The Dialysis of Brain Pyrophosphatase

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It was previously reported (Gordon, 1953) that when rat-brain homogenates were dialysed against distilled water at 0° for 18 hr., about 90 % of their Mg^{2+} ion-activated pyrophosphatase activity was lost. The activity could be partly restored by addition of cysteine, which also had a slight activating effect upon the fresh, undialysed enzyme. This phenomenon does not appear to have been encountered with yeast pyrophosphatase, as the methods used in its purification have included dialysis (Bailey & Webb, 1944; Heppel & Hilmo, 1951). The nature of the dialysis effect in brain pyrophosphatase was of some interest, for during attempts to purify the enzyme by methods involving fractional precipitation, it became desirable to remove ammonium sulphate by dialysis; this could not be done without practically complete destruction of the enzyme in every case. There appeared at first to be some possibility that the inactivation could be due to the loss of a diffusible cofactor (in addition to magnesium). Further work, however, led to the conclusion that the effect was due to the extreme lability of the enzyme under mildly acid conditions, the fall in pH at the membrane of the dialysis sac being apparently sufficient to cause the observed changes. The experiments which led to this conclusion are described in this paper.

EXPERIMENTAL

Enzyme preparation. Aqueous homogenates of rat brain were made by homogenizing the whole brain with ice-cold 0.9 % NaCl (2-3 ml.) in a Potter & Elvehjem (1936) apparatus, and diluting with water to approximately 10 mg./ml.

Activity measurements. These were carried out at 37° as previously described for fresh rat-brain homogenates (Gordon, 1953). Each estimation was carried out on at least two tissue concentrations (1.25-2.5 mg./ml.) at pH 7.4 in veronal buffer containing 0.008 M- $MgCl_2$. The total volume of the reaction mixture was always 4 ml., including the substrate (0.001 M sodium pyrophosphate). Appropriate blanks showed that none of the substances added to the reaction mixtures interfered with the Fiske-Subbarow phosphate estimation except 2,3-dimercaptopropanol (BAL). This substance was, however, readily removed by adding 1 ml. of $CuSO_4$ solution (1 %, w/v) with the trichloroacetic acid used as protein precipitant. The BAL was

thereby precipitated as a copper complex and was removed together with the protein on filtration; control phosphate estimations then gave completely satisfactory results. Activities are expressed as m-moles of substrate/30 min./100 g. of tissue.

Dialysis against water. About 15 ml. of brain homogenate was placed in a Visking cellophane tube and dialysed against distilled water (6 l.) at 0° for 18 hr.

Dialysis against mixed resins. This was carried out as described by Gilbert & Swallow (1950), except that the experiments were done at 0°, and the dialysis time was 18 hr. The resins used consisted of Amberlite IR-100 (H^+ form) and Amberlite IR-4B (OH^- form).

RESULTS

As previously found (Gordon, 1953), when brain homogenates were dialysed at 0° against distilled water for 18 hr., at least 90 % of the Mg^{2+} ion-activated pyrophosphatase activity was lost, whereas storage at 0° for the same time resulted in only 15 % loss. Similar results were obtained when erythrocyte pyrophosphatase (a 1:100 dilution of washed, lysed rat erythrocytes) was subjected to the same treatment.

Activation by thiol compounds

It was confirmed that the addition of cysteine to the dialysed enzyme caused appreciable reactivation and was able also to cause slight activation of the undialysed enzyme. Two other thiol compounds, glutathione and BAL, showed similar effects. The relative efficiencies of the three thiol compounds are indicated in Table 1. The optimum reactivating concentrations differed greatly for the three compounds. BAL showed its maximum effect at a concentration approximately one-tenth of that of cysteine or glutathione, though the extent of reactivation was appreciably less than for cysteine. At optimum concentration, however, cysteine was the most effective in restoring activity, but still only reactivated the dialysed enzyme to the extent of about 42 %.

