Properties of Brain Pyrophosphatase

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A preliminary report (Gordon, 1949) has described some of the properties of rat-brain pyrophosphatase, and quoted experiments which point to the probability that the enzyme contains active thiol groups, resembling in that respect the yeast (Bailey & Webb, 1944) and the erythrocyte (Naganna & Menon, 1948) pyrophosphatases. The present communication gives a full account of the experimental work upon which the previous report was based, and provides additional data bearing on the behaviour of the enzyme towards inhibitors, especially thiol inhibitors. Activities of brain pyrophosphatase in several species have also been investigated, and compared with those of the corresponding acid and alkaline brain phosphomonoesterases and erythrocyte pyrophosphatase.

EXPERIMENTAL

The brains of mature rats were mainly used as source of enzyme, but preparations were also made from sheep and rabbit brain, and from human cerebral cortex. All results are expressed as mmol. substrate decomposed/30 min./100 g. original tissue. These units were used instead of Roche P units in order that activities of pyrophosphatase and other phosphatases could be more readily compared.

Preparation of enzymes

Brain phosphatase. Fresh whole rat brains, specimens of minced rabbit or sheep brain (1–2 g.), or specimens of human cerebral cortex (1–2 g.) were ground and washed according to the procedure described by Fleischhacker (1938), using ice-cold reagents throughout. The tissue was homogenized with 2–3 ml. of acetone in a hard-glass homogenizer, shaken with acetone (10–15 vol.) and centrifuged. The acetone washing was repeated twice, and the tissue similarly washed successively three times each with 70% (v/v) acetone-water, pure acetone, and ether, and finally dried in a vacuum desiccator at 0°. Weights of dried powder (mg./g. original tissue) finally obtained were 143–154 (rat), 149 (sheep), 116 (rabbit) and 103 (human cerebral cortex). Activity is retained for several weeks.

Erythrocyte pyrophosphatase was prepared from freshly drawn blood by the procedure of Naganna & Menon (1948). Attempts were made also to prepare a pyrophosphatase-containing powder from sheep erythrocytes, by shaking the washed packed cells with ice-cold acetone (15 vol.) and then proceeding in the same manner as for brain. A dry powder was obtained containing the equivalent of 3.3 μ l. packed cells/mg.

Measurement of enzyme activity

Acetate-veronal buffer solutions were used throughout.

Pyrophosphatase. Pure $\text{Na}_4\text{P}_2\text{O}_7$ (free from orthophosphate) was used as substrate. Enzyme powder (10–20 mg.) was ground with glycerol (0.5–1.0 ml.) to a fine paste, and made to a suspension with water. A 2 mg./ml. dilution was suitable with rat or sheep brain, but stronger suspensions were necessary with rabbit brain (20 mg./ml.) and human cerebral cortex (6 mg./ml.). The suspension (1 ml.) was incubated at 37° with 1 ml. of veronal buffer (0.1 M, pH 7.4) in presence of MgCl_2 (0.02 M final concentration), diluting to 3 ml. with distilled water. When inhibitors were used, they were included in the mixture, which was then incubated for 30 min.; otherwise a few minutes were allowed for thermal equilibration. Substrate (1 ml., 0.002 M), previously incubated at 37°, was then added, and the mixture shaken. Enzyme and substrate blanks were also set up. The reaction was stopped after 30 min. by addition of 10% (w/v) trichloroacetic acid (2 ml.), and the mixtures allowed to stand 20 min. at room temperature and then filtered. Orthophosphoric acid was estimated in 3 ml. of the filtrate by the method of Fiske & Subbarow (1925).

Optimal conditions were established by a series of experiments in which variations were made separately in time of incubation, substrate concentration, MgCl_2 concentration and pH value.

Erythrocyte pyrophosphatase activity was estimated by the procedure used for brain, with a lysed cell preparation (0.1–0.3 ml.) or a dried powder suspension (1 ml. of 20 mg./ml., prepared as for brain).

