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 adenyl acid by frog muscle extract whilst, using mammalian tissue extracts, Conway & Cooke (1939), working with adenosine and muscle adenyl 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 vivo. 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-NaHCO₃ in equilibrium with a mixture of O₂ and 5% CO₂, pH 7.1, or 0.1 m-glycine-NaOH (Koltchoff, 1932), pH 9.2, were used, although in a few experiments Ringer’s solution replaced the buffer solution. Mg++ was added as MgSO₄.7H₂O or MgCl₂.5H₂O and Ca++ as CaCl₂.

`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 adenosine 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 adenyl 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 NH₃ formation. Under our test conditions it could not be assumed that any NH₃ 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 NH₃ 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.

**RESULTS**

**The effect of magnesium on deamination**

Under these conditions Mg²⁺ 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 Mg²⁺ 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
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 effective 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++ (MgCl2) levels. Reaction carried out in flasks under air containing 100 mg. minced muscle in Ringer’s solution. Initial concentration in each case approx. 0-0004 M. Total volume in each case 6-0 ml. Black columns at 3-0 mg. Mg++/100 ml. (0-0013 M); open columns at 6-0 mg. Mg++/100 ml. (0-0026 M).

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 MgCl2 in 11 out of 14 experiments with little obvious effect in the remaining three (Fig. 5). When MgSO4 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 NH₃ 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 intraperitoneally 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 group 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 MgCl₂ on the dephosphorylation of adenosine triphosphate give some support to this view. The variable results seen with MgSO₄ may perhaps be explained by the work of Barrenscheen & Lang (1932), who found that the stimulant action of the SO₄²⁻ 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 MgCl₂, inhibited the dephosphorylation of adenosine triphosphate but, as MgSO₄, 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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REFERENCES

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, CH₅(CH₂)₆NH₂ and CH₅(CH₂)₁₀NH₂ and five diamines, NH₂(CH₂)₆NH₂, NH₂(CH₂)₈NH₂, NH₂(CH₂)₁₀NH₂, NH₂(CH₂)₁₂NH₂ and NH₂(CH₂)₁₄NH₂. 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 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 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 10⁻⁴M concentration, that of diamine oxidase 0·5 x 10⁻⁴M-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 μl. in 15 min. with 0·5 x 10⁻⁴M-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 10⁻⁴M-tyramine alone gave 90 μl. of oxygen consumed in the first 15 min., but with 10⁻⁴M-tyramine plus 10⁻⁴M-dodecylamine the oxygen uptake was only 4 μl. 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 x 10⁻³M-octadecylamine. This was due to a lack of affinity of the amine for amine oxidase: the oxygen uptake with 10⁻³M-tyramine was the same in the presence and in the absence of 10⁻³M-octadecylamine.