Failure of various other factors to activate the dialysed enzyme

The dialysed enzyme was completely uninfluenced by various amino acids which do not contain thiol groups, e.g. DL-alanine, L-glutamic acid, DL-aspartic acid, L-tyrosine, DL-tryptophan, or

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Table 1. *Activation of dialysed and undialysed brain pyrophosphatase by thiol compounds*

Activity of control = 31.0 m-moles/30 min./100 g.

Expt.	Enzyme	Thiol compound	Concn. (M)	Activity (% of control)
1	Undialysed	Cysteine	0.010	118.0
	Dialysed	Nil	—	12.9
	Dialysed	Cysteine	0.003	37.8
	Dialysed	Cysteine	0.006	49.6
	Dialysed	Cysteine	0.010	49.6
2	Undialysed	Glutathione	0.005	106.0
	Dialysed	Nil	—	10.7
	Dialysed	Glutathione	0.003	28.2
	Dialysed	Glutathione	0.005	28.2
3	Undialysed	BAL	0.0015	107.0
	Dialysed	Nil	—	10.3
	Dialysed	BAL	0.0005	25.7
	Dialysed	BAL	0.0015	25.7

DL-methionine, at concentrations similar to those used for the thiol compounds. Negative results were also obtained on adding either a concentrated form of the diffusate, or some enzyme preparation which had been inactivated by heating at 100° for 15 min.

The effects of FeSO_4 , ZnSO_4 and MnSO_4 (0.04×10^{-6} to 0.1×10^{-6} M) were also tested, since it was reported by Pett & Wynne (1933, 1934) that bacterial pyrophosphatases were appreciably activated by low concentrations of Zn^{2+} and Fe^{2+} ions. King, Wood & Delory (1945) reported activation of erythrocyte acid phosphatase by Mn^{2+} ions, whereas Hove, Elvehjem & Hart (1940) found that purified alkaline phosphatase could be activated by low concentrations of Zn^{2+} or Mn^{2+} ions in combination with certain amino acids. In the present work on brain pyrophosphatase, completely negative results were obtained with Fe^{2+} , Mn^{2+} and Zn^{2+} ions, either alone or in combination with α -amino acids.

Since it appeared possible that the reactivating effect of thiol compounds was due to their reducing properties, those reducing agents used by Purr (1935) to reactivate papain after oxidative inactivation, i.e. NaCN (0.004 M), ascorbic acid (0.15 M) combined with FeSO_4 (4×10^{-3} M), and sodium thiosulphate (0.004 M), were examined. (It was confirmed that at these concentrations the substances did not inhibit the undialysed enzyme.) No reactivation of the dialysed enzyme was observed in any case. The above results made it seem unlikely that the dialysis effect could be due to the loss of a diffusible cofactor, and some other explanation of the phenomenon was sought. An experiment was carried out in which the enzyme was stored, but not dialysed, at 0° for 18 hr. in a cellophan sac. Under these conditions the enzyme lost only 30 % activity. Though this is greater than the loss which normally occurs on storage in the usual way at 0°, it was evident that the almost complete inactivation which

takes place on dialysis is dependent on the actual process of dialysis, and not merely on the presence of the membrane; the inactivation could not, for instance, be due to the adsorption of a cofactor on the surface of the sac.

Lablity of the enzyme under mildly acid conditions

The factors influencing the stability of the enzyme were next considered. Pillai (1938) concluded that pyrophosphatase in muscle is a very labile enzyme. Brain pyrophosphatase in acetone-dried powders is quite stable at 0° for several weeks (Gordon, 1950), but in aqueous dispersions of fresh tissue it is slightly inactivated (up to 15 %) on standing at 0° for 24 hr. Naganna (1950) found that erythrocyte pyrophosphatase was rapidly inactivated on incubation at 38° with acetate-veronal buffer at neutral pH. It seemed worth while to investigate the effect of pH on the stability of the enzyme at 0°, especially as a fall in pH may be expected during dialysis (Gilbert & Swallow, 1950).

