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The Effect of Magnesium and Calcium Ions on Adenosine Triphosphatase in the Nervous and Vascular Tissues of the Brain

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During the course of histochemical work on the distribution of adenosine triphosphatase in brain tissue, it has been found that the enzyme is present in both the vascular and the nervous elements (Naidoo & Pratt, 1951). It is therefore considered valuable, in the study of properties of brain adenosine triphosphatase, to take account of the existence of the enzyme in tissues of widely different embryological origins within the same organ. Since the brain is well supplied with blood vessels, the proportion of mesodermal tissue is substantial.

A detailed study of brain-adenosine triphosphatase activity was made by Gore (1951). Enzyme activity showed two maxima with variation of pH—one at pH 7.4 and the other at pH 8.2. The enzyme was activated by magnesium but inhibited by calcium ions. There is some variation in the properties ascribed to the enzyme by different workers. In previous histochemical work optimum activity has been found at a lower pH, 6.5 (Naidoo & Pratt, 1951). Some workers report activation of brain adenosine triphosphatase by magnesium ions, with inhibition or no activation by calcium ions (Epelbaum, Sheves & Kobylin, 1949; Gordon,

1950, 1953; Lowry, Roberts, Wu, Hixon & Crawford, 1954; Cseh, Hermann & Zombori, 1954). On the other hand, the enzyme has been reported as activated by calcium ions (DuBois & Potter, 1943; Feldberg & Mann, 1945). The work of Gore (1951) suggests that there are adenosine triphosphatase fractions in brain which differ in solubility in various aqueous media. According to Lowry *et al.* (1954) brain adenosine triphosphatase releases only the terminal phosphate group and is inhibited by adenosine diphosphate.

The purpose of the present work is to investigate the extent to which the properties of adenosine triphosphatase are likely to depend on the cytological origin of the enzyme, and whether there is any evidence to suggest that some of the inconsistencies in the reported properties of the enzyme are in fact due to its origin from both the vascular and the nervous tissue. This paper reports a study, by histological and by quantitative methods, of the comparative effect of magnesium and calcium ions on the enzyme of the vessels, on the one hand, and on that of the nerve and glial cells, on the other.

EXPERIMENTAL

Normal adult male and virgin female Wistar laboratory-bred rats, aged 100–120 days, weighing 180–230 g., were used. The animals were fed on a commercial rat diet (no. 41; Associated Flour Millers, London) in unrestricted quantity, and were housed in a room with controlled heating at 22°.

The adenosine triphosphate (ATP) was either the chromatographed sodium salt (Sigma Chemical Co., St Louis, Mo.) or the barium salt (Boots Pure Drug Co. Ltd., Nottingham) reprecipitated as previously described (Pratt, 1954).

Histochemical. Frozen-dried paraffin sections from one hemisphere were prepared and processed according to the procedure previously described (Naidoo & Pratt, 1951, 1952, 1954). The other hemisphere was used for quantitative work. The composition of the medium for incubating sections was: 50 mM sodium succinate; 0.5 mM lead acetate; 2 mM ATP; with or without 5 mM-MgCl₂ or -CaCl₂.

Quantitative method. Blood vessels were separated from the nervous tissue of the hemisphere by microdissection and by maceration working at 5°. Microdissection was carried out by means of a Singer geodetic stereoscopic micromanipulator. Maceration was effected by the slow application of pressure to the hemisphere placed between two 3 in. × 3 in. pieces of plate glass. The glasses were separated, the position of the flattened brain was altered and the pressure reapplied. This process was repeated several times. The tissue was diluted in 0.9% NaCl solution and the suspension shaken. Clumps of vessels were removed on a small Pt wire hook, drained on a glass surface, weighed and ground in a small measured volume of 0.9% NaCl. About 10–15 mg. of vascular tissue was separated from a brain weighing 0.8–1.2 g. This fraction was regarded as 'mesodermal'. An 'ectodermal' fraction was obtained by filtering the macerated brain through three layers of surgical gauze prepared by washing in 5N-HCl, followed by distilled water. The filtrate, representing the 'ectodermal' fraction, was weighed and ground in a measured volume of 0.9% NaCl. Samples from the separated 'mesodermal' and 'ectodermal' fractions were examined microscopically in smear preparations fixed in Schaudin fixative and stained with haematoxylin and eosin. Only a small proportion of vessels were still present in the 'ectodermal' fraction, and similarly only a little brain tissue was found in the 'mesodermal' sample.

The suspension was added to the following medium at 0°: 0.1M glycylglycine, pH 7.4; 2 mM ATP. The fresh tissue dilution was 1.5 mg./ml. for the 'ectodermal' and 0.33 mg./ml. for the 'mesodermal' fraction. Portions were pipetted at 30 sec. intervals into glass-stoppered tubes, which were previously placed in the thermostat both at 37° and contained the MgCl₂ or CaCl₂ additions. Incubations were carried out for 15 min. with rapid agitation, and the inorganic phosphate content was estimated by a procedure using phosphomolybdic acid extraction as previously described (Pratt, 1953). Under these conditions the rate of reaction was proportional to the tissue concentration and linear with time up to 30 min. Increasing the substrate concentration above 1 mM did not increase the activity appreciably. Activity was not critically dependent on pH at pH 7.4.

