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THE BIOLOGICAL SIGNIFICANCE OF THE LINKAGES IN ADENOSINE TRIPHOSPHORIC ACID.

By J. H. GILLESPIE.

(*From the Department of Physiology, Queen's University, Belfast.*)

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For some years before 1929 interest had been aroused in the adenyl group of compounds by the recognition of the important part played by them in the glycolytic processes of muscular activity. Since then, additional significance has been attached to them because of the demonstration by Drury and Szent-Györgi [1929] of certain characteristic biological actions. After having studied the activity of simple saline extracts of cardiac muscle, they isolated from the trichloroacetic acid extract of bullock's heart a crystalline substance which their analysis showed to be adenylic acid. This acid was undoubtedly derived from the adenosine triphosphoric acid (a.t.p.) present in the original extract.

They found that adenylic acid produced a typical heart block in the guinea-pig, dilatation of the coronary arteries, lowering of blood-pressure in the dog, and diminution in the amplitude of contraction of isolated strips of intestine.

Lindner and Rigler [1930] found that extracts of the sinus, node of Tawara, and bundle of His from the calf's heart, prepared in a manner not specified, stimulated an hypodynamically acting frog's heart. They attempted to fractionate their extracts, and eventually prepared a substance which dilated the coronary arteries in the mammalian heart, and had no effect on the frog's heart. This substance they found to contain a pentose and a purine base.

Work of this nature, including that of Rigler and Schaumann [1930] and of Rothmann [1930], began to make it clear that the biological activity of many of the tissue extracts which had been studied by earlier workers was really due to their adenosine content.

Zipf [1930] showed clearly that the depressor substance which can be isolated from defibrinated blood, and which had been described by Freund [1920] as "Frühgift," was adenylic acid.

Bennet and Drury [1931] further investigated the production of heart block in the guinea-pig by injection of the adenyl compounds, and concluded that the amounts required to produce block were independent of the presence of other substances, and therefore might be used as a method for the biological assay of the adenylic acid present in tissue extracts. Adenylic acid was thus recognized in nearly all tissues, especially in heart and voluntary muscle.

Wedd [1931] investigated the action of adenosine and allied compounds on the coronary flow of the perfused rabbit's heart. He noticed the difficulty of correlating this action with that upon the musculature of the heart, which he concluded must be of a different nature. The maximal dilatant effects could be obtained with amounts which caused little or no cardiac slowing. He also noticed that adenosine was a more powerful coronary dilator than adenylic acid obtained from muscle or yeast.

Hochrein and Keller [1931] compared various tissue extracts by their effects on the blood-pressure and the electrocardiogram, and concluded that the active principle of most of them was the same, but no details were given regarding the chemical nature of the active substances.

Subsequently, Drury [1932 *a*] revised his view that deamination was the essential factor in the activity of the adenyl compounds, and he attempted to explain the effects as partly due to the presence of phosphate radicles in the molecule. He found, for example, that orthophosphate increased the amplitude of the mechanogram, while pyrophosphate first decreased and then increased it. Both forms of inorganic phosphate, however, reduced the coronary flow. Drury therefore supposed that the beat might be influenced by (1) the adenyl complex acting on the coronary vessels, and (2) the phosphate radicles acting on the musculature. This, however, is not sufficient to account for the fact that adenylic acid and even adenosine may produce heart block indistinguishable from that caused by a.t.p. Moreover, in producing effects with inorganic phosphates, Drury had to employ doses far outside the range of those used in the case of the adenyl derivatives, so that the effects are scarcely comparable.

Ostern and Parnas [1932] estimated the adenosine content of various tissue extracts by perfusion of the frog's heart. They stated that a.t.p. was three times as active as adenosine, judging by the time taken for heart block to develop. Deuticke [1932] compared the activities of the adenyl compounds on the virgin guinea-pig's uterus. Following a short latent period, contraction was caused by adenosine, adenylic acid, and a.t.p. as well as by yeast adenylic acid and a related nucleotide

recently isolated by Embden from the heart. He emphasized that the maximum height of contraction was reached more rapidly with substances which contained more phosphorus in their molecule.

