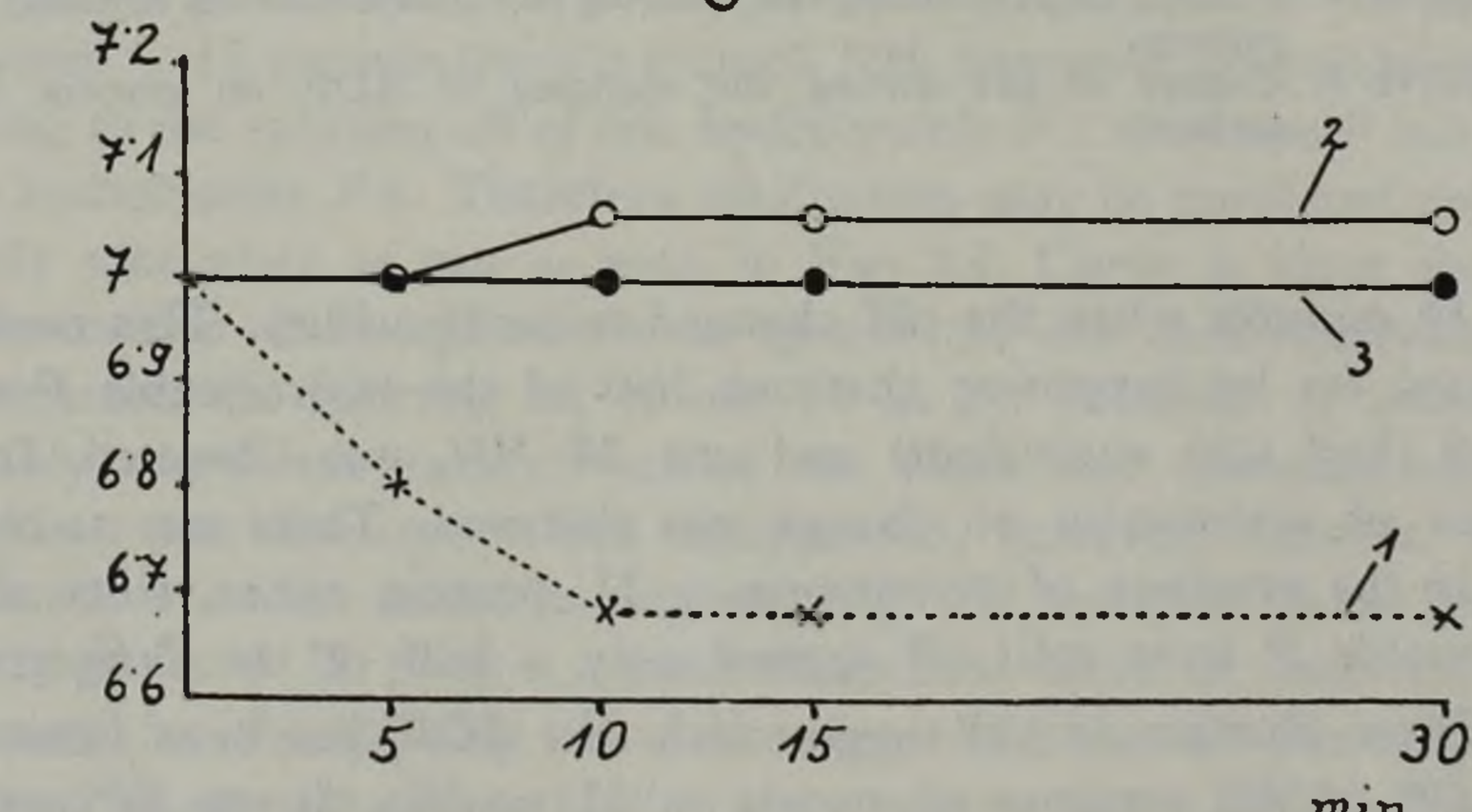
Fig. V—VIII. show the pH changes of *ATP*, *DNP* and of ADP_1, Fig. V. in the presence of myosin, Fig. VI. in the presence of myosin + II. protein, Fig. VII. in the presence of actomyosin, and Fig. VIII. in the presence of actomyosin + II. protein.

Fig. VII.



Curve 1. Change of pH during the splitting of ATP on actomyosin.
Curve 2. Change ot pH during the splitting of dinucleotide on actomyosin.
Curve 3. Change of pH during the splitting of ADP_1 on actomyosin.

Fig. VIII.



Curve 1. Change of pH during the splitting of ATP on actomyosin + II. protein.
Curve 2. Change of pH during the splitting of dinucleotide on actomyosin + + II. protein.
Curve 3. Change of pH during the splitting of ADP_1 on actomyosin + II. protein.

SUMMARY

My experiments have shown that as product of the splitting of *ATP* by crystallised myosin, two products can be isolated: a dinucleotide and an ADP_1. The latter has been marked with an index figure since I had to suppose it is an isomer of *ADP*. The dinucleotide however is actually a dinucleotidepentaphosphate, as indicated by the *N/P* ratio as well as by its enzymatic reaction. Dinucleotidepentaphosphate, as opposed to *ATP* is not split by myosin; in the presence of myosin + II. protein 25—30% of its hydrolysable *P* is split off initially, and after a latent time of 10 minutes commences the splitting off of the total hydrolysable *P*. Half of the NH_2-group is split off already at the beginning of the reaction. In the presence of actomyosin + II. protein the *P* and the NH_2 are split off immediately.

The hydrolysable *P* of ADP_1 splits off with a measurable velocity only in the presence of actomyosin + II. protein.

The pH changes observed during the splitting of *ATP*, of *DNP* and of ADP_1, correspond to the pH changes which might be supposed to take place during the simultaneous splitting off of *P* and NH_2.

LITERATURE

1. *I. Banga* and *Gy. Josepovits:* This Vol. page 67. 1946.
2. *I. Banga:* Studies from the Inst. of Med. Chem. Univ. Szeged. Vol. III. page 72. 1943.
3. *A. Szent-Györgyi:* ibid. Vol. III. page 76. 1943.
4. *K. Laki:* ibid. Vol. III. page 16. 1943.
5. *F. B. Straub:* ibid. Vol. III. page 23, 1943.

