The Action of Magnesium and Calcium on the Enzymic Breakdown of Certain Adenine Compounds

By H. B. STONER AND H. N. GREEN, *Department of Pathology, The University, Sheffield*

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The initial breakdown of adenosine and its phosphorylated derivatives is brought about by deamination or by dephosphorylation. Deaminases for these compounds are widely distributed in the tissues although in some organs (e.g. liver) they act only after dephosphorylation (Conway & Cooke, 1939). The influence of Mg^{++} on such deaminases has not been studied in great detail. Ostern & Mann (1933) claimed that Mg^{++} inhibits the deamination of adenosine triphosphate and of muscle adenylic acid by frog muscle extract whilst, using mammalian tissue extracts, Conway & Cooke (1939), working with adenosine and muscle adenylic acid, and Brady (1942), working with adenosine, found Mg^{++} to be without effect on the deamination of these compounds. The effect of Ca^{++} on these deaminases has not, to our knowledge, been investigated.

The effect of Mg^{++} and Ca^{++} on the dephosphorylation of adenosine triphosphate has been studied more closely. The liberation of the terminal phosphate group by myosin is activated by Ca^{++} (Needham, 1942; Bailey, 1942) and is inhibited by Mg^{++} (Lyubimova & Pevsner, 1941). The removal of the second phosphate group by myokinase is activated by Mg^{++} (Kalckar, 1943); the effect of Ca^{++} has not been tested.

Our interest in this subject arose from a study of adenosine triphosphate as a metabolic factor possibly responsible for some of the general reactions to tissue trauma (Green, 1943; Bielschowsky & Green, 1943), a recent development of which was the finding that Mg^{++} potentiates the shock-inducing action of adenosine triphosphate (Green & Stoner, 1944). The present work had the object of explaining the *in vivo* findings by a study of the effects of Mg^{++} and its pharmacological antagonist Ca^{++} on the breakdown of adenosine triphosphate and related compounds *in vitro*. No attempt was made to separate the enzyme systems concerned since it was desired to approximate the conditions as closely as possible to those within the body.

METHOD

The tissues used were the liver and muscle from the albino rat. In most experiments liver slices and muscle pulp (prepared by means of a Latapie mincer) were used. The tissues were suspended in Ringer's solution prepared according to the directions of Krebs & Henseleit (1932),

pH 7.4. A few experiments with watery extracts of minced liver and muscle were also performed. With the extracts, buffers of 0.01 M- $NaHCO_3$ in equilibrium with a mixture of O_2 and 5% CO_2 , pH 7.1, or 0.1 M-glycine- $NaOH$ (Kolthoff, 1932), pH 9.2, were used, although in a few experiments Ringer's solution replaced the buffer solution. Mg^{++} was added as $MgSO_4 \cdot 7H_2O$ or $MgCl_2 \cdot 5H_2O$ and Ca^{++} as $CaCl_2$. 'Analar' reagents and glass-distilled water were used. The reactions were carried out in Warburg vessels or small conical flasks suspended in a water-bath at 37°. The adenine compound was added in the case of the Warburg vessel from the side-arm; otherwise directly. The compounds used were the Na and Mg salts of adenosine triphosphate, the Na salts of adenosine diphosphate and muscle adenylic acid and adenosine (British Drug Houses Ltd.). All the salts were prepared from barium adenosine triphosphate and were at least 98% pure as judged by their N, pentose, total P contents and P released on acid hydrolysis for 7 min. The initial concentration in the reaction mixture was approximately 0.0004 M in every case.

After the required period of incubation the reaction was stopped by the addition of 1.0 ml. of 30% trichloroacetic acid. The precipitated proteins were filtered off immediately. The amount of adenosine radicle remaining was determined by assaying a neutralized portion of the filtrate against adenosine on the guinea-pig auricle preparation of Drury, Lutwak-Mann & Solandt (1937) as modified by Stoner & Green (1944). A biological, in preference to a chemical method, was chosen for estimating these small amounts, largely because we had the facilities available and an extensive experience of its reliability. It is highly sensitive, detecting 1 μg . of adenosine, and highly specific, for the related inosine compounds have no effect upon the guinea-pig auricle. Loss of this specific biological activity occurs after deamination and Drury *et al.* (1937), in a study of the breakdown of adenosine by blood, have already shown that loss of this biological activity is accompanied by NH_3 formation. Under our test conditions it could not be assumed that any NH_3 released would be derived from the adenine compound alone, and in view of the small quantity of the compound relative to the weight of tissue used it would be unlikely to be so. However, working with higher concentrations (0.001–0.003 M) of the adenine compounds we have found that NH_3 production runs almost parallel with loss of biological activity (Fig. 1). Consequently, under our test conditions, biological inactivation would appear to represent deamination, and throughout the paper the terms have been used as synonymous. The extent of dephosphorylation was determined by estimating the inorganic phosphate in a portion of the filtrate by Brigg's method (Peters & Van Slyke, 1932). The initial concentrations of the adenosine radicle and 7 min. P were determined by the difference between controls in which the

purine solution was replaced by an equal volume of 0.9% NaCl and those in which the reaction was stopped at zero time. The amount of deamination and dephosphorylation, i.e. the decrease in the amount of adenosine radical present and the increase in the inorganic phosphate, could then be expressed as percentages of the initial concentrations.