Marcou [1932] examined the cause of the lowering of blood-pressure by adenosine, and found that it was evidently largely due to dilation of intestinal and peripheral vessels. The volumes of the spleen and kidney were at the same time diminished.

Gaddum and Holtz [1933] tested adenosine, adenylic acid, and a.t.p. on the vessels of the lung. They found the cat more sensitive than the dog: in both, constriction occurred, with fall in lung volume.

There is, therefore, a considerable accumulation of knowledge about the biological actions of adenyl compounds, which ought to make possible a definite statement as to their rôle in the activity of any given tissue extract. Unfortunately, the substances tested by most of the authors quoted have been obtained from widely divergent sources, and it is difficult to correlate the changes which occur in the biological activity of the adenyl compounds with variations in their chemical constitution. For example, Drury suggested that ease of deamination is not the essential factor in their biological activity from a comparision of yeast adenylic acid and muscle adenosine. The former cannot be deaminated by enzymes, while the latter can, although only with difficulty, and under special conditions. In several of the papers the exact source of the compound tested has not been stated.

A series of experiments was therefore carried out using the various derivatives from the same mother substance, the naturally occurring adenosine triphosphate of skeletal muscle. This substance was prepared from rabbits' muscle as a calcium salt. The distribution of the phosphorus per mg. dried salt was as follows:

Free (inorganic) phosphorus	Nil
Labile P hydrolysable in 10 min.	0·096 mg.
Total P liberated by total hydrolysis	0·143 mg.

The ratio of labile to stable phosphorus is the theoretical one of 2 to 1 and the N : P ratio was found to be the theoretical one (5 : 3).

As the changes most easily produced in the natural nucleotide are deamination and the removal of the two labile phosphoric acid molecules, it is necessary to compare the biological activities of adenosine—and inosine—triphosphoric acids (a.t.p. and i.t.p.), and of adenylic and inosinic acids respectively. It is evident, however, from the earlier work, that so long as the pentose linkage with adenine is retained, certain of the

characteristic effects produced by the adenyl derivatives are still obtainable even when the ester linkage between the sugar and phosphoric acid has been broken. Although doubts have been expressed regarding the possibility of the removal of NH_2 from a.t.p., it is possible to obtain i.t.p. by nitrous acid deamination. The body so formed has a 4N : 3P ratio and retains the two labile phosphoric acid molecules. Adenylic and inosinic acids may be readily obtained by weak alkaline hydrolysis of a.t.p. and i.t.p. respectively. Adenosine may be obtained in the usual way by alkaline hydrolysis at 170°, while the purine-pentose linkage can be readily broken by acid hydrolysis.

For purposes of injection, salts were used which were obtained either as hydrolysed solutions of a.t.p., analysed for their content of the derivatives, or in the dried purified form, made up to the required watery dilution. In the former case, the procedure was briefly as follows.

A weighed amount of the pure a.t.p. was suspended in the hydrolysing solution, and heated in sealed glass tubes. When it was desired to obtain adenylic acid as the sole derivative of alkaline hydrolysis, the water was made sufficiently alkaline to neutralize the two labile phosphoric acid molecules which would be set free. The tubes were then heated at 100° C. for 2 hours. The hydrolysed solution showed on analysis that the rise in inorganic phosphate was fully accounted for by the liberation of the labile H_3PO_4, without encroachment on the stable H_3PO_4 of the adenylic acid molecule.

It was thus easy to obtain the adenylic acid content of the solution, which also contained the labile H_3PO_4 of the original a.t.p. in the form of inorganic phosphate. In experiments carried out for the purpose, it was found that this inorganic phosphate was quite inactive in the amounts in which it occurred in the hydrolysed solutions.

When it was desired to obtain adenosine by hydrolysis, a weighed amount of a.t.p. suspended in 16 p.c. ammonia was heated at 170° C. for 2 hours. Analysis showed that this procedure set free all three H_3PO_4 molecules and, since the glucoside linkage remains unbroken under these conditions, it could reasonably be assumed that the solution contained only adenosine plus inorganic phosphate.

