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The Activation of Tryptophanase Apo-enzyme by Potassium, Ammonium and Rubidium Ions

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Pyridoxal phosphate is the coenzyme to the tryptophanase apo-enzyme (Wood, Gunsalus & Umbreit, 1947; Dawes, Dawson & Happold, 1947), but its addition to dialysed enzyme preparations only effects a partial reactivation of the apo-enzyme, and this capacity to reactivate decreases as dialysis proceeds, as shown in the present communication. In previous studies in these laboratories we observed an apparent activation of tryptophanase by treatment with certain antisera other than anti-tryptophanase and then observed that this activating effect was shared with normal rabbit sera (Dolby, Hall & Happold, 1951); this activating effect was not destroyed by heating the serum to 100° and was not due to any deficiency of pyridoxal phosphate. Two possibilities have been investigated in the present studies: (1) that removal of the coenzyme by dialysis allowed an irreversible inactivation of the apo-enzyme to occur, (2) that the enzyme complex requires other dialysable activators besides pyridoxal phosphate to complete the system. We have observed that ammonium, potassium and rubidium ions activate dialysed cell-free preparations of tryptophanase prepared from *Escherichia coli*.

EXPERIMENTAL

The enzyme preparations used were obtained from acetone-dried cells of *Esch. coli* which had been grown for 18 hr. at 37° on casein digest agar medium (Dawes *et al.* 1947). The dried cells were extracted overnight at 37° with a solution of 0·8 M-KCl (40 mg. dried cells/ml. solvent). The activity of the enzyme was determined by measuring the initial rate of indole formation in a total volume of 2·5 ml. comprised of 0·5 ml. enzyme preparation, 1 ml. 0·1 M sodium phosphate buffer (pH 7·8), 2 mg. L-tryptophan and to which was added distilled water with or without different ions and with or without 10 μg. pyridoxal phosphate. When conditions have been varied the variation is stated. The enzymic activity of the preparations was determined by the method of Gooder & Happold (1954) and is expressed in terms of the indole produced in the above system during the initial 7 min. at 37°. The reaction was stopped by the addition of two drops of 40% formaldehyde instead of the trichloroacetic acid previously used. The cations tested were added as chlorides (0·5 ml. solution); the final concentration of the cation was therefore one-fifth of that added. Enzyme preparations were dialysed in cellophan bags rotated at an angle of 45° in the dialysing liquid; an air bubble and a few glass beads were provided in the bag to increase the circulation of its contents (Spiegelman, Reiner & Morgan, 1947). As dialysis of these preparations at room temperature resulted in rapid inactivation of the enzyme, this procedure was carried through at 2°.

RESULTS

Dialysis of the enzyme against distilled water. The results presented in Fig. 1 shows that the rate of inactivation of the enzyme increased progressively as the period of dialysis was prolonged. Inactivation was complete in 3 hr. and in some experiments much sooner. After 45 min. dialysis, complete activity was restored by the addition of pyridoxal phosphate; after 90 min., 80% of the original activity could be recovered, after 3 hr. a mere trace and after 4 hr. no reactivation occurred. The results presented in Fig. 2 show that 45 min. dialysis resulted in a loss of 84% of the original activity, and the addition of pyridoxal phosphate only raised the activity to 29% of the initial value. These results show that there is some factor causing loss of tryptophanase activity other than the loss of pyridoxal phosphate; moreover, additions of pyridoxal phosphate to the dialysed enzyme over the basic amount did not increase the activity.