Fig. 1. Comparison of the biological inactivation and deamination, as calculated from NH_3 production, of adenosine by rat muscle. Reaction carried out in flasks under air. 300 mg. minced muscle suspended in Ringer's solution. Initial concentration of adenosine 0.0028 M. Total volume 6.0 ml. ●—● % inactivation; x—x % deamination.

RESULTS

The effect of magnesium on deamination

Under these conditions Mg^{++} proved to be a powerful inhibitor of the deaminases of adenosine triphosphate present in liver slices and minced muscle. This is shown in Fig. 2, where an increase in the Mg^{++} concentration from 3.0 mg./100 ml. (i.e. about the normal plasma level) to twice this concentration significantly delayed the deamination of adenosine triphosphate by minced muscle. A similar effect for liver extract in Ringer's solution is shown in Fig. 3, where the percentage deamination occurring in a

Fig. 2. Effect of Mg^{++} on the deamination of adenosine triphosphate by rat muscle. Reaction carried out in Warburg vessels under $\text{O}_2 + 5\% \text{CO}_2$. 200 mg. of minced muscle suspended in Ringer's solution. Initial concentration of sodium adenosine triphosphate 0.0003 M. Total volume 6.0 ml.

Mg^{++} (MgCl_2) concentration:

- 3.0 mg./100 ml. (0.0013 M).
- 6.0 mg./100 ml. (0.0026 M).

Fig. 3. Effect of different Mg^{++} (MgCl_2) levels on the amount of deamination of adenosine triphosphate by rat liver extract occurring in 10 min. Reactions carried out in flasks under air. Each flask contained liver extract equivalent to 200 mg. liver made up in Ringer's solution and the Mg^{++} concentration varied for each flask. Initial concentration of sodium adenosine triphosphate 0.0003 M. Total volume 6.0 ml.

standard time at different Mg^{++} levels is compared. In similar experiments with liver slices and minced muscle, curves closely resembling that of Fig. 3 were obtained. The Na and Mg salts of adenosine triphosphate were equally affected by a raised Mg^{++} level and the sulphate and chloride of Mg were equally effective as inhibitors. When no additional Mg^{++} was added to the Ringer's solution (i.e. at a Mg^{++} level of 3.0 mg./100 ml.), minced muscle deaminated magnesium adenosine triphosphate more slowly than sodium adenosine triphosphate. This may be due to a slight increase in the Mg^{++} concentration of the Ringer's solution as a result of dissociation of magnesium adenosine triphosphate. It would appear that relatively small increases in the Mg^{++} concentration above the normal plasma level can produce relatively large decreases in the rate of deamination.

Tissue extracts in the buffer solutions used were not so suitable for the demonstration of the effect. Reduction in the rate of deamination by bicarbonate (Conway & Cooke, 1939) is accentuated by the presence of Mg^{++} . Deamination diminishes with increasing pH (Conway & Cooke, 1939; Ostern & Mann, 1933). Using the glycine buffer, pH 9.2, we found little if any deamination of adenosine triphosphate by either liver or muscle extracts.

Fig. 4. Comparison of the amount of deamination of adenosine triphosphate (ATP), adenosine diphosphate (ADP), muscle adenylic acid (A5MP) and adenosine (A) occurring in 15 min. at two Mg^{++} ($MgCl_2$) levels. Reaction carried out in flasks under air containing 100 mg. minced muscle in Ringer's solution. Initial concentration in each case approx. 0.0004M. Total volume in each case 6.0 ml. Black columns at 3.0 mg. Mg^{++} /100 ml. (0.0013M); open columns at 6.0 mg. Mg^{++} /100 ml. (0.0026M).

Of the other adenine compounds tested, the rate of deamination of adenosine and muscle adenylic

acid by liver and muscle was inhibited by Mg^{++} , whereas under these experimental conditions, the deamination of adenosine diphosphate was unaffected (Fig. 4). The differences in the degree of Mg inhibition of adenosine triphosphate, muscle adenylic acid and adenosine cannot be stressed because of the small number of experiments.