RESULTS

Quantitative. The adenosine triphosphatase activity of the 'ectodermal' or 'mesodermal' brain fractions was determined separately. The effect of varying the Ca²⁺ or Mg²⁺ concentration was studied.

The activity of the 'mesodermal' fraction was low in the absence of added bivalent cations, but rose rapidly with small additions of either Ca²⁺ or Mg²⁺ (Fig. 1). The highest activity resulted from

Fig. 1. Effect of varying concentrations of Mg²⁺ or Ca²⁺ on mesodermal ATPase. Enzyme activity in μmoles of inorganic phosphate/g. hr. 0.1M glycylglycine buffer, pH 7.4; 2 mM ATP; tissue 0.33 mg. (fresh wt.)/ml. Incubation 15 min. at 37°.

Fig. 2. Effect of varying concentrations of Mg²⁺ or Ca²⁺ on ectodermal brain ATPase. Enzyme activity in μmoles of inorganic phosphate/g. hr. Conditions as in Fig. 1, except that the tissue concentration is 1.5 mg./ml.

the addition of about 10 mM-CaCl₂, but concentrations in the range 3–20 mM gave nearly maximal activity. The concentration of MgCl₂ giving high activity, about 4 mM, was rather critical. The maximal Mg²⁺ activation was rather less than that of Ca²⁺, and the activity fell sharply as the MgCl₂ concentration was increased to about 8 mM.

The activity of the 'ectodermal' fraction was low in the absence of added bivalent cations, being of a similar order to that of the 'mesodermal' fraction. Small additions of either Ca^{2+} or Mg^{2+} increased the activity, but the maximal values reached were considerably lower than those of the 'mesodermal' fraction (Fig. 2). Ca^{2+} activation amounted to only about 50–70%, reached with concentrations greater than about 3 mM. A greater activation of about 200% was reached with 8–10 mM- MgCl_2 , and the activity fell only slightly at higher MgCl_2 concentrations.

Histological. Good vascular staining was seen without added Ca^{2+} or Mg^{2+} . The cells of the cerebellum were only moderately stained (Fig. 3), but

Fig. 3. Cerebellum. ATPase showing deep staining of the pia-arachnoidal vessels but only moderate staining of the cerebellar cells. $\times 90$.

Fig. 4. Cerebellum. ATPase. Mg^{2+} added. Showing deep staining of the pia-arachnoidal vessels as well as of glial cells of the molecular layer, Purkinje cells and granules. $\times 90$.

the vessels of the pia arachnoid were deeply stained. The addition of Mg^{2+} (Fig. 4) increased considerably the rate of staining of the glial cells of the molecular layer, the Purkinje cells and the granular layer cells.

The vascular staining in all regions of the cerebrum in media with or without Mg^{2+} , examined over a wide range of incubation times, was similar. The parenchymal vessels of the brain and the larger extracerebral vessels developed staining at

the same rate (Fig. 5) and were readily demonstrable at incubation periods of only 3 min. The pia-arachnoidal cells did not respond to Mg^{2+} addition. The addition of Mg^{2+} increased considerably the rate at which nuclei of the ectodermal elements stained, but did not alter the rate of staining of the mesodermal elements (Fig. 6). Nuclei of ectodermal elements were evident after 5 min. incubation, whereas without Mg^{2+} a similar intensity of staining required 40 min. incubation.

Fig. 5. Cerebrum. ATPase. Showing deep staining of the intra- and extra-cerebral vessels and pia-arachnoidal cells. The neuronal and glial elements are lightly stained. $\times 64$.

Fig. 6. Cerebrum. ATPase. Mg^{2+} added. Greater activation of ectodermal elements than of mesodermal elements. $\times 64$.

With the addition of Ca^{2+} the major differences are demonstrable at short incubation times. Sections incubated in media containing Ca^{2+} showed much more deeply staining vessels than in sections incubated without it (Fig. 7). The ectodermal elements showed little change whether or

Fig. 7. Cerebrium. ATPase. Ca^{2+} added. Greater activation of mesodermal elements than of ectodermal elements. $\times 64$.

not Ca^{2+} was added to the medium. At long incubation periods the Ca^{2+} incubated sections closely resembled the control sections.

In some experiments the Pb^{2+} concentration in the incubation medium was increased to 4 mM. Glial and neuronal nuclei showed only very faint staining, even in the presence of Mg^{2+} , but the staining of vessels was affected only slightly by the raised Pb^{2+} concentration.

DISCUSSION

Comparison of brain adenosine triphosphatase in the nervous and non-nervous tissue in respect of activation by calcium or magnesium ions has been carried out by two separate methods. Although the quantitative and the histological results are not directly comparable they are mutually confirmatory. The histological picture shows qualitatively the activation of the enzyme in the various ectodermal and mesodermal elements *in situ*, whereas assay of enzyme activity in separated-tissue samples has given a quantitative indication of the differences.