THE ENZYMATIC SPLITTING AND DESAMIDATION OF ATP.

BY I. BANGA AND G. JOSEPOVITS

FROM THE INST. OF. BIOCHEMISTRY OF THE UNIVERSITY OF BUDAPEST.

RECEIVED FOR PUBLICATION 15. XI. 1946.

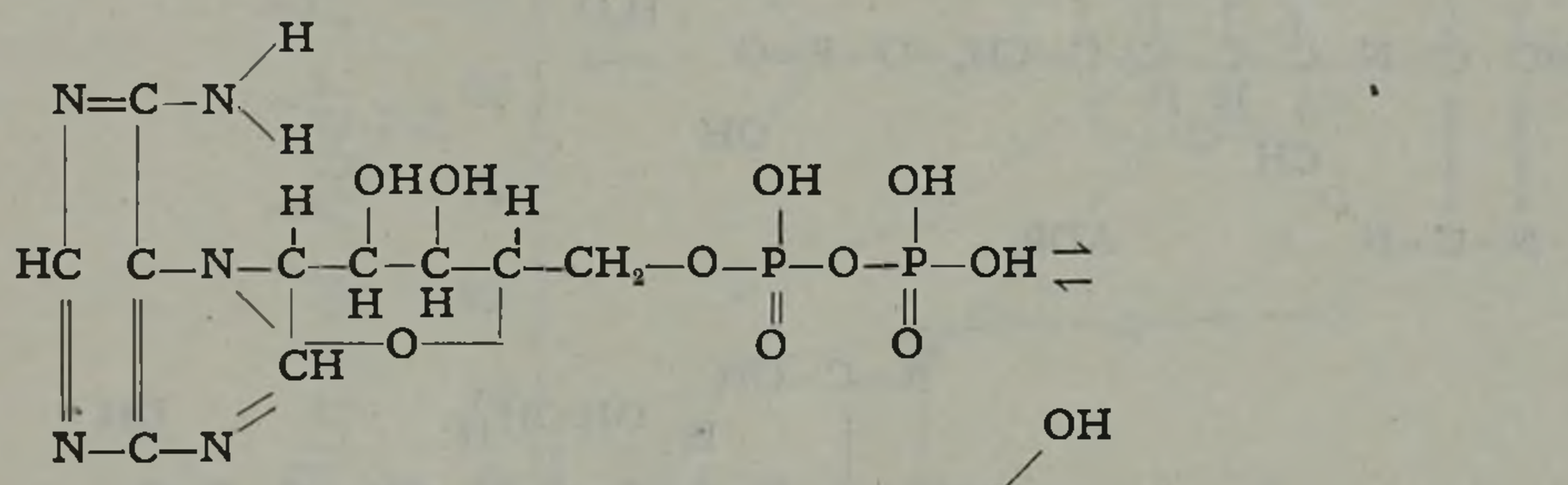
II. protein[1] added to crystallised myosin forms an enzyme complex. The enzymatic effect of this complex consists in the splitting off of both labile *P*-s of *ATP* as well as of the NH_2-group. Myosin alone splits off one *P* only from the *ATP* molecule but it does not liberate NH_3. The II. protein in itself does not catalyse the splitting off of *P* or NH_3.

In the course of our investigations we tried to establish whether the two processes, viz. the liberation of amino-*N* and the splitting off of *P* from *ATP*, are catalysed by the same enzyme complex, or whether there are two different soluble proteins present. None of the experiments have corroborated the latter conjecture and II. protein could not be decomposed into two different enzymes. In the course of isolation the two enzymatic activities could not be separated. After prolonged storage in the ice-box the II. protein is inactivated but the degree of the inactivation has always been the same with the two enzymatic activities. 0,01 M $CaCl_2$ activates the splitting off of the second *P* from *ATP* as well as the desamidation by 60—80%. Both enzyme reactions have the same pH curve identical with the curve of K. Laki[2] established for the splitting off of the second *P* from *ATP*, the maximum being at pH 8,18. All the experiments have proved that the same enzyme complex is necessary for the splitting off of the second *P* as for the desamidation of *ATP*.

The second question we were interested in, in connection with the mechanism of *ATP* splitting, was how the splitting of the two labile *P*-s took place in the presence of a myosin + II. protein enzyme complex. Are they split together in the form of pyrophosphate or does myosin display its enzymatic activity on *ATP* by splitting off one *P* forming *ADP*, so that the myosin + II. protein enzyme complex acts on the *ADP*, decomposing it into *P*, NH_2 and inosinic acid.

Experiments to be described in this paper, show that myosin + II. protein enzyme complex splits the second *P* and liberates NH_2 only after

