INHIBITION OF COBAMIDE COENZYME ACTIVITY BY INTRINSIC FACTOR*

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A spectrophotometric assay for cobamide coenzymes has been described (Barker et. al., 1960a) which is based upon their catalytic activity in accelerating the glutamate isomerase reaction, the conversion of glutamate to β-methylaspartate. The latter compound is then converted by an excess of the enzyme β-methylaspartase to mesaconate which absorbs light strongly at 240 mμ. Intrinsic factor (IF) preparations have been found to inhibit the conversion of glutamate to mesaconate, presumably by combining with the added coenzyme and decreasing its effective concentration. The degree of inhibition with a given concentration of IF preparation has been found to depend greatly upon the specific coenzyme analog used. A much greater percentage inhibition is obtained with the benzimidazolyl cobamide (BC) coenzyme (Barker et. al., 1960b) in rate limiting concentration than with the 5,6-dimethylbenzimidazole analog (DBC coenzyme, coenzyme B_{12}). This difference is probably attributable to the fact that the affinity of the BC coenzyme for the apoenzyme system is about 80 times greater than that of coenzyme B_{12} and consequently the concentration of BC coenzyme that permits a convenient reaction rate is much lower. At a lower absolute coenzyme concentration, a higher percentage of the coenzyme is presumably bound by a given concentration of IF preparation, and consequently the percentage inhibition is correspondingly greater.

The assumption that inhibition is caused by the binding of coenzyme is supported by two observations: (a) the percentage inhibition is much less with the DBC coenzyme than with the BC coenzyme and (b) the inhibition by IF

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preparations can be completely reversed by increasing the concentration of coenzyme.

The inhibition tests were done under the conditions previously described for the spectrophotometric coenzyme assay (Barker et. al., 1960a) except that the BC coenzyme was added at a concentration of 1.7×10^{-7} M. With the enzyme preparations used in these tests, this coenzyme concentration permitted a rate of absorbancy change of 0.05 to 0.08 units per minute at 240 mμ in the absence of inhibitor. The reaction was generally started by addition of enzyme after mixing the other components of the assay system. However, essentially the same results were obtained when the reaction was started by addition of coenzyme indicating that preincubation of coenzyme and inhibitor was not a significant factor. In order to obtain a measure of inhibitor activity, the concentration of each IF preparation required to cause 50% inhibition was determined. This was done by using two or three inhibitor concentrations that gave rates between 25% and 80% of that of the control. By interpolation the concentration of inhibitor causing 50% inhibition could be estimated generally with an accuracy of $\pm$ 10%. The inhibitor concentration was expressed in μg. protein per ml. The reciprocal of this concentration, multiplied by 100, was used as a measure of the specific inhibitor activity of an IF preparation. Protein was determined by the method of Lowry et. al. (1951) using human blood serum as a standard.

All of the assays reported were done with a single enzyme preparation and the results are directly comparable. A few assays done with another enzyme preparation gave somewhat different absolute values for the amount of inhibitor causing 50% inhibition. This is not surprising since the enzyme preparations we have used are only slightly fractionated extracts and undoubtedly vary in their properties. Until more highly purified enzyme preparations are available, the assay conditions must be carefully standardized to give comparable results.

The IF preparations were assayed by the slight modification (Ellenbogen and Williams, 1958) of the urinary excretion test of Schilling (1953). The stated activities are at the level which the urinary excretion test gave values comparable to a standard IF preparation.

Fig. 1 shows the influence of the concentration of three IF preparations upon the rate of the isomerase reaction. It can be seen that the decrease in rate is roughly proportional to the concentration of inhibitor. This relation facilitates the estimation of the concentration for 50% inhibition.

Table 1 gives a comparison of eight IF preparations with respect to their clinical activity and specific activity as inhibitors of the glutamate isomerase reaction. Considering the difficulty of accurately measuring IF activity, the correlation is reasonably good except for preparation 16. This latter preparation is very crude and the binding of this sample by vitamin B_{12} is almost nil.

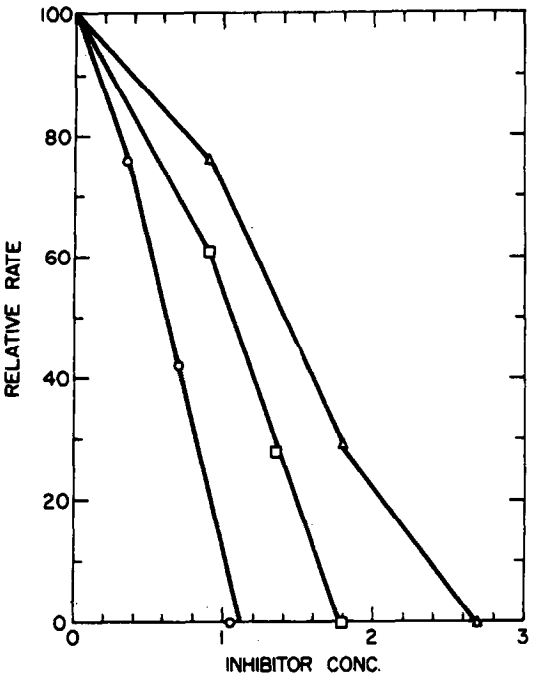

Fig. 1. Influence of the concentration of IF preparation on the rate of the glutamate isomerase reaction. The abscissa gives the concentration of IF preparation; each unit represents 100 µg. of protein per ml. The ordinate gives the rate expressed in percent of the uninhibited control. IF preparation 1 (Table 1), ⊙ ; prep. 2, △ ; prep. 5, □ .

Table 1

Comparison of Inhibitor and Intrinsic Factor Activities of Intrinsic Factor Preparations

IF Prep.	Protein Conc. at 50% Inhibition µg./ml.	Spec. Activity as Inhibitor 100 µg./ml. (a)	Intrinsic Factor		Activity Ratio (a)/(b)
			Min. Effective Dose mg.	Spec. Act. 1/mg. (b)	
1	61	1.64	1.0	1.0	1.6
2	139	0.72	2.5	0.4	1.8
3	136	0.74	2.5	0.4	1.9
4	60	1.67	1.0	1.0	1.7
5	105	0.95	1.5	0.7	1.4
6	106	0.94	2.5	0.4	2.3
9	316	0.32	8.0	0.12	2.6
16	390	0.25	100.0	0.01	25.0

The correlation of the inhibition with IF activity is similar to the correlation of vitamin B_{12} binding by IF with IF activity (Ellenbogen and Williams, 1960). This further suggests that the inhibition is due to the binding of the coenzymes. The measurement of the cobamide coenzyme activity in the presence of IF may serve as a useful *in vitro* assay method for purified IF preparations. Preparations 2 and 3 (Table 1), obtained by partial heat inactivation of preparation 1, showed a reduction in clinical activity to approximately the same extent as the reduction in specific activity as inhibitors of the glutamate isomerase reaction.

Table 2 gives some data on the inhibition of the glutamate isomerase system by heparin and several proteins. Both lysozyme and heparin, reported to bind vitamin B_{12}, (Meyer *et. al.*, 1950; Okuda *et. al.*, 1955), inhibit to a small extent, but the specific activity is relatively low. Bovine serum albumin and gelatin are virtually inactive in this assay. This demonstrates that the inhibitory activity is not a general property of proteins.

Table 2

Inhibitor Activity of Various Substances

Substance	Conc. Tested mg./ml.	Observed Inhibition %
Lysozyme	0.50	21
Heparin	2.5	21
Bovine serum albumin	2.5	6
Gelatin	5.0	0

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