The ammonia used in the solutions was removed by aeration, and the reaction was brought to *p*H 7·3 by a few drops of dilute hydrochloric acid.

Inosine triphosphate was prepared by deamination of a.t.p. with nitrous acid, and the doses referred to in the text were the weights of pure dry sodium salt used for injection. The salt may have had some very

slight admixture with non-deaminated a.t.p., but the ratio of N to P was approximately 4 : 3 instead of 5 : 3.

Inosinic acid was also used after separation as a pure barium salt, prepared in the laboratory after the method of Ostern. For some confirmatory tests, a sample of pure dry barium inosinate, kindly sent by Dr Levene, was also used. In all cases the barium was removed before the salt was used biologically, the sodium salt being that employed. Adenosine was also tested in the form of "Lacarnol," kindly supplied by Messrs Bayer. Contrary to the statement of Rothmann [1930] we found on repeated analysis that the lacarnol in our possession was phosphorus-free, and therefore could not be a nucleotide. Whatever other components it may contain, adenosine can be recovered from it in practically pure form.

The actions of the salts on different test objects will now be described in order.

Blood-pressure.

(1) *Adenosine triphosphate and inosine triphosphate.*

The rabbit was largely used, but occasionally also the cat, the actions in the two animals being very similar. The blood-pressure was measured by a cannula tied in the carotid artery, and injection was made slowly

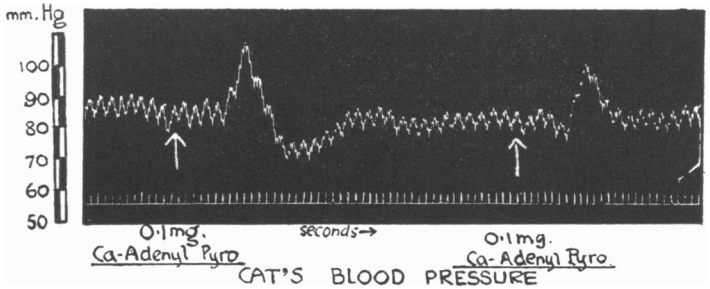

Fig. 1. Effect of injection of 0·1 mg. adenosine triphosphate on cat's blood-pressure. On repetition of the injection, the blood-pressure rise alone is seen.

into a cannula in the jugular vein. The time taken to inject was gauged so as not to affect the blood-pressure, and was about 7 sec.

The usual fall in blood-pressure follows injection of both these substances, but so long as the labile phosphoryl linkage is retained in the molecule, there is frequently to be seen a transient rise in pressure preceding the fall. These effects persist after atropinization.

A typical example may be given (Fig. 1). On injection of 0·1 mg. of adenosine triphosphate into the jugular vein of a cat after a latency of 7 sec., there was a rise in pressure of 20 mm. of mercury, followed at once by a fall of 18 mm. below the normal level. When a second injection is given rapidly after the first one, the rise in blood-pressure alone may be obtained.

The deaminated derivative is much less active, and doses five to ten times as great as those used with the mother substance are necessary to produce the effects. But the transient rise and subsequent fall are both evident when sufficient amounts (say 1 mg. of the dried salt) are injected.

(2) *Adenylic acid and inosinic acid.*

The well-known fall in blood-pressure was seen following injection of adenylic acid, but rarely with any evidence of the initial rise, which generally appears to indicate the presence of the labile phosphate in the molecule. After deamination the same decrease in activity is to be observed as in the case of the mother substance. Inosinic acid resembles adenylic acid in producing only the fall in pressure. The most inactive specimen of inosinate was that prepared by Dr Levene, which only produced a very slight fall in doses of 2 mg. Bennet and Drury [1931] described inosinic acid as quite inactive in their experience.

(3) *Adenosine.*

The typical fall in blood-pressure was obtained after injection of 0·1 mg. of pure adenosine and, judging by the duration of the fall, it appeared to be the most active of all the non-deaminated derivatives.