Alkaline phosphatase activity was estimated in brain-powder suspensions (1 ml. of 10 mg./ml.) at pH 9.4 with sodium α -glycerophosphate (0.006 M final concentration) as substrate, in presence of 0.001 M- MgCl_2 , the incubation period being 1 hr. The procedure otherwise was the same as for pyrophosphatase.

Acid phosphatase activity was estimated at pH 5.0 in the same manner as alkaline phosphatase, but with no MgCl_2 present. A 2 hr. incubation period was employed.

The above methods of estimation of acid and alkaline phosphatase activity were based on the optimal conditions established by Giri & Datta (1936), but a substrate concentration of 0.006 M was found to be adequate, whereas these workers used β -glycerophosphate in 0.02 M concentration.

RESULTS

Optimal conditions of activity

The effects of time of incubation, substrate concentration, magnesium ion concentration and pH of medium on the activity of brain pyrophosphatase

preparations are illustrated in Figs. 1–4. The following conclusions may be drawn:

(1) The rate of decomposition of pyrophosphate is linear up to 81 % hydrolysis.

Fig. 1. Progress curve of enzymic hydrolysis of $\text{Na}_4\text{P}_2\text{O}_7$ by brain at 37° . Rat brain suspension with 0.0005M $\text{Na}_4\text{P}_2\text{O}_7$ and 0.02M MgCl_2 , at pH 7.4.

Fig. 2. Effect of substrate concentration on pyrophosphatase activity of brain at 37° . Rat brain suspension with 0.02M MgCl_2 at pH 7.4.

Fig. 3. Effect of Mg^{++} concentration on pyrophosphatase activity of brain at 37° . Rat brain suspension with 0.0005M $\text{Na}_4\text{P}_2\text{O}_7$ at pH 7.4.

(2) The activity is optimal when a substrate concentration of the order of 0.0005M is reached: further increase results finally in a fall of activity at 0.0015M .

(3) Enzyme activity is slight in absence of magnesium ions, which give optimal activation at 0.015M .

(4) Maximum activity is in the pH range 6.5–7.4.

It is of interest to compare the above conditions with those previously reported for pyrophosphatases obtained from other sources. Yeast pyrophosphatase shows an optimal concentration of 0.0001M for substrate and 0.002M for magnesium, and a pH optimum 6.9–7.0 (Bailey & Webb, 1944); this pH optimum is also shown by muscle pyrophosphatase (Lohmann, 1928). The erythrocyte enzyme has optimum substrate concentration 0.0005M and magnesium concentration 0.02M , and pH optimum 7.4–8.2 (Naganna & Menon, 1948).

Fig. 4. Effect of pH of medium on pyrophosphatase activity of brain at 37° . Rat brain suspension with 0.0005M substrate. A, without Mg^{++} ; B, with 0.02M Mg^{++} .

Effects of inhibitors

The effects of different enzyme inhibitors on rat brain pyrophosphatase are indicated in Table 1. Substantial inhibition is evidently brought about by calcium ions at an order of concentration similar to that necessary to inhibit erythrocyte pyrophosphatase (Naganna & Menon, 1948).

It is important that the enzyme is sensitive to low concentrations of substances known to oxidize or react with thiol groups, i.e. alloxan, copper sulphate, iodoacetate and mapharside (*m*-amino-*p*-hydroxyphenylarsenoxide hydrochloride); this suggests that thiol groups are necessary for the activity of the enzyme. This conclusion is supported by experiments indicating that the inhibitions can be reversed by excess of cysteine. The inhibition due to alloxan was considerably reduced in presence of cysteine, an effect previously demonstrated for succinic dehydrogenase (Hopkins, Morgan & Lutwak-Mann, 1938). The effect of mapharside, which inhibited strongly at $7.4 \times 10^{-6}\text{M}$, was abolished by cysteine; this arsenic compound showed a similar behaviour towards a number of enzymes known to contain active thiol groups, but not towards certain other enzymes which do not contain such groups (Gordon & Quastel, 1948).

Table 1. *Effects of enzyme inhibitors on rat brain pyrophosphatase*

(Brain suspension incubated with inhibitor in presence of MgCl_2 for 30 min. at 37° before addition of substrate. Final concentration of MgCl_2 0.02 M, of substrate 0.0005 M. Temp. 37° ; pH 7.4.)