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Dialysis of the enzyme against salt solutions. When enzyme preparations (in 0·8M-KCl) were dialysed against 0·01M sodium phosphate (pH 7·6) containing 0·25M-NaCl, rapid inactivation occurred; but when 0·25M-KCl was substituted for NaCl, the activity increased by 20% after 30 min. dialysis and even after 15 hr. 90% of the initial activity still remained in the preparation. This increase in activity which has been frequently observed in the early stages of dialysis against solutions of 0·25M-KCl might be due to the fact that 0·8M- and 0·25M-KCl are on opposite sides of the optimum salt concentration for maximum enzymic activity. Additional experiments demonstrated that dialysis against stronger solutions of KCl (up to 1·25M) resulted not only in increased tryptophanase activity but in a stabilization of this heightened activity over a period of days, whereas complete inactivation occurred with 1·25M-NaCl in 4 hr. A tryptophanase preparation (in 0·8M-KCl) remained stable for 41 hr. and retained 60% of its original activity after 83 hr. (Table 1). A 25 ml. sample of the same preparation dialysed against 4 l. of 0·8M-KCl, which was changed daily, showed no loss of activity after 19 hr.; after 41 hr. it retained 88% of its original activity, which was raised to 96% when 10 μg. pyridoxal phosphate was added to the system. Progressive decay of the enzyme system then occurred with increasing time of dialysis, this decay being perhaps slightly reduced by the addition of pyridoxal phosphate.

The effect of the addition of potassium ions and pyridoxal phosphate to the dialysed enzyme. An enzyme preparation (40 ml.) was dialysed against 4 l. of distilled water (final K^+ concentration approx. 0·008M). Samples were removed from the dialysis sac at intervals during a 3 hr. period and their activities compared with the original enzyme

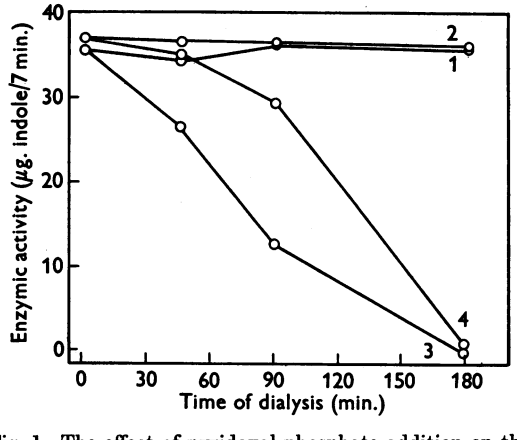

Fig. 1. The effect of pyridoxal phosphate addition on the activity of dialysed and undialysed tryptophanase. 1 ml. of 0·1M sodium phosphate buffer (pH 7·8); 0·5 ml. enzyme; 2 mg. L-tryptophan; water to 2·5 ml. 1, original enzyme; 2, original enzyme + 10 μg. pyridoxal phosphate; 3, dialysed enzyme; 4, dialysed enzyme + 10 μg. pyridoxal phosphate.

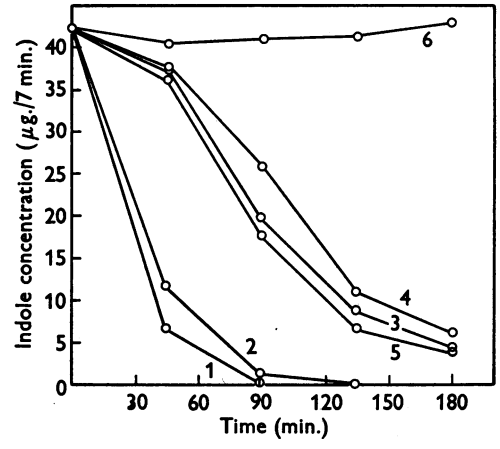

Fig. 2. The effect of the addition of pyridoxal phosphate and potassium chloride singly and combined and of concentrated dialysate to dialysed tryptophanase. 1 ml. of 0·1M sodium phosphate buffer (pH 7·8); 0·5 ml. enzyme; 2 mg. L-tryptophan; water and other additions to 2·5 ml. 1, dialysed enzyme; 2, dialysed enzyme + 10 μg. pyridoxal phosphate; 3, dialysed enzyme + KCl to 0·166M; 4, dialysed enzyme + pyridoxal phosphate + KCl combined; 5, dialysed enzyme + equivalent of concentrated dialysate; 6, original undialysed enzyme.

Table 1. *Effect of dialysis against* 0·8M *potassium chloride on activity of tryptophanase*

1 ml. 0·1M sodium phosphate buffer (pH 7·8); 0·5 ml. enzyme preparation; 2 mg. L-tryptophan; water to 2·5 ml.; pyridoxal phosphate, 10 μg.