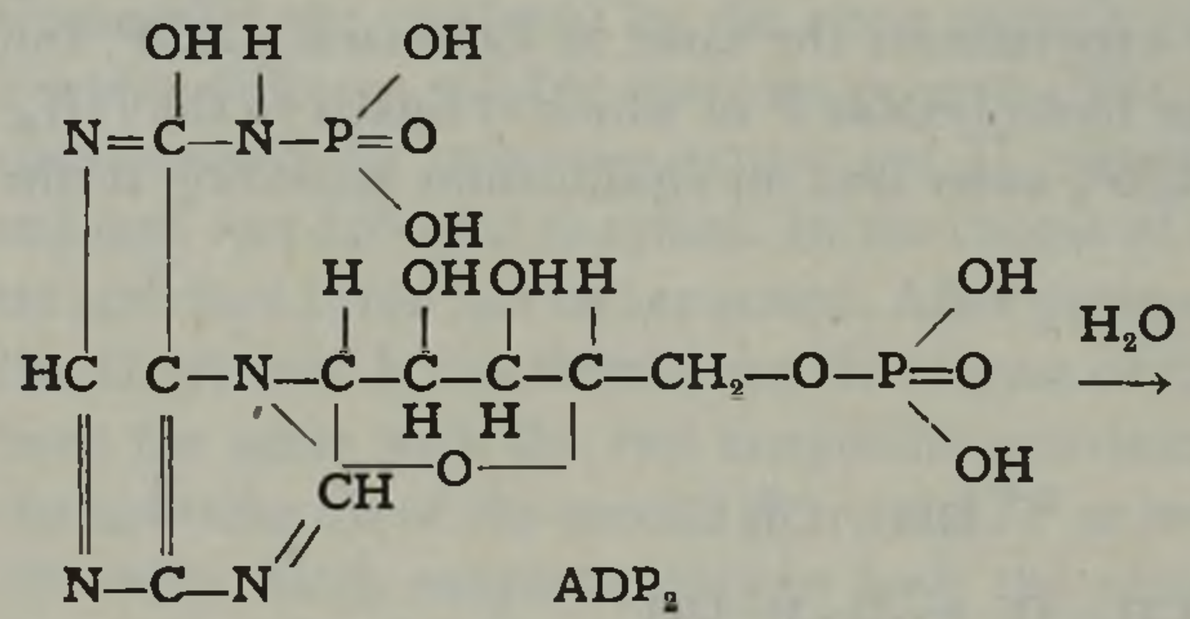
the first *P* has already been split off. It follows that *ADP* arising from *ATP* is the only product which reacts directly on a myosin + II. protein enzyme complex. But as we have shown in a previous paper,[1] ADP_1 is split and desamidated in the presence of myosin + II. protein but very slowly. On the other hand, if we start with *ATP* then the breaking down of *ATP* into inosinic acid in the presence of a myosin + II. protein enzyme complex, goes on very quickly. In order to clear up this contradiction, we examined the products of splitting off of *ATP* in the presence of myosin + II. protein as they successively arose in the course of the reaction. As we have already described[1] dinucleotide and ADP_1 arise when *ATP* is split by myosin but none of the two react further at any considerable velocity on a myosin + II. protein enzyme complex. If we carry out the experiment with *ATP* in presence of myosin + II. protein enzyme complex, dinucleotide does not arise at all, but there is always present some free *ATP* even after an incubation of 30 minutes, when *ATP* has already been broken down to inosinic acid almost about 75%. Beside this free *ATP* there has arisen an *ADP* which differs from ADP_1 since it reacts in the presence of myosin + II. protein. We conclude from this that ADP_1 arising when *ATP* splits in presence of myosin, is isomerised to ADP_2. ADP_1 is, according to our experiments the same as Lohmann's *ADP*, but ADP_2 must be an *ADP*, the hydrolysable *P* of which is linked to the NH_2-group.[1] Thus ADP_1 and ADP_2 enter into an equilibrium according to the following equation:

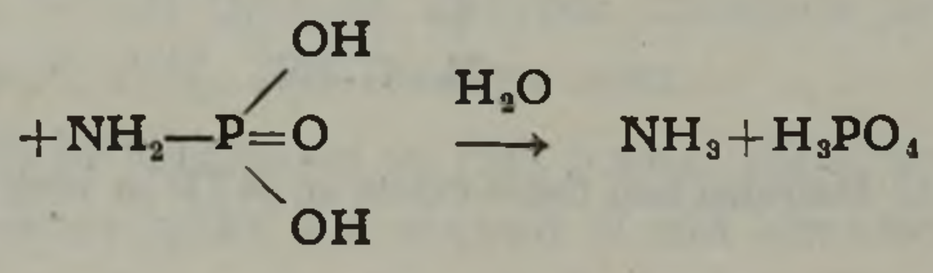


[1] According to Barrenschen there exists an ATP in which the P is linked to the NH_2-group.

We suggest that the transformation of ADP_1 to ADP_2 already takes place on myosin itself becaouse the *ADP* compound in dinucleotidepentaphosphate is accarding to our experiments:[1] ADP_2. If this is the case then the equilibrium in myosin between ADP_1 and ADP_2 is probably 50—50%. In the presence of a II. protein this equilibrium shifts in the direction of ADP_2 since the myosin + II. protein enzyme complex reacts only on ADP_2, so that all ADP_1 must be converted into ADP_2. 0,001 M $MgCl_2$ decreases, in the presence of crystallised myoisn, the splitting off of the first *P* from *ATP*, shown by *I. Banga and A. Szent-Györgyi*.[3] Therefore if the first *P* could not be split off, then myosin + II. protein enzyme complex could not cause desamidation either, since if our supposition was right, the latter reacted only on *ADP*. Experiments show in Fig. II. that in the presence of 0,001 M $MgCl_2$, no NH_3 is liberated and no *P* is split off.

Our experiments seem to prove, that a myosin + II. protein enzyme complex shows substantially only one enzymatic effect i. e. it splits off the aminophosphate group from ADP_2 where the following reaction takes place:





EXPERIMENTAL PART

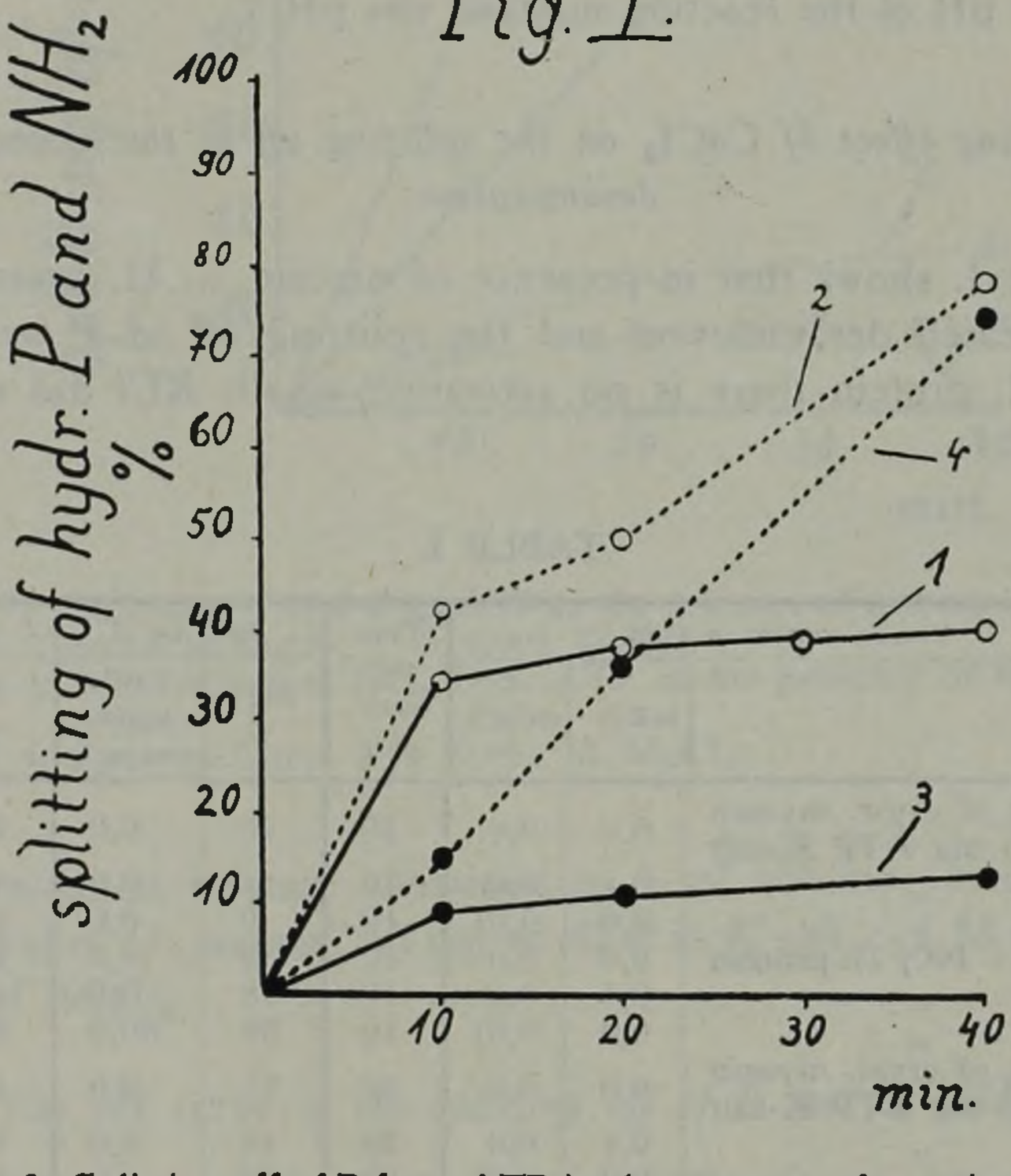
Crystallised myosin : Myosin twice crystallysed after the method of A. Szent-Györgyi.[4]

II. protein : Prepared as described by K. Laki.[2] By further purification we intended to separate this preparation into two compounds by different adsorption methods, but all the preparations show the same enzymatic activity in regard to the splitting off the second *P* and by the liberation of the NH_2-group.

The time-curve of the splitting off of the second P and of NH_2.

A myosin + II. protein enzyme complex splits off the second *P* and the NH_2 from *ATP* only after the first *P* has already been split off. In Fig. I. we give the quantity in per cent of the *P* and NH_2 which is split off at different times of incubation in the presence of myosin and of a myosin + II.

Fig. I.



Curve 1. Splitting off of P from ATP in the presence of myosin.
Curve 2. Splitting off of P from ATP in the presence of myosin + II. protein.
Curve 3. Splitting off of NH_2 from ATP in the presence of myosin.
Curve 4. Splitting off of NH_2 from ATP in the presence of myosin + II. protein.

protein enzyme complex. The percentage of NH_2 was calculated from *N/5* of *ATP*. This Fig. I. clearly shows that the splitting off of the second *P* does not commence until the splitting off of the first *P* has been completed. There is a period of 10 minutes which may be considered as latent period, during which only very small quantities of *P* are liberated. After that period the splitting off of the second *P* proceeds with great velocity. (The 50% on Fig. I. means the complete splitting off of the first *P*, while 100% means the splitting off of both *P*-s.)

The liberation of NH_3 proceeds simultaneously. In the first ten min. there is hardly any liberation of NH_3 to be observed. The liberation of NH_3 commences only in the second ten minutes when the first *P* of *ATP* has already been split off. From the twentieth minutes onwards the two curves i. e. the curves of the splitting off of second *P* and that of NH_3, proceed parallelly.

The reaction mixture contained in 5 ml volume 10 mg of cryst. myosin, 100γ of II. protein, 15 mg of *ATP* as *K*-salt, 0,1 M *KCl* + 0,01 M $CaCl_2$. The pH of the reaction mixture was pH 7.

The activating effect of $CaCl_2$ on the splitting off of the second P and on desamidation.

Table I. shows that in presence of myosin + II. protein 0,01 M $CaCl_2$ increased desamidation and the splitting off of *P* by 70—80%. Without II. protein there is no activation at all. *KCl* did not replace $CaCl_2$.