(4) *Adenine and hypoxanthine.*

These were tested after neutralization of the acid hydrolysed solutions of adenosine and inosine triphosphate. They were always found to be inactive.

HEART.

Rabbits' hearts were perfused using a modification of Gunn's apparatus, the coronary flow being estimated by allowing the fluid to fall into a bucket which tipped when it contained 1·5 c.c. In order to construct suitable graphs to show the relative activities of the various bodies as coronary dilators, the reciprocal of the time taken for 1·5 c.c. to perfuse was used as a measure of the rate of flow. The rate measured before injection of the drug was taken as 100, and the increase in this rate during

every second following injection was calculated as a percentage. The figures so obtained were plotted in the manner seen (Fig. 2), and all the graphs shown were constructed from the average figures of numerous experiments.

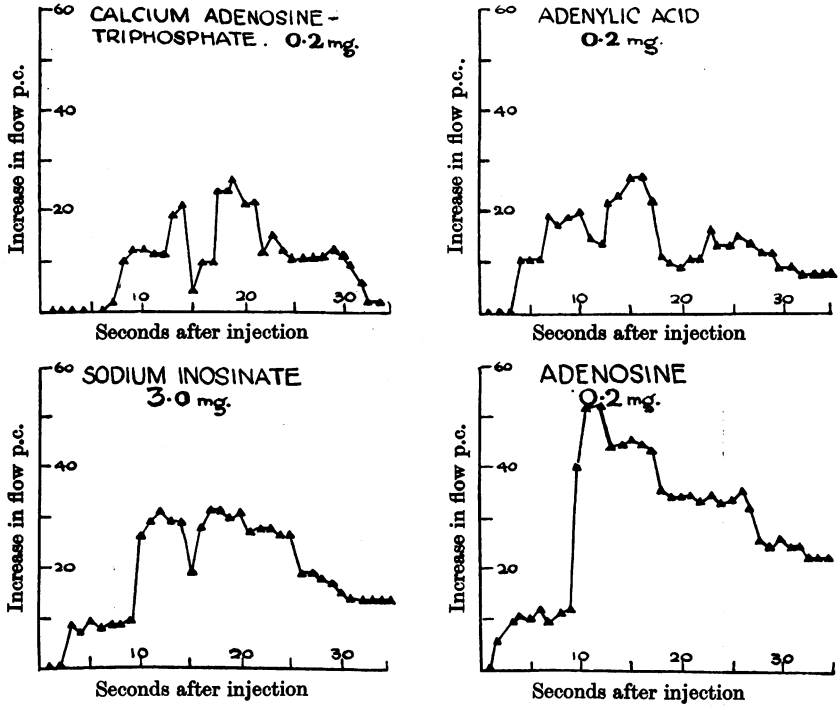

Fig. 2. Graphs showing the percentage increase in coronary outflow in perfused rabbits' hearts after injection of adenyl derivatives.

(1) *Adenosine triphosphate and inosine triphosphate.*

When 0·2 mg. of a.t.p. was injected into the side-piece of the cannula there was a latent period of 7 sec., after which an increase in the flow commenced which reached its maximum in 19 sec. The maximum increase was 26 p.c. above the normal, and in 33 sec. the flow had almost returned to normal. Very similar figures were obtained, using i.t.p., except that about ten times the dose was necessary to produce the effect. The graph is not shown because only five experiments were carried out with this drug.

Some workers have described improvement of the action of the heart by the adenyl compounds, while others only mention inhibition. This

appears to be because the action on the musculature and conducting system is really always depressant, while the improvement in the beat is secondary to the increased coronary flow. The reasons on which this statement is based are the following.