Age of enzyme or period of dialysis (hr.)	Activity of enzyme (μg. indole/7 min.)			
	Original enzyme		Dialysed enzyme	
	Alone	+ Pyridoxal phosphate	Alone	+ Pyridoxal phosphate
0	14·1	—	—	—
17	14·1	14·1	14·1	14·1
41	14·1	14·1	12·45	13·5
83	8·25	8·5	6·10	6·5
179	—	—	3·65	4·0

Table 2. *Effect of the addition of monovalent cations and magnesium on the activity of dialysed tryptophanase*

1 ml. of 0·1 M sodium phosphate buffer (pH 7·8); 0·5 ml. dialysed enzyme; 2 mg. L-tryptophan; 10 μg. pyridoxal phosphate; 0·5 ml. 0·1 M appropriate chloride (except $MgCl_2$ which was 0·02 M); water to 2·5 ml. In the controls 0·5 ml. water was substituted for the added chloride.

Dialysed enzyme (preparation no.)	Activity of system (μg. indole produced in 7 min.)							
	Control	NaCl	KCl	RbCl	LiCl	CsCl	NH_4Cl	$MgCl_2$
1	9·4	7·3	22·1	19·1	8·6	—	—	6·6
2	Trace	Trace	5·0	2·5	Trace	Trace	6·2	—
3	3·5	3·5	10·8	8·5	—	3·5	21·0	—

preparation. Tests were made with the following additions to the dialysed enzyme: (1) pyridoxal phosphate (10 μg.); (2) KCl (to its initial concentration in the undialysed enzyme); (3) pyridoxal phosphate and KCl together; (4) no additions; (5) concentrated dialysate. The last series was included to see whether any factor other than pyridoxal phosphate which might influence enzymic activity was removed by dialysis. The results are recorded in Fig. 2. They show a slight restoration of the activity of the dialysed enzyme when pyridoxal phosphate is added, a much greater one by the addition of K^+ and a small further increase when K^+ and pyridoxal phosphate are added jointly. In this experiment, whilst the rate of inactivation on dialysis is more rapid than that shown in Fig. 1, the loss in activity is less dependent upon loss of pyridoxal phosphate. The final concentration of K^+ in the experiment recorded in Fig. 1 was not determined but was almost certainly less than the concentration (0·008 M) in the present experiment. The addition of concentrated dialysate produced an effect indistinguishable from that obtained with K ions alone, suggesting that no additional cofactor is removed on dialysis. The degree of re-activation with added K^+, which was approximately 50% after 90 min., drops rapidly as dialysis proceeds.

The effect of addition of other cations on the re-activation of dialysed tryptophan. In early studies we had observed that tryptophanase preparations precipitated with $(NH_4)_2SO_4$ retained their activity for long periods until the salt was removed, and it therefore seemed desirable to us to test the action of other monovalent cations. Enzyme preparations were consequently dialysed for 2 hr. at 5° against water and 1 ml. samples tested for their initial activity rates in the presence of sodium phosphate buffer (pH 7·0), pyridoxal phosphate and various cations added as chlorides. The results (Table 2) make it clear that ability of ions to reactivate the dialysed enzyme follows the order $NH_4^+ > K^+ > Rb^+$, while Na, Li and Mg ions appear to inhibit slightly. Eight series of experiments were next made to determine the range of concentration at which K, NH_4 and Rb ions were effective in the activation of dialysed enzyme. When the final concentration of KCl was 0·001 M or less no activation occurred, but

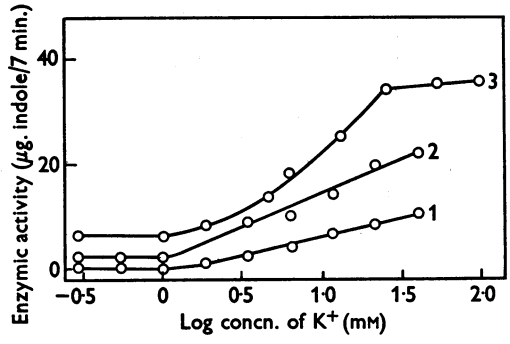

Fig. 3. The effect of K^+ on the activity of dialysed enzyme in the absence and in the presence of pyridoxal phosphate. 1 ml. of 0·1 M sodium phosphate buffer (pH 7·8), 0·5 ml. dialysed enzyme, K^+ as KCl to appropriate concentration and water to 2·5 ml. Curves 1 and 2 are for the same enzyme preparation. Curves 2 and 3 have had 10 μg. pyridoxal phosphate added to each tube.