The effect of magnesium on dephosphorylation

In this case the results were not so clear-cut. The dephosphorylation of adenosine triphosphate by liver slices and muscle pulp was definitely inhibited by $MgCl_2$ in 11 out of 14 experiments with little obvious effect in the remaining three (Fig. 5). When

Fig. 5. Effect of different Mg^{++} ($MgCl_2$) levels on the amount of dephosphorylation of adenosine triphosphate by rat muscle occurring in 10 min. Reactions carried out in flasks under air containing 100 mg. of minced muscle suspended in Ringer's solution. Initial concentration of sodium adenosine triphosphate 0.0002M. Total volume 6.0 ml.

$MgSO_4$ was used, the results were erratic; in seven experiments an increase of the Mg^{++} level from 3.0 to 6.0 mg./100 ml. of Ringer's solution produced slight acceleration in four, well-marked inhibition in two and no change in one. The same type of variation was seen with both liver and muscle in the form of either whole tissues or extracts and with both the Na and Mg salts of adenosine triphosphate. When no additional Mg^{++} was added to the medium (i.e. at a Mg^{++} level of 3.0 mg./100 ml.), the rate of dephosphorylation of the Na and Mg salts was the same. Compounds other than adenosine triphosphate were not studied in detail.

*The effect of calcium on deamination
and dephosphorylation*

Briefly stated, it was found that increasing the Ca^{++} level from 12.0 to 24.0 mg./100 ml. of Ringer's solution had no effect either upon the rate of deamination or dephosphorylation of adenosine triphosphate by liver slices or muscle pulp. Compounds other than adenosine triphosphate were not studied.

DISCUSSION

Since we have shown a direct relationship between biological inactivation and deamination as judged by NH_3 production, our results would seem to indicate that under the described conditions Mg^{++} is a potent inhibitor of the liver and muscle deaminases for adenosine triphosphate, muscle adenylic acid and adenosine. The finding of Bielschowsky, Green & Stoner (1945) that larger amounts of adenosine triphosphate and adenosine given intraportally survived their passage through the liver after Mg^{++} administration suggests that the inhibition of liver deaminases produced by Mg^{++} *in vitro* may also occur *in vivo*. The cardiovascular effects of these compounds are possibly brought about during the process of deamination and we have already suggested that the diminution in these effects produced by Mg^{++} may in some degree be due to its inhibitory action on deamination (Bielschowsky *et al.* 1945). The results reported here provide some experimental support for this hypothesis.

On the other hand, our *in vitro* findings do not materially assist in explaining why Mg^{++} increases the shock-inducing effect of adenosine triphosphate and allied compounds (Green & Stoner, 1944). It was postulated (Green, 1943) that the pyrophosphate grouping in adenosine triphosphate might be responsible for its shock-inducing properties, and Bielschowsky & Green (1944) have shown that the potency of phosphorylated adenosine compounds increases with the number of phosphate groups. It seemed possible, therefore, that Mg^{++} might increase the shock-inducing effect of such compounds by delaying dephosphorylation. The *in vitro* results obtained with MgCl_2 on the dephosphorylation of adenosine triphosphate give some support to this view. The variable results seen with MgSO_4 may perhaps be explained by the work of Barrenscheen & Lang (1932), who found that the stimulant action

of the SO_4^- ion on dephosphorylation masked the slight inhibitory action of Mg^{++} ion. So far, numerous attempts to obtain consistent results in these experiments have not succeeded and until that can be done it would be unwise to interrelate the *in vitro* and *in vivo* findings more closely. The work of DuBois, Albaum & Potter (1943), in which increased yields of adenosine triphosphate were obtained from animals killed by Mg^{++} injections, suggests, however, that its action on dephosphorylation may be more pronounced *in vivo* than would seem the case *in vitro*.

Ca^{++} also increases the shock-inducing properties of adenosine triphosphate, although to a less extent than Mg^{++} , and reasons have been given (Green & Stoner, 1944) for believing that this is not a specific effect. The finding that Ca^{++} does not affect the dephosphorylation of adenosine triphosphate is not opposed to this view. Ca^{++} also potentiates the cardiovascular effects of these compounds, but again it was not thought, on physiological grounds, that enzymic processes were involved (Bielschowsky *et al.* 1945). The finding that Ca^{++} has no effect on the deamination of adenosine triphosphate is not contrary to this idea.

SUMMARY

1. The effect of Mg^{++} and Ca^{++} on the deamination (determined by a biological method) and dephosphorylation of adenosine triphosphate and other adenine compounds by liver and muscle was studied.

2. Under the described experimental conditions, Mg^{++} inhibited the deamination of adenosine triphosphate, muscle adenylic acid and adenosine, but not of adenosine diphosphate.

3. In most experiments Mg^{++} , as MgCl_2 , inhibited the dephosphorylation of adenosine triphosphate but, as MgSO_4 , it had a variable effect, producing chiefly acceleration.