Inhibitor	Concentration of inhibitor (M)	Enzyme activity (mmol. substrate/30 min./100 g. tissue)		Inhibition (%)
		Without inhibitor	With inhibitor	
CaCl_2	0.5×10^{-4}	4.88	3.54	27.4
CaCl_2	2.0×10^{-4}	4.88	2.06	57.8
CaCl_2	20.0×10^{-4}	4.88	0.17	96.7
CuSO_4	0.5×10^{-6}	4.31	3.12	27.6
CuSO_4	2.0×10^{-6}	4.31	1.41	67.3
CuSO_4	10.0×10^{-6}	4.31	0.296	93.2
Na iodoacetate	0.1×10^{-3}	3.54	2.48	30.0
Na iodoacetate	2.5×10^{-3}	3.54	1.26	64.4
Alloxan	0.1×10^{-3}	3.50	2.66	24.0
Alloxan	2.0×10^{-3}	3.50	0.39	88.9
Alloxan	0.5×10^{-3}	4.52	0.92	79.6
Alloxan	0.5×10^{-3}	9.26	8.02	13.4
Alloxan + cysteine	1.5×10^{-3}			
Mapharside	0.74×10^{-6}	3.42	2.40	29.9
Mapharside	7.4×10^{-6}	4.52	1.03	77.3
Mapharside	7.4×10^{-6}	5.86	5.84	0.0
Mapharside + cysteine	5×10^{-3}			
Formaldehyde	0.25×10^{-3}	5.00	3.18	36.4
Formaldehyde	2.5×10^{-3}	5.00	2.40	52.0
NaF	0.5×10^{-5}	4.32	3.70	14.3
NaF	2.0×10^{-5}	4.32	2.26	47.7
NaF	5.0×10^{-5}	4.32	1.06	75.5
NaCN	0.4×10^{-2}	3.10	3.44	0
NaCN	1.5×10^{-2}	3.10	1.78	42.6
Na tauroglycocholate	2.4×10^{-3}	3.54	2.90	18.1
Na cholate	0.5×10^{-3}	2.98	3.08	0

Table 2. *Pyrophosphatase activities in brain tissue of various species, compared with brain phosphomonoesterase and erythrocyte pyrophosphatase*

(Activities in mmol. substrate/30 min./100 g. brain tissue or mmol. substrate/30 min./100 ml. whole blood.)

Species	Brain pyrophosphatase (pH 7.4)	Phosphomonoesterase (substrate, Na α -glycerophosphate)		Erythrocyte pyrophosphatase (pH 7.4)	
		Acid enzyme (pH 5.0)	Alkaline enzyme (pH 9.4)	Lysed cell preparation	Acetone powder preparation
Rat	6.22	0.354	0.899	3.58	—
Rabbit	0.260	0.228	1.58	2.59	—
Sheep	2.78	0.300	1.42	0.733	0.0196
Man	1.17	0.138	1.08	3.84	—

The effects of fluoride and cyanide are similar to those noted for the erythrocyte enzyme (Naganna & Menon, 1948): the enzyme is highly sensitive to low concentrations of fluoride, but relatively high concentrations of cyanide are necessary to produce inhibition. Definite toxic effects are also produced by formaldehyde in 2.5×10^{-3} M concentration; this may indicate that the enzyme contains active amino groups (Gould, 1944). Cholate and tauroglycocholate were also tried, since it has previously been found that salts of bile acids may inhibit the alkaline phosphatase of brain (Fleischhacker, 1938). Brain pyrophosphatase, however, is relatively unaffected by these salts at the concentrations used.

Comparison of phosphatases in different species

Results of a series of estimations of the brain pyrophosphatase levels of several species, as compared with the brain phosphomonoesterases and erythrocyte pyrophosphatases, are shown in Table 2. Erythrocyte pyrophosphatase is expressed in terms of activity per 100 ml. whole blood. The activity of acetone-dried sheep erythrocyte preparation was also estimated, and it will be seen that a strong suspension (20 mg./ml.) of this powder did not exhibit any marked effect; the activity was apparently destroyed by the acetone treatment.