(1) In the mammalian heart, since the dose required to produce blocking of conduction is greater than that which will dilate the coronary vessels, it is sometimes possible to obtain an improvement in amplitude with a small dose, and the reverse effect with a larger dose. This is seen in Fig. 3, where injection of 0·1 mg. of adenosine triphosphate induced a

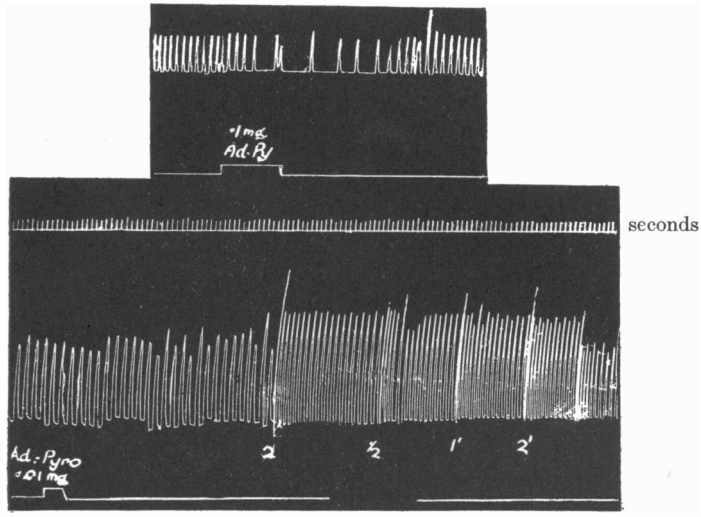

Fig. 3. 0·1 mg. adenosine triphosphate produced "heart block," while in the same heart 0·01 mg. dilated the coronary vessels and improved the beat.

"heart block," while in the same heart a dose of 0·01 mg. dilated the coronary vessels and improved the beat. Unfortunately a record of the coronary output was not kept in this case. Some of the improvement in amplitude in this record is doubtless due to the increased rate of beating, as pointed out by Dale.

(2) An increase in amplitude of the beat was only seen after the coronary outflow had been increased. The improvement followed a short latent period during which there was no coronary dilatation, or the beneficial effects of the dilatation had not been felt. During this period the "blocking" effect might appear (Fig. 4).

(3) In a heart where the coronary arteries were already fully dilated

no increase in amplitude of the beat has been seen to follow injection of the adenyl compounds.

(4) In the course of a large number of experiments in which the frog's heart was perfused with the Straub-Führer cannula the adenyl compounds were always seen to produce interference with conduction of the beat, and diminution in amplitude, and never an improved action of the heart.

It may here be mentioned that a.t.p. was the most active of the adenyl group in producing heart block in the frog's heart.

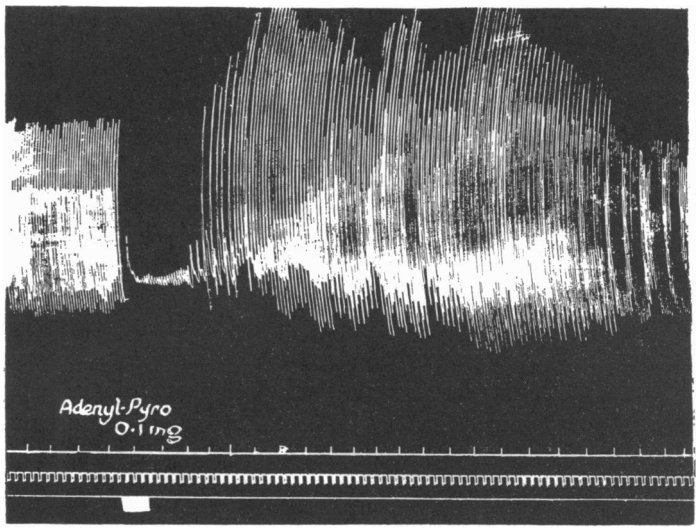

Fig. 4. The typical effect of adenosine triphosphate on the perfused mammalian heart.

The typical action of a.t.p. on the mammalian heart is seen in Fig. 4. This shows the combination of the blocking which is a direct action on the conducting system, and the improvement in the beat following the increased coronary flow. In this case there is very little alteration in the rate of beating, and any increase which there may be is not sufficient to account for the improved amplitude.

A similar picture, using ten times the dose of a.t.p., is given by i.t.p.