above this value the activity increased steadily with concentration up to 0·07 M and there was no diminution of this heightened activity up to a final concentration of 0·4 M in the complete enzyme–substrate system. The NH_4^+ and Rb^+ activating effect was also absent below 0·001 M and reached an optimum around 0·06 M, that is, the range of concentration effective for reactivation was similar with all these ions. The NH_4 ions were the most powerful activators and the Rb ions the least. The effect of increasing concentrations of K ions on the activity of dialysed tryptophanase is shown in Fig. 3.

DISCUSSION

It is shown that pyridoxal phosphate is not the only factor responsible for the reactivation of dialysed tryptophanase and that substantial activation is achieved with NH_4, K and Rb ions, whereas Na, Li and Mg ions may increase enzyme degradation. Activation commences at 0·001 M and reaches its optimum at 0·07 M, this activity range being almost identical with that recorded by Rothstein & Demis (1953) for the stimulation of yeast fermentation by different concentrations of K^+. These results are consistent with the view that K ions play an im-

portant part in the structure of the holo-enzyme, binding the coenzyme to the apo-enzyme. This composite unit is stable. But if K ions are removed, the complex dissociates and coenzyme may be lost by dialysis. Restoration of K^+ and coenzyme can lead to reformation of the complete active holo-enzyme if added without undue delay. But the free apo-enzyme appears to be unstable and undergoes some irreversible change, so that after 3 hr. or so it can no longer be reformed into active holo-enzyme by K ions and coenzyme.

The studies on the effect of K ions on carbohydrate fermentation and storage in yeast by Pulver & Verzár (1940) caused Evans, Handley & Happold (1942) to study the effect of K ions upon the rate of development of tryptophanase activity in washed cells of *Esch. coli*, which had been grown on glucose–bouillon agar and then suspended in solutions of tryptophan buffered with phosphate. Such cells only produced indole after a period of 1–3 hr., but this period could be greatly reduced and the rate of indole production thereafter increased by increasing the concentration of K ions; these effects were reversed when Na ions were substituted for the K ions. We now know from the more effective bacterial crushing obtained through the Hughes press (Hughes, 1951) that tryptophanase is present in cells grown in glucose–casein-digest medium or glucose–bouillon, although they do not produce indole immediately. This failure may be due to a permeability effect whereby the exogenous tryptophan does not enter the cell, for the tryptophan content of glucose–bouillon does not change during growth so long as fermentable carbohydrate is present (Happold & Hoyle, 1936). Alternatively, if K ions are preferentially utilized in the fermentation of glucose they will not be available for the activation of the tryptophanase system. The utilization of the available pyridoxal phosphate by systems such as the amino acid decarboxylases is a further possibility. These alternatives are being investigated and it is not unlikely that the two latter possibilities may play a joint part which results in the exogenous tryptophan not being utilized. Von Korff (1953) has shown that K, NH_4 and Rb ions activate the acetate-activating enzyme obtained from animal tissues. Similar effects have been noticed for K ions by Nachmansohn & John (1945) for the choline-acetylating system, by Kachmar & Boyer (1953) for pyruvate phosphopherase, by Stadtman (1952) for phosphotransacetylase and by Black (1951) for aldehyde dehydrogenase.

SUMMARY

1. Apotryptophanase is activated by ammonium, potassium and rubidium ions in the presence of pyridoxal phosphate. Activation occurs at concentrations between 0·001 M and 0·07 M.

2. The dissociation of pyridoxal phosphate from the tryptophanase complex occurs only slowly if it is dialysed against 0·25 M potassium chloride, but is rapid if dialysis is made against distilled water or 0·25 M sodium chloride. Potassium chloride and ammonium sulphate exert a stabilizing action on tryptophanase.

3. It is suggested that potassium, ammonium and rubidium ions play an important part in the structure of the holo-enzyme.

4. The known effect of potassium ions on the inhibition of indole production by *Escherichia coli* grown on glucose–bouillon is discussed.

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