Values of brain pyrophosphatase activities in the different species, both absolutely and relatively to the other phosphatases, showed wide variations. Generally, however, the levels of pyrophosphatase were higher than those of the phosphomonoesterases, except with the rabbit. A strict comparison between pyrophosphatase levels of brain tissue and of erythrocytes was not possible, but values of approximately the same order were found, except with the rabbit, where the erythrocyte enzyme was clearly present in considerably greater strength than in the brain.

DISCUSSION

The present work suggests that the pyrophosphatase of brain tissue resembles the pyrophosphatases of yeast and erythrocytes in many respects, especially in the effects of inhibitors, and to some extent in the factors of magnesium activation and optimal pH range, in spite of certain small differences. The difference between the brain and erythrocyte enzymes is, however, evident from the fact that the former can be isolated by acetone precipitation, while such treatment destroys the erythrocyte activity.

The effects of inhibitors show that the brain pyrophosphatase contains active thiol groups within its structure, and possibly active amino groups also.

It is important to consider the possible physiological role played by pyrophosphatase in brain function. No deductions regarding its relative importance may be drawn from the values given in Table 2, as these vary widely. It is conceivable,

however, that the enzyme may exercise some function in connexion with phosphorylation processes. Cori (1942) has mentioned experiments in which inorganic pyrophosphate was detected in rat-liver preparations after aerobic oxidation of glutamate, pyruvate and succinate, while Cross, Taggart, Covo & Green (1949) have deduced that inorganic pyrophosphate is formed as a decomposition product in the phosphorylation reactions taking place under the influence of the cyclophorase system. It remains to be seen, however, what part is played by pyrophosphatase itself in the chain of reactions governing phosphorylation mechanisms.

SUMMARY

1. A description is given of the properties of the magnesium-activated pyrophosphatase in preparations from brain tissue.

2. The enzyme is strongly inhibited by various substances which react with thiol groups, and by calcium ions, formaldehyde and fluoride. It is also inhibited to some extent by cyanide, but is relatively unaffected by cholate and tauroglycocholate.

3. Activities of brain pyrophosphatase in several species are compared with those of the brain phosphomonoesterases and with corresponding erythrocyte pyrophosphatases.

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REFERENCES

- Bailey, K. & Webb, E. C. (1944). *Biochem. J.* **38**, 394.
Cori, C. F. (1942). *Symposium on Respiratory Enzymes*, p. 175. Madison, U.S.A.: University of Wisconsin Press.
Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Fleischacker, H. H. (1938). *J. ment. Sci.* **84**, 947.
Giri, K. V. & Datta, N. C. (1936). *Biochem. J.* **30**, 1089.
Gordon, J. J. (1949). *Nature, Lond.*, **164**, 579.
Gordon, J. J. & Quastel, J. H. (1948). *Biochem. J.* **42**, 337.
Gould, B. S. (1944). *J. biol. Chem.* **156**, 365.
Hopkins, F. G., Morgan, E. J. & Lutwak-Mann, C. (1938). *Biochem. J.* **32**, 1829.
Lohmann, K. (1928). *Biochem. Z.* **203**, 172.
Naganna, B. & Menon, V. K. N. (1948). *J. biol. Chem.* **174**, 501.

Are Phospholipins Transmitted through the Placenta?

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It was shown in a previous investigation (Popják, 1947), with the aid of ^{32}P , that all foetal tissues in the rat, rabbit and guinea pig synthesize phospholipins. The results suggested that most, if not all, the foetal phospholipins are obtained by synthesis within the foetus. The possibility, however, that a small amount of phospholipin preformed in the mother might pass through the placenta could not be ex-

cluded. The experiments reported here were designed to probe this question. It will be shown that, although the foetal placenta takes up large amounts of phospholipins from the maternal circulation, it does not transmit to the body of the foetus detectable amounts of phospholipins. A preliminary account of this work has been given to the Biochemical Society (Popják & Beeckmans, 1949).