(2) *Adenylic acid and inosinic acid.*

The dilatation of the coronary vessels produced by adenylic acid resembles that caused by a.t.p. (Fig. 2), except that the increase in flow began 3 sec. earlier, and after 35 sec. was still 8 p.c. above the original

value. This may be taken as evidence of somewhat greater dilatant activity.

The graph obtained with sodium inosinate is very similar, but the effective dose was 3 mg., while 0·2 mg. was the dose employed with the non-deaminated compounds.

Adenylic acid and inosinic acid both produce heart block in the perfused amphibian and mammalian heart, but the loss of the labile phosphate from the molecule causes a loss of activity in this respect, as compared with a.t.p. and i.t.p. The criteria of this were the duration of the block in the rabbit's heart, and the time taken for the block to develop in the frog's heart.

(3) *Adenosine.*

Removal of both labile and stable phosphate groups from the molecule of adenosine triphosphate still further increases the dilatant activity upon the coronary arteries. Thus the most active of our series was found to be the adenosine prepared by alkaline hydrolysis (Fig. 2). The increased flow begins after 1 sec., reaches 52 p.c. above the normal in 12 sec., and after 35 sec. is still 22 p.c. above normal.

Using the same criteria as in the case of adenylic acid, adenosine appears to be the least active of the series in producing heart block. If its action in the intact animal be similar, this may help to explain the clinical popularity of adenosine, since it has the greatest margin of safety between the dose which dilates the coronary vessels and that which produces heart block. Nevertheless, Honey, Ritchie and Thompson [1930] have described dangerous interference with conduction in the human heart following intravenous injection of 50 mg. of adenosine.

(4) *Adenine and hypoxanthine.*

These were quite inactive on amphibian and mammalian hearts.

ISOLATED SMALL INTESTINE.

(1) *Adenosine triphosphate and inosine triphosphate.*

A.t.p. has a unique action on the small intestine which does not appear to have been mentioned in the literature. Like the other adenyl compounds it produces a fall in tone in concentrations as low as 1 in 500,000. But in many cases, and specially if the tone be initially low, it produces and maintains a subsequent rise in tone (Fig. 5). This increase in tone is sometimes very striking, and has been seen in a gut which showed no sign of life until the a.t.p. was added to the solution.

During the action of the drug the rate of contraction is not affected and, with the general increase in tone, the amplitude of the single contractions is decreased. Usually slightly higher concentrations (say 1 in 50,000) are required to bring out the tonic effect.

I.t.p. inhibited the gut in concentrations ten times as great as those in which the mother substance was active, but was never seen to produce increase of tone.

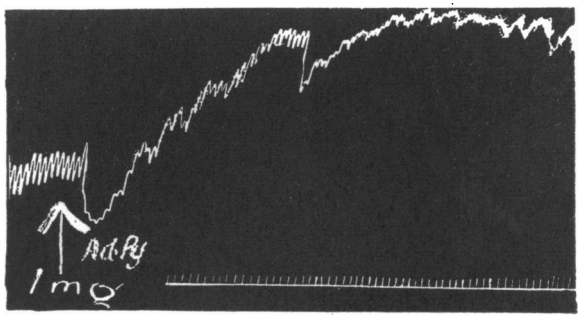

Fig. 5. Increased tone caused by adenosine triphosphate acting on small intestine in concentration of 1 in 50,000.