In the histochemical study the lead concentration has been kept low in order to avoid differential inhibition. It has been necessary, therefore, to work at a lower adenosine triphosphate concentra-

tion than the optimum in order to avoid technical difficulties arising from local exhaustion of lead ions at the sites of high activity. The pH is kept rather lower than the optimum in order to avoid precipitation of lead carbonate. In the quantitative study of activity in the separated tissue, on the other hand, optimum conditions can be used. Contamination of the 'ectodermal' fraction by a small proportion of vessels and of the 'mesodermal' sample by some adhering brain tissue tends to reduce or obscure differences in enzyme properties. Notwithstanding the modifications in the histological conditions and the incomplete separation of tissue for quantitative study, there is general agreement between the two sets of results. The greater activation of the 'mesodermal' fraction adenosine triphosphatase by calcium salts, and its inhibition by higher magnesium-salt concentrations, conform with the greater staining of vessels when calcium but not magnesium salts are added to the medium. The activation of the 'ectodermal' fraction adenosine triphosphatase by magnesium ions, which is only slight with calcium ions, conforms with the greater staining of neuronal and glial nuclei when magnesium but not calcium ions are added to the medium.

It has been suggested that calcium ions increase the permeability of mitochondria (Cleland, 1952), releasing latent adenosine triphosphatase activity. However, Potter, Siekevitz & Simonson (1953) did not find clear evidence of such latency in tissues other than liver. In the present work, a considerable activation by calcium ions has been found in the 'mesodermal' fraction, rather than in the 'ectodermal' fraction in which the enzyme is probably largely mitochondrial. According to Lowry *et al.* (1954) calcium ions compete with magnesium ions and inhibit the activity when both are present.

The effect of lead ions on adenosine triphosphatase differs from that on other phosphatases, for this cation probably competes with magnesium ions for the substrate (Pratt, 1954). The work of Neuberg & Mandl (1949) suggests that adenosine triphosphate forms complexes with various cations, including magnesium, calcium and lead. Evidence for the existence of a magnesium-adenosine triphosphate complex has been given by Spicer (1952). Brain adenosine triphosphatase is inhibited by chelating agents that bind magnesium and calcium ions (Pappius & Elliot, 1954; Maxwell & Nickel, 1954), but the inhibition can be overcome by an excess of magnesium ions. The preferential poisoning by lead ions suggests that the magnesium-dependent enzyme is different from the calcium-activated one.

Skeletal muscle has been reported to contain both a calcium-activated adenosine triphosphatase

associated with the actomyosin complex and a magnesium-activated enzyme associated with lipoprotein granules (Kielley & Meyerhof, 1948). The main activity observed in the brain 'mesodermal' fraction may be due to a myosin-like enzyme from smooth-muscle cells. Pappius & Elliot (1954) find that a suspension prepared from the whole cerebral hemisphere of the rat contains an adenosine triphosphatase activated by calcium ions and also a magnesium-activated enzyme which is strongly inhibited by calcium ions. From studies of the activation of adenosine triphosphatase by calcium and magnesium ions in the cytoarchitectonic layers of rat cerebral cortex, Hess & Pope (1954) suggest that separate enzymes with different cytological locations are present.

The differences between the activation by bivalent cations of adenosine triphosphatase of brain vessels and of brain parenchyma suggest that separate consideration of the enzymes of the ectodermal and mesodermal tissue may help to explain some of the conflicting findings about brain adenosine triphosphatase. Stability of the enzyme is likely to be important here. Earlier results suggest that the activity of the adenosine triphosphatase in glial and neuronal nuclei declines more rapidly than that in the vessels in stored tissue (Naidoo & Pratt, 1954). Efficient separation of the tissue into nervous and non-nervous tissue fractions may prove to be a valuable step in the preparation of purified enzymes.

SUMMARY

1. Two tissue fractions have been separated from rat brain, one fraction consisting mainly of tissue of mesodermal origin and the other mainly of tissue of ectodermal origin. Adenosine triphosphatase activity has been studied quantitatively in saline suspensions prepared from these fractions and qualitatively by histological staining of enzymically active sites in frozen-dried sections from rat brain.

2. The adenosine triphosphatase activity of both fractions is of a similar order in the absence of added bivalent cations. The activity of the 'mesodermal' fraction is increased more than 15-fold by the presence of 10 mM calcium ions, and to a lesser extent by 3 mM magnesium ions. The adenosine triphosphatase of the 'ectodermal' fraction is more sensitive to magnesium ions, an activation of about

200 % being obtained at 8 mM concentration, than to calcium ions, which activate only about 60 % at 3 mM concentration.

3. The staining of brain vessels was accelerated much more by calcium ions than by magnesium ions. The staining of neuronal and glial nuclei was accelerated by magnesium ions but not by calcium ions. A lead-salt concentration of 4 mM preferentially inhibited the staining of nervous-tissue elements.

4. The significance of these results is discussed in relation to the preparation, properties and function of brain adenosine triphosphatase.

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