4. Ca^{++} was without effect on either the deamination or dephosphorylation of adenosine triphosphate.

5. The bearing of these results on the action of Mg^{++} and Ca^{++} on these compounds *in vivo* is discussed.

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Substrate Specificity of Amine Oxidases

By H. BLASCHKO* AND RUTH DUTHIE,* *Department of Pharmacology, Oxford*

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It has been known for some time that diamines such as putrescine and cadaverine are not oxidized by amine oxidase (Blaschko, Richter & Schlossmann, 1937). This is due to their lack of affinity for the enzyme (Blaschko, 1939). Their breakdown in the mammalian organism, first studied by Udranszky & Baumann (1891), is brought about by the enzyme diamine oxidase (Zeller, 1938a).

The molecular grouping which is present in all substrates of amine oxidase is the amino-group attached to a terminal carbon atom. That the diamines with two such groups have no affinity to the enzyme shows that the presence of the second group in some way interferes with the ability of the first group to react with the enzyme. The nature of this interference is unknown.

We have recently had the opportunity of examining the oxidation of a number of long-chain mono- and diamines by tissue extracts and our results are described in this paper. They represent a contribution to the problem of substrate specificity.

MATERIAL AND METHODS

We wish to thank Dr H. King, F.R.S., for giving us the substances which were examined. They included two monoamines, $\text{CH}_3(\text{CH}_2)_{11}\text{NH}_2$ and $\text{CH}_3(\text{CH}_2)_{17}\text{NH}_2$ and five diamines, $\text{NH}_2(\text{CH}_2)_6\text{NH}_2$, $\text{NH}_2(\text{CH}_2)_8\text{NH}_2$, $\text{NH}_2(\text{CH}_2)_{14}\text{NH}_2$, $\text{NH}_2(\text{CH}_2)_{16}\text{NH}_2$ and $\text{NH}_2(\text{CH}_2)_{18}\text{NH}_2$. As typical substrates of amine oxidase we used tyramine, and of diamine oxidase, cadaverine. The amine oxidase preparation was from rabbit's liver (Blaschko & Duthie, 1945). As source for diamine oxidase we used pig's kidney; two preparations were employed, first, an aqueous extract of the organ dialyzed against distilled water overnight, with one-tenth the volume of $\text{M}/15$ sodium phosphate buffer of pH 7.4 added, and secondly, an acetone-dried preparation. The latter preparation was obtained by passing the organ through a meat mincer and grinding the mince in a cooled mortar. The brei obtained was extracted with cold acetone,

three times the weight of the tissue, and this treatment was repeated three times. The last traces of acetone were pressed out and the preparation was kept in a vacuum desiccator overnight. Coarse particles were removed from the dry powder by passing it through a no. 40 mesh sieve. 1.5 g. of the resulting powder were incubated with 15 ml. of $\text{M}/15$ sodium phosphate buffer, pH 7.4, at room temperature for 50 min. with frequent stirring. The preparation was centrifuged for 5 min. and the supernatant, strained if necessary, in order to remove suspended particles, was used.

The manometric plan need not be discussed in detail. The standard substrate of amine oxidase was tyramine hydrochloride, used in 10^{-2}M concentration, that of diamine oxidase was $0.5 \times 10^{-2}\text{M}$ -cadaverine dihydrochloride. The temperature in all experiments was 38° . The gas phase was oxygen in the amine oxidase experiments and air in those with diamine oxidase. The total reaction volume in each flask was 2.0 ml.

EXPERIMENTAL

(1) Experiments with amine oxidase

(a) *Monoamines*. The compound with 12 carbon atoms had a marked affinity for amine oxidase, and it was a substrate of the enzyme. In an experiment in which 1.6 ml. rabbit liver extract were used there was an additional oxygen uptake of $43 \mu\text{l}$. in 15 min. with $0.5 \times 10^{-2}\text{M}$ -dodecylamine. That this was due to the amine oxidase present in the extract is supported by the observation that the amine strongly depressed the oxygen uptake due to tyramine. For instance, 0.5 ml. extract with 10^{-2}M -tyramine alone gave $90 \mu\text{l}$. of oxygen consumed in the first 15 min., but with 10^{-2}M -tyramine plus 10^{-3}M -dodecylamine the oxygen uptake was only $4 \mu\text{l}$. in the same period.

Octadecylamine, the other monoamine tested, gave entirely different results. There was no oxygen uptake in excess of the blank with $2 \times 10^{-3}\text{M}$ -octadecylamine. This was due to a lack of affinity of the amine for amine oxidase: the oxygen uptake with 10^{-2}M -tyramine was the same in the presence and in the absence of 10^{-3}M -octadecylamine.

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