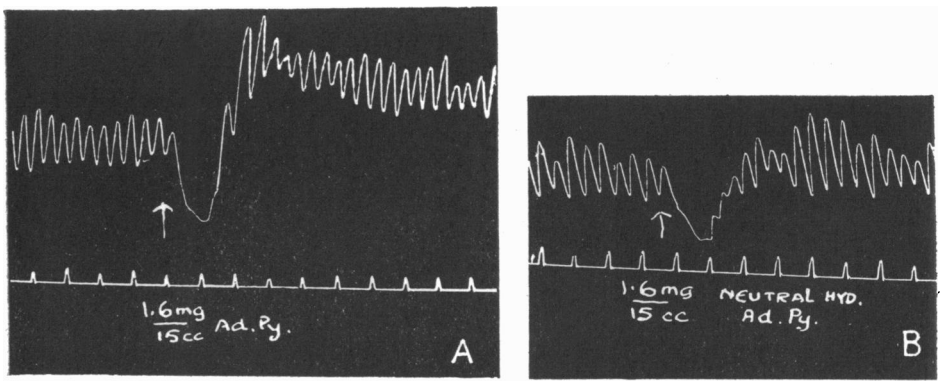

Fig. 6. A. Shows the inhibition followed by increase in tone of the intestine caused by a.t.p. in a concentration of about 1 in 10,000. Time = 5 sec. B. Shows how after hydrolysis in neutral solution (adenylic acid being formed) only the inhibition appears.

(2) *Adenylic acid and inosinic acid.*

Removal of the labile phosphate from the molecule of a.t.p. leaves the power of inhibiting the gut slightly weaker, while the subsequent increase in tone is no longer to be observed except very rarely with larger

doses. Comparison of Fig. 6 A and B shows how hydrolysis brings about this alteration in activity.

Inosinic acid gave results similar to adenylic acid in doses five times as great, only the inhibition of the gut being seen.

(3) *Adenosine.*

Adenosine inhibits the gut in concentrations of 1 in 50,000. It is the least active of the non-deaminated adenyl derivatives in this respect. It has only once been seen to produce increase in the tone of the gut, and that was with an unusually excitable specimen.

(4) *Adenine and hypoxanthine.*

These substances showed no action on the gut.

UTERUS.

The isolated virgin guinea-pig's uterus was used after the technique of Sawasaki [1925], as recommended by Deuticke [1932].

(1) *Adenosine triphosphate and inosine triphosphate.*

With a tension of 6 g. on the cornu, a.t.p. produces tonic contraction in concentrations of 1 in 500,000, or even less. The action does not appear to be as completely reversible as Deuticke suggests. Often the cornu starts to contract at a high level of tone after the specific action of the substance has passed off, and it is difficult to abolish these rhythmic contractions even by washing out the fluid. Since the uterus must be quiescent before a test can be made, the contractions may make it impossible to use the same preparation for testing a second specimen.

I.t.p. must be present in ten times the concentration of a.t.p. before it can produce increase in tone.

(2) *Adenylic acid, inosinic acid, and adenosine.*

Removal of phosphate from the molecule decreases the activity of these compounds on the uterus, so that a.t.p. is the most active of the non-deaminated series, and adenosine the least active.

Inosinic acid appears to have about one-tenth the activity of adenylic acid on this test object.

(3) *Adenine and hypoxanthine.*

These showed no activity on the uterus.

Some experiments were made in order to discover whether the adenyl compounds are freely dialysable in active form from the tissues where they naturally occur. Analyses of the fluids inside and outside a collodion tube containing a saline muscle extract, which had been allowed to dialyse against a Ringer-Locke solution for varying periods, showed that equilibrium was reached in the case of the adenyl compounds in about 2 hours, and that this time was unaffected by the presence of the colloid material in the muscle extract. Of course, if the glycolytic ferment were not inactivated by boiling, the ordinary enzymic changes would go on in the extract, and the a.t.p. would lose at the least some of its labile phosphate before it could escape into the outer fluid.

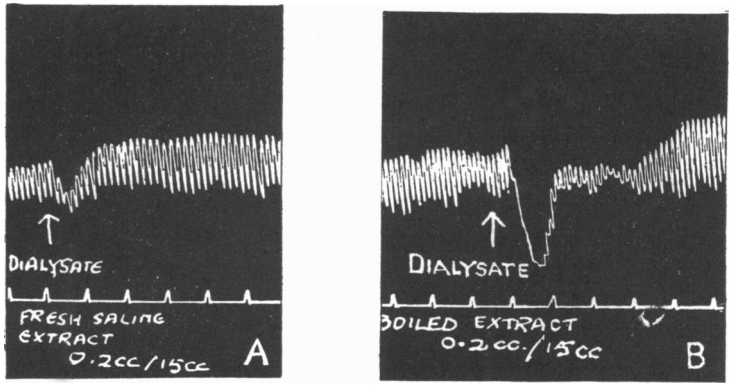

Fig. 7. A. Shows the effect of adding 0·2 c.c. of a 2 hours' dialysate of fresh saline extract of rabbit's muscle to 15 c.c. Ringer-Locke solution in which intestine was contracting. Time = 5 sec. B. Shows the effect of adding 0·2 c.c. of a dialysate of a similar extract in which ferment changes had been inhibited by boiling.
Note the much stronger activity seen in B.