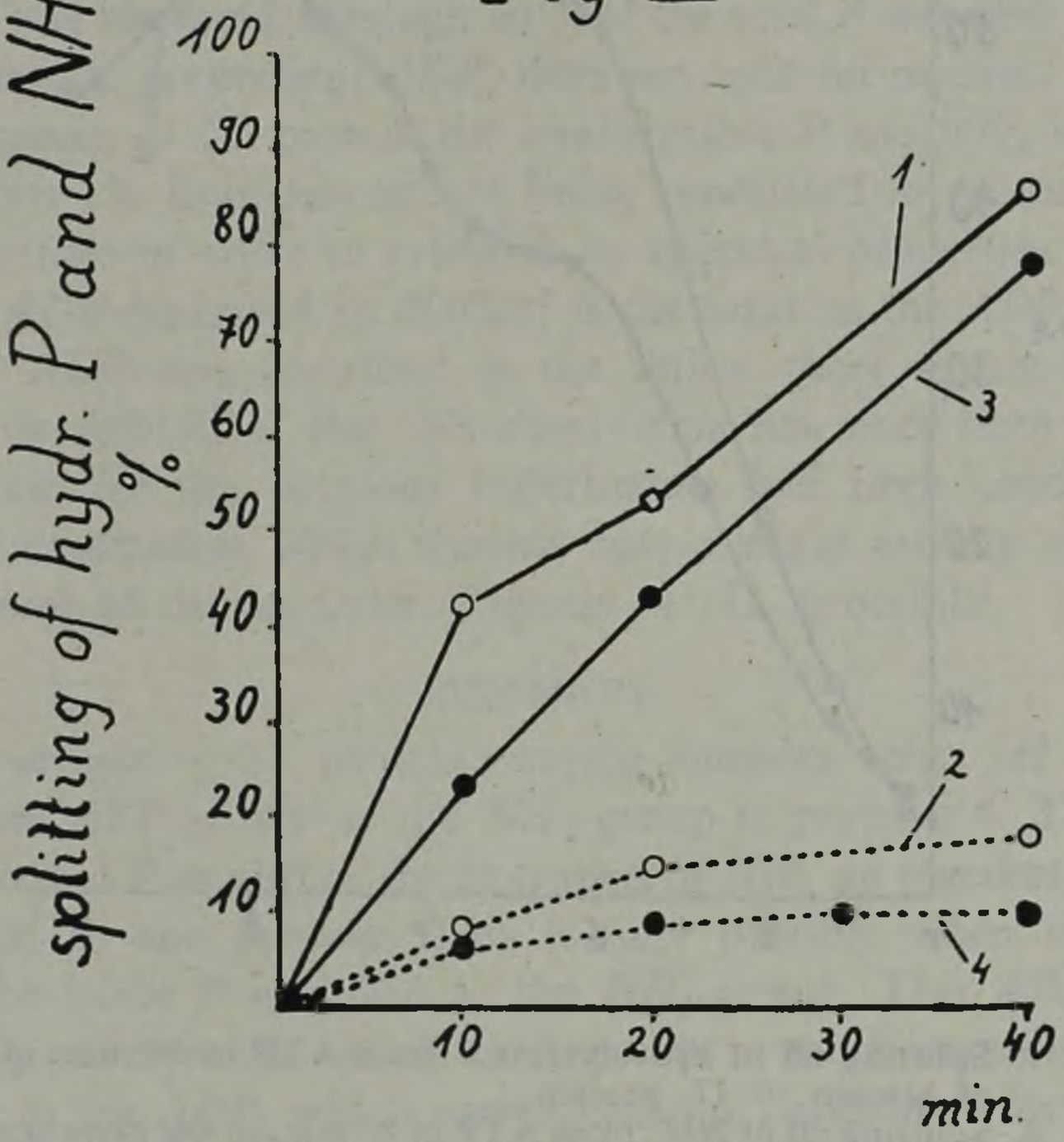
TABLE I.

No.	Vessels contain in 5 ml	mKCl	mCaCl	Time of incub. min.	Splitting of NH_2 %	Splitting of $CaCl_2$ activation in %	Splitting of P %	Splitting of $CaCl_2$ activation in %
1.	10 mg of cryst. myosin + 15 mg ATP K-salt	0,0	0,0	10	15	0,0	43	0,0
2.	,,	0,1	0,0	10	12	0,0	43	0,0
3.	,,	0,0	0,01	10	9	0,0	44	0,0
4.	As 1./+ 100γ II. protein	0,0	0,0	10	33	0,0	40	0,0
5.	,,	0,1	0,0	10	38	0,0	42	0,0
6.	,,	0,0	0,01	10	59	80,0	68	68,0
7.	10 mg of cryst. myosin + 15 mg ATP K-salt	0,0	0,0	20	21	0,0	43	0,0
8.	,,	0,1	0,0	20	18	0,0	44	0,0
9.	,,	0,0	0,01	20	16	0,0	44	0,0
10.	As 7./+ 100γ II. protein	0,0	0,0	20	43	0,0	45	0,0
11.	,,	0,1	0,0	20	47	0,0	49	0,0
12.	,,	0,0	0,01	20	73	70,0	81	80,0

The decreasing effect of $MgCl_2$ on the splitting off of the second P and on desamidation.

Fig. II. shows that 0,001 M $MgCl_2$ decreased the splitting off of *P* and of NH_2.

Fig. II.



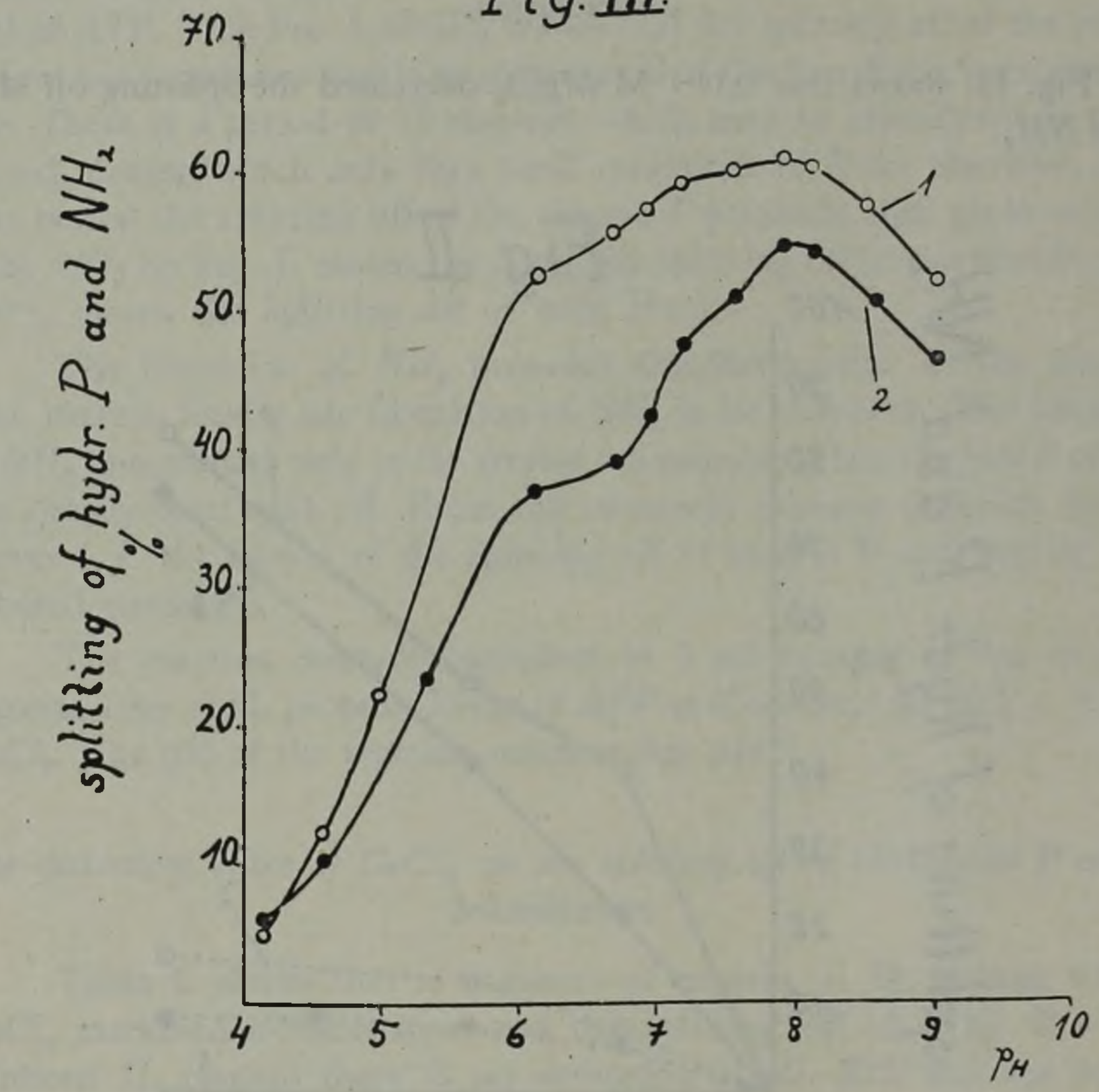
Curve 1. Splitting off of P from ATP in the presence of myosin + II. protein.
Curve 2. Same as Curve 1, + 0,001 $MgCl_2$.
Curve 3. Splitting off of NH_2 from ATP in the presence of myosin + II. protein.
Curve 4. Same as Curve 3 + 0,001 M $MgCl_2$.