Samples of a fresh enzyme preparation were adjusted to various pH values by adding 0.02 N-HCl, and allowed to stand at 0° for 18 hr. No buffering substances were added, but the pH was determined at the end of the storage period, and no perceptible change was found. After re-adjusting to pH 7.4 with dilute NaOH, the activities were measured as usual. (It was confirmed that NaCl in low concentrations did not inhibit the untreated enzyme.) The results showed that the enzyme was extremely labile in the presence of acid, losing 43 % of its activity at pH 5.9 and 73.6 % at pH 5.4. At pH 4.9, the enzyme was 85.3 % inactivated (comparable with that produced by dialysis). Similar experiments were carried out in which cysteine (0.01 M) was added to the enzyme after storage, and before proceeding with the activity

Table 2. *Effect of cysteine on brain pyrophosphatase after exposure to acid*

Control activity without cysteine = 18.7; with cysteine = 23.9 m-moles of substrate/30 min./100 g.

pH	Inactivation (% of control activity)	
	Without cysteine	With 0.01 M cysteine
7.4 (Control)	0	0
5.9	41.2	5.5
5.4	64.7	10.9
4.9	76.0	29.6

measurements; these results are given in Table 2. Cysteine appreciably reactivated the enzyme, as in the dialysis experiments.

The above results were consistent with the possibility that the dialysis effect could be due to changes in the pH of the homogenate during dialysis. An attempt was made to stabilize the pH by using 0.05 M acetate-veronal buffer (pH 7.4) instead of water as the dialysis medium, but under these conditions the enzyme was, as usual, inactivated to the extent of 90 %. The pH of a brain homogenate, dialysed in the usual way against distilled water, was tested before and after dialysis, but the pH fall was found to be only slight: from about 7.3 to 7.1. These results seemed surprising, but in view of the high acid-sensitivity of the enzyme it was still thought possible that more pronounced temporary changes of pH at the membrane during dialysis could be sufficient to inactivate the enzyme. To clarify this point, experiments were carried out in which the enzyme was dialysed against ion-exchange resins.

Dialysis against ion-exchange resins

The use of ion-exchange resins has been investigated by Gilbert & Swallow (1950), who were investigating methods of freeing protein and enzyme solutions from electrolytes by dialysis without denaturation of protein and inactivation of enzyme. They found that a urease solution, when dialysed through a cellophan sac against mixed resins, was inactivated; this was ascribed to a fall in pH on the membrane. On further investigation, these authors were able, with the mixed resins, to devise three methods of protecting the system against a fall in pH. In the present work it was thought that interesting data would be produced by the use of the Gilbert & Swallow (1950) techniques of dialysis. Experiments were carried out under the conditions described by these authors, with the slight modifications already referred to in the Experimental section.

The results of these experiments confirmed the conclusions already drawn, that the loss of enzymic activity on dialysis is due to a fall in pH value on the

membrane. When the enzyme was dialysed against the mixed resins, with no precautions taken to prevent a fall in pH, it was inactivated to the extent of 92.5 %, as in ordinary dialysis. Gilbert & Swallow (1950) described three methods by which the fall in pH could be prevented, and each of these methods was applied. When the membrane was pretreated with *m*-phenylenediamine resin, only 33.5 % of the activity was lost. When the mixed resin system was pretreated with NaOH, only 3 % of the enzyme activity was lost, and when a layer of anion-exchange resin was interposed between the resin mixture and the membrane, there was 18.1 % inactivation. Each of these methods of preventing a fall in pH, therefore, gave also a substantial measure of protection against inactivation of the enzyme. The least protection was given by the resin-treated membrane; this confirms the observations of Gilbert & Swallow (1950), who indicated that this method was the least effective.

DISCUSSION

It is evident from the results described that dialysis effects are due to an actual denaturation of the enzyme, resulting from its extreme instability under slightly acid conditions. This ease of inactivation by acid may have some bearing on evidence already reported (Gordon, 1950) that active thiol groups are probably present in the enzyme. The essential thiol groups would be susceptible to spontaneous oxidation under various conditions; this apparently takes place very slowly even at 0° in neutral solution, and an acceleration of the process may be brought about by alteration in the pH. The partial reversal of the effect by thiol compounds is in agreement with such a mechanism, though it is difficult at present to see why the other reducing agents tried did not have some effect too. However, the results on the whole are consistent with the previous conclusion that brain pyrophosphatase probably has active thiol groups in its structure.

