IMMUNOLOGICAL STUDY OF ENZYMES WHOSE COENZYME IS PYRIDOXAL PHOSPHATE

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It was during the last decade of the nineteenth century, that numerous studies were made on the effect of specific anti-enzyme sera on the activities of their respective enzymes. Among the anti-enzymes studied were: anti-gelatinase, anti-rennin, anti-trypsin, etc. One of the outstanding contributions in the recent years has been the observation that there is a marked similarity between the specificity of immunological and enzymic reactions. Sevag (1954) observed that the anti-body prepared against malt α-amylase completely inhibited the enzymic activity, but had no effect on the activity of β–amylase obtained from the pancreas and saliva. It was also demonstrated that the production of inhibitory anti-serum against an enzyme was dependent on the presence of active catalytic groups in the enzyme. Dolby, Hall and Happold (1952) showed that the enzyme preparation containing tryptophanase was capable of producing tryptophanase antiserum, and the activity of tryptophanase was completely inhibited by the antiserum; there was marked precipitation and no tryptophanase activity was present in the supernatant. The antiserum against the tryptophan-inactive extract caused marked precipitation with tryptophan-active extract, but did not inhibit tryptophanase activity and full tryptophanase activity was found in the centrifuged supernatant. On the basis of these observations it may be suggested that the specific antigenic property of a protein molecule depends on its potential catalytic centres, which further emphasizes the analogy between enzymic and antigenic specificities. The next question arises is whether or not specific antigenic and enzymic properties of a protein molecule arise from the same active catalytic centres. The specificity of – SH, – COOH, phenolic OH group, etc. has been amply demonstrated in enzymic and antigen-antibody reactions (Sevag, 1954). Therefore, there is some evidence that the active catalytic centres in a protein molecule responsible for enzymic and antigenic properties may be the same.

Tryptophanase, kynureninase, glutamic acid decarboxylase, etc., require pyridoxal phosphate as coenzyme for their activity. Attempts to produce

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anti-enzymes to pyridoxal phosphate-linked enzymes with a view to observing cross immunological reactions between the enzymes and their anti-bodies did not meet with much success, but some interesting observations were made which are being reported here.

MATERIALS AND METHODS

Kynureninase

Extraction of the enzyme. The enzyme was prepared from *Neurospora Crassa* (74q—4b) by the technique of Jakoby and Bonner (1953a) with slight modification. The modification being that 50 g. of wet mycelium was cut into small pieces and homogenized with 200 ml. 0·1 M phosphate buffer pH 7·8 for 5 minutes in an Auto Mix (M. S. E.). The homogenized suspension was divided equally between two bottles, each containing 300 glass beads (3mm. in diameter), and shaken on a Tower Extra-Rapid shaker, Model E, for 2 hr. It was strained through cheese-cloth and centrifuged for 35 min. at 17,800 g. (approximate gravitational field at the bottom of tube). The volume of supernatant was made up to 200 ml. by 0·1 M phosphate buffer pH 7·8. The 200 ml. extract was submitted to ammonium sulphate fractionation. Amounts of ammonium sulphate of 36, 24·4, 8·4 and 9·2 g. were added consecutively to give concentrations of 25,42,48 and 54% saturation respectively. The precipitate after each addition was removed by centrifugation. The precipitate between 42 — 48% and 48—54% saturation were dissolved together in 20 ml. distilled water and dialyzed for 2 hr. against 4 l. distilled water. The precipitate which appeared during dialysis was removed by centrifugation and the enzyme preparation was stored in a deep freeze until required. The enzyme was active for more than a month.

Method of measuring the enzyme activity. Tubes containing 2·4 μ moles DL—kynurenine, 0·5 ml. of 0·1 M phosphate buffer pH 7·7 and 0·25 ml. enzyme in a final volume of 2·0 ml. were incubated at 37°C for 1 hr. At the end of the incubation period, the reaction was stopped by the addition of 1 ml. 30% trichloracetic acid and the precipitate removed by centrifugation; the suparnatant was neutralized by suitable