The reaction mixture contained in 5 ml volume 10 mg of cryst. myosin, 100γ of II. protein, 15 mg of *ATP* as *K-salt*, 0,1 M of *KCl* and 0,001 M of $MgCl_2$.

The pH curve of the splitting off of P and of NH_2.

Fig. III. shows the pH curve of the splitting off of P and of NH_2 in the presence of myosin + II. protein. As can be seen the two curves are very close to each other.

Fig. III.



Curve 1. Splitting off of hydrolysable P from ATP in 30 min. in the presence of myosin + II. protein.
Curve 2. Splitting off of NH_2 from ATP in 30 min. in the presence of myosin + + II. protein.

The reaction mixture contained in 5 ml 2 ml M/7 veronal-acetate buffer at different pH., 10 mg of cryst. myosin, 100γ of II. protein, + 15 mg *ATP* as *K*-salt. Time of incubation 30 minutes.

P was determined in the course of these experiments in an aliquot part of the trichloroacetic filtrate according to Fiske—Subbarow; the NH_2 was determined according to the method of mikro-Kjeldahl; distillation took place in a Wagner—Parnas apparatus.

ADP_2.

The great difficulty of preparing ADP_2 is caused by the fact that in a mixture of myosin + *ATP*, ADP_2 is only present in a free state if a II. protein is also being added. But in the presence of the II. protein ADP_2 splits with great velocity to inosinic acid. In one case ADP_2 was found

in a purity of 80—85% when in the presence of actomyosin + II. protein the product was isolated without incubation. The actomyosin + II. protein + *ATP* was mixed by shaking without incubation. Then 20% of trichloroacetic acid was added for deproteination and ADP_2 was isolated from the filtrate in the water soluble *Ba*-salt fraction.

Analytical data for ADP_2 : $N/P = 1{,}2$ (theoretical value is 1.12). The number of acid-equivalents was 4. After acid hydrolysis no acid equivalent was liberated although 50% of the total *P* was split off.

Enzymatic properties: ADP_2 does not split on myosin. In the presence of myosin + II. protein the hydrolysable *P* and 90% of the NH_2-group is split off. Experiments are being conducted to produce ADP_2 in larger quantities in order to establish its chemical properties.

The *ADP* described in *Nature*[5] is the same as the ADP_2 dealt with here. This *ADP* was described in the article there as not undergoing enzymatic desamidation. But this observation has since been proved not to be correct for the previous experiments had been conducted with Schmidt's[6] desamidase which showed only a slight activity as compared to our system of desamidation (myosin + II. protein).[1]

SUMMARY

The myosin + II. protein enzyme complex splits off the second labile *P* from *ATP* as well as the NH_2-group in position 6. The splitting off of the second *P* as well as the liberation of NH_3 go together and can be looked upon as one process. This is only possible when in the *ADP* molecule the labile *P* is linked to the NH_2-group. This *ADP* has been called ADP_2 and it is this which corresponds to the Barrenschen formula as opposed to the ADP_1 which corresponds to Lohmann's formula.

The activation with $CaCl_2$ and the inhibition with $MgCl_2$ as well as the pH curve prove that in *ATP* the splitting off of the second hydrolysable *P* and the liberation of NH_3 go together.

LITERATURE

1. *I. Banga :* present volume page 72.
2. *K. Laki :* Studies from the Inst. of Med. Chem. Univ. Szeged, Vol. III. page 16, 1943.
3. *I. Banga* and *A. Szent-Györgyi :* Studies, from the Inst. of Med. Chem. Univ. Szeged. Vol. III. page 72, 1943.
4. *A. Szent-Györgyi :* Studies from the Inst. of Med. Chem. Univ. Szeged. Vol. III. page 76, 1943.
5. *I. Banga :* Nature, June, 8, 1946.

[1] On account of difficulties in the postal service I was unable to receive proofsheets of my article which appeared in Nature June 8, 1946, and thus the following misprints have not been corrected. Instead of adenosin-diphosphate in line 4, read „adenosin-triphosphate" and instead of adenosin-diphosphate-isomerase in line 10 read „adenosine-triphosphate-isomerase". I. Banga.

DESAMIDATION OF ATP.

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RECEIVED FOR PUBLICATION 13. XI. 1946.

It has been shown in a previous paper,[1] that in presence of myosin + II. protein the *ATP* molecule is dephosphorylated and desamidated to inosinic acid. The liberation of NH_3 goes parallel with the splitting off of the second *P* and it seemed that the two reactions were catalysed by the same enzyme system. It will be shown in this paper that there exists a direct desamidation of *ATP* also, which is independent from the splitting off of *P* though it also requires myosin and a soluble protein, which is present in the II. protein. The quantity of myosin which is necessary for the direct desamidation of *ATP* is much smaller as the quantity required for dephosphorylation. On the other hand the quantity of II. protein of our purest preparation, necessary for direct desamidation of *ATP*, is much greater as the quantity necessary for dephosphorylation. For the simultaneous splitting off of *P* and NH_3 from *ATP* 1 mg/ml of myosin and 10—20γ/ml of II. protein was necessary, for direct desamidation of *ATP* 40γ/ml of myosin and 160—200γ/ml of II. protein. The desamidase which goes together with the splitting off of the second *P* from *ATP* will be called *ADP*-desamidase and the enzyme which catalyses the direct desamidation of *ATP*, *ATP*-desamidase. As will be shown the two kinds of desamidation can be separated owing to the difference of the relation of myosin and II. protein.

EXPERIMENTAL PART

Crystallised myosin and II. protein was made by the methods described in a previous paper.[1] *P* was determined by the method of Fiske—Subbarow and NH_3 by micro-Kjeldhal controlled with Nessler's reagent.

Table I. shows that by decreasing the myosin concentration the splitting off of *P*- (labile *P*-s) from *ATP* comes to an end while the desamidation goes on independently from the dephosphorylation of *ATP*.

TABLE I.

Samples contained in 5 ml volume 0,1 M of KCl, 0,01 M of $CaCl_2$, 15 mg of ATP as K-salt, 1 mg of II. protein and variable quantities of myosin. Time of incubation 20 minutes at 38° C. (P_7 means the labile P of ATP.)

Myosin mg	Splitting off of P_7 from ATP %	Splitting off of amino-N %
4	54	62
1	11	41
0,5	8	38
0,25	3	38

Table II. shows that the II. protein without myosin does not split off the amino-*N* from *ATP;* in the presence of relatively small quantity of myosin *ATP* is desamidated to 50%.

TABLE II.

Samples contained in 5 ml volume 0,05 M of KCl, 0,01 M of $CaCl_2$ 15 mg of ATP as K-salt. 38° C.

Myosin mg	II. protein mg	Time of incub. min.	Splitting off of P_7 %	Splitting off of amino-N %
0,0	1	5	0,0	0,0
0,0	1	10	0,0	0,0
0,0	1	40	0,0	0,0
0,0	1	80	0,0	0,0
0,25	0,0	80	4	14
0,25	1	5	2	24
0,25	1	10	3	35
0,25	1	20	4	41
0,25	1	40	8	46
0,25	1	80	11	49

The experiments show that the factor, which catalyses the *ATP* desamidation in II. protein is different from that of *ADP* desamidation. For *ATP* desamidation 4 times as much II. protein was required as from myosin so the relation between myosin to II. protein is 1 : 4. For

ADP desamidation and for the splitting off of the whole labile *P* from *ATP* a 1/20—1/100 times smaller quantity of II. protein was required as from myosin, so the relation between myosin to II. protein was 1 : 0,01. The difference between the two relation is 1 : 400.

In Table III. it can be seen that in the presence of a great quantity of myosin, which dephosphorylates *ATP* with maximal effects, on adding II. protein in decreasing quantities the decrease in desamidation is much stronger as that in dephospharylation.

TABLE III.

Samples contained in 5 ml volume 0,05 M KCl, 0,01 M $CaCl_2$, 15 mg of ATP as K-salt and 6 mg of cryst. myosin and various quantities of II. protein. Incubation 20 min. at 38°C.

II. protein mg	Splitting off of P_7 %	Splitting off of amino-N %
0,0	38	14
0,4	85	100
0,2	85	100
0,1	80	80
0,05	60	45

To prove that it was the *ATP* molecule which underwent desamidation we examined the direct desamidation of *ATP* at various *ATP* concentrations. If the NH_3 comes from *ATP* then the % splitting of amino-*N* of *ATP* must be the same at different *ATP* concentrations. As Table IV. shows this was really the case. We observed a little fall in percentage only at high concentrations of *ATP*.

TABLE IV.

Samples contained in 5 ml volume 0,05 M of KCl, 0,01 M of $CaCl_2$, 200 γ of cryst. myosin, 800 γ of II. protein and various quantities of ATP. Incubation 10 min. at 38° C.

ATP mg	Splitting off of amino-N %	Splitting off of P_7 %
0,0	0,0	0,0
3	27	0,0
6	27	0,0
10	27	0,0
15	20	0,0
20	20	0,0

ATP-desamidase is an acid-stable protein the action of which could not be increased by boiled muscle extract. The quantity in muscle is the same as that of the *ADP*-desamidase. By purification it goes parallel with the *ADP* desamidase so that in the purest II. protein preparation both enzymes has the same purity. It can be activated with 0,01 M of $CaCl_2$ and the pH maximum of the reaction lies at pH 7,2—7,6. As we have shown[1] the pH maximum of the *ADP*-desamidase is at pH 8,18.

SUMMARY

ATP can be desamidated without dephosphorylation. In this process two factors are involved: myosin and a soluble protein. There are two kinds of desamidases: an *ATP*- and an *ADP*-desamidase. The soluble protein necessary for direct desamidation of *ATP* is present in our purest II. protein preparation.

LITERATURE

1. *I. Banga* and *G. Josepovits*: Present volume page 82.

THE TYPES OF ABSORBTION OF VARIOUS COMMERCIAL PENICILLINS.