The observations reported here are also of interest with reference to the important problem of purifying the enzyme. Repeated attempts at purification have so far met with only slight success, and the readiness with which inactivation can take place imposes serious limitations on the kind of processes which can be applied. The stabilization of pH, and probably the necessity for operating under ice-cold conditions, are two important factors to be taken into account in devising suitable methods for the purification of the enzyme, the achievement of which will be necessary in order both to investigate more conclusively the chemical nature of this interesting system, and to attempt to elucidate its role in the metabolism and function of the brain.

SUMMARY

1. Brain pyrophosphatase is readily inactivated on standing at 0° in slightly acid solution.

2. The fall in activity which takes place when the enzyme is dialysed is not observed when the dialysis is carried out under conditions in which the pH is prevented from falling.

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Potassium Uptake by *Alcaligenes faecalis*

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In experiments on the metabolism of the bacterium *Alcaligenes faecalis* it was noted that the capacity to respire is much reduced, often to almost zero, on washing the cells with distilled water, but is restored on addition of yeast extracts. The essential component of yeast extracts was found to be potassium. This organism differs from many others in that it can lose its intracellular potassium readily to the medium if the potassium concentration of the latter is low. The cells can recover potassium if its concentration in the medium is raised. This recovery is in part due to active transport, depending on respiration, but appreciable amounts of potassium can be taken up against concentration gradients without a supply of energy. This paper is concerned with a study of the potassium distribution between cells and medium under a variety of experimental conditions.

EXPERIMENTAL

Growth conditions. The basal medium contained 1% (w/v) of Difco peptone, 0.5% of Difco yeast extract, 1% (w/v) of casein hydrolysate (Pronutrin or Tryptone), and 1% (w/v) of glucose. In some of the later experiments glucose was omitted when it had been found that it was not oxidized. The pH was adjusted with NaOH to 7.0. The inoculum consisted of a 3–5 ml. liquid subculture of the same medium. Roux bottles containing 100 ml. of medium and 2% of agar were incubated at 30° for about 18 hr. The cells were washed on the centrifuge with three portions of water. After washing, the cells from one Roux bottle were suspended in 15 ml. of water and stored at +2°. This suspension con-

tained generally 11–12 mg. of dry cells/ml. In the later experiments agar was omitted to avoid contamination of cell suspension with pieces of agar gel, and in order to reduce the path of diffusion of oxygen 50 ml. of medium was used/Roux bottle.

Organisms. Three strains of *A. faecalis* were examined. They were: strains 415 and 1347 of the National Collection of Type Cultures; and strain 19 obtained from the Department of Bacteriology, University of Sheffield. The organism is a Gram-negative rod incapable of fermenting sugars. Unless otherwise stated strain 1347 was used.

Ionic distribution. The distribution of potassium and sodium between medium and cells was determined as follows. At the end of the incubation period the suspension was thoroughly shaken and 3 or 5 ml. of the suspension was transferred to a weighed centrifuge tube of 6 ml. capacity. If the conditions of incubation were aerobic the suspension was gassed for 10 min. with oxygen to prevent anaerobic conditions in subsequent manipulations and the suspension was then spun for 10 min. in a refrigerated centrifuge at 20 000 g. As much as possible of the supernatant was removed with pipettes or by decanting and collected for analysis. The amount of cell residue was ascertained by reweighing the centrifuge tubes with the residue. The cell volume was calculated on the assumption (based on measurements) that the specific gravity of the residue was 1.017–27°. The specific gravity of the standard medium was 1.011. Examples of the calculations are contained in Table 3.

For analysis by flame photometry the supernatant was diluted usually to 10 ml., but less if the amounts of potassium or sodium were small. The residue was mixed with 0.05 ml. of conc. HNO₃ and placed for 30 min. in a boiling-water bath. This resulted in a clear solution which was diluted to a known volume, usually 10 ml. The diluting material