The figure shows that, in the extract in which ferment action had been checked by boiling, the adenyl compounds can escape in biologically active form (Fig. 7). It is therefore possible that they may do so in the living animal, and, as we have pointed out, Zipf [1930] has shown clearly that the action of defibrinated blood plasma in lowering blood-pressure is due to its content of adenylic acid, which must consequently be always present there in small quantities during life.

The question thus arises as to whether the biological activities which the adenyl bodies show under experimental conditions have any counterpart in their normal activity in the living animal.

At the present time we can do no more than guess at the answer to this question. If we consider broadly the actions of all these compounds, two

facts are made fairly clear. In the first place complete loss of biological activity does not occur until the pentoside linkage in the molecule has been broken down. This has been pointed out recently by Ostern and Mann [1933], who note that the presence of phosphate in the molecule is not essential for the possession of biological activity. These authors say, however, in common with most workers, that deamination of the substances inactivates them completely. This we do not believe to be always the case. In fact, as far as we can see, the adenyl compounds never become completely inactive in the tissues, since deamination and removal of the phosphate is the greatest amount of destruction which they are likely to undergo.

But it is true in the second place that these compounds do suffer changes which very markedly alter their biological activity, and these changes occur constantly during muscular activity. The addition of phosphate to the molecule increases the activity of the compounds where tonic and muscular effects are concerned. Examples of this are the effects seen on the heart muscle, the intestine, and the uterus.

On the other hand, the removal of phosphate from the molecule seems to increase the activity of the substances as regards ability to dilate blood vessels. This has been seen in the case of the general blood-pressure, and most strikingly in that of the coronary vessels.

Possibly the tonic properties of the a.t.p. are made available for the muscle during anabolism and rest, while the early removal of the labile phosphate from its molecule during activity brings into play the more powerful dilatant actions of adenylic acid and adenosine. We should thus have a local adjuvant to the vaso-dilatation of activity in the normal breakdown products of metabolism.

It would seem, by reason of the extreme rapidity of all the biological actions, that the effects are really produced by alterations in the colloids at the surfaces of the cells concerned. A hundredth part of a milligramme of a.t.p. which succeeds in dilating one of the coronary arteries as it is whirled past at high pressure in a great volume of fluid has certainly not had time to take part in an elaborate metabolic process. It is this extraordinary rapidity of response, as well as of chemical change, which makes the approach to the problem so very difficult.

Summary.

1. Adenosine triphosphate, the naturally occurring adenyl compound, is more active than any of its derivatives in producing interference in conduction in the heart, and increased tone in the isolated virgin guinea-

pig's uterus. The removal of phosphate from its molecule does not lessen its activity so markedly as deamination.

2. A.t.p. has a tendency, which rarely appears in any of its derivatives, to increase the tone of the small intestine, after the preliminary depression which is also produced by the other active adenyl compounds. Deamination, and, to a less extent, the successive breaking off of the labile and stable phosphate linkages, lessen its depressant activity on the intestine.

3. Removal of phosphate from the molecule of a.t.p. increases its ability to lower blood-pressure and to dilate the coronary vessels. Thus the order of activity of the adenyl series is adenosine, adenylic acid, and a.t.p. Deamination greatly lessens, but does not destroy, this vasodilatant power.

4. A combination of the heart block and coronary dilation may give an explanation of some of the inconsistent behaviour of the so-called "heart hormones."

5. Biological activity finally disappears from the compounds when the pentose is split off from the purine base.

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