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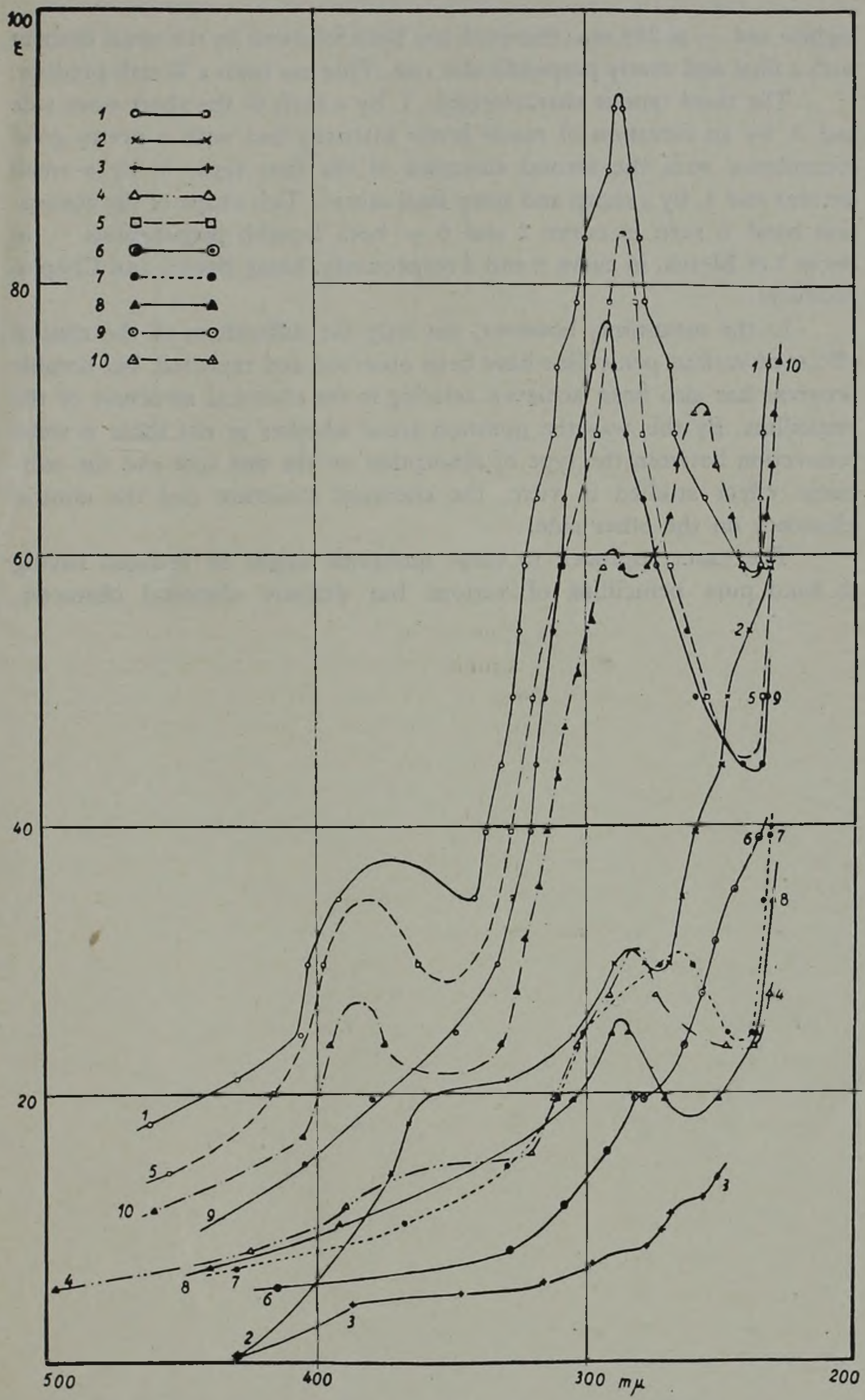
The study of the spectrographic behaviour of penicillin was initiated following the fact that in the literature accessible at that time no mention was made of this question and it seemed to be attractive to have to hand a method by means of which it might be possible to follow the fate of the drug even without carrying out an antobiotic experiment. Soon it was observed that the administration of penicillin was not always responded by an identical or similar reaction: by this way the question relating to the possible differences between the various brands of the drug arose, all the more as in those cases significant differences in the character of the infection, in its qualitative and quantitative relations, were not to assume.

Spectrographic examination of the absorption band in the ultraviolet range has been carried out with the apparatus E3, Judd-Lewis of Hilger. Diluting 100,000 O. U. s of penicillin in 20 ml of apyrogenic water, at first an absorption was observed with a small wave at 375 mμ and a high peak at 288 mμ, the descent of which was followed by a third elevation nearly perpendicular (see curve 1). The behaviour of another batch was similar with a somewhat smaller second peak and a descent to a deeper level before the final perpendicular rise (curve 5). A Boot's product exhibited a similar graph with the lower wave at 375 and the high peak at 278 mμ. The character of the two first specimens is to be seen in the following graph (curve 9) with the difference that the first wave was not present, while the point of the high peak is practically at the same wave-lenght. The two first have been products of Pfizer and the latter a drug of the C. S. C.

A second type of absorption band has been noted in a product yielding three waves: the first at 385, a second at 290 and a third — the


100
ε
80
60
40
20
1
2
3
4
5
6
7
8
9
10
500
400
300 mμ
200

highest one — at 258 mμ; this peak has been followed by the usual descent with a final and nearly perpendicular rise. This has been a Wyeth product.

The third type is characterized: 1. by a shift to the short wave side and 2. by an elevation of much lower intensity but with a pretty good coincidence with the second elevation of the first type, 3. by a small descent and 4. by a sharp and steep final ascent. This shape of the absorption band is seen in curve 2 and 6 — both Squibb preparations — in curve 7 of Merck, in curve 8 and 4 respectively, being Bristol and Cheplin products.

In the meantime, however, not only the differences in the clinical effects of various penicillins have been observed and reported, but notable progress has also been achieved relating to the chemical structure of the penicillins. By this way the question arose whether or not there is some connection between the type of absorption on the one side and the antibiotic effect studied in vitro, the chemical structure and the clinical efficiency on the other side.

The exact approach to these questions might be realized having to hand pure penicillins of various but definite chemical character.

A szerkesztésért Mansfeld Géza, a kiadásért Szent-Györgyi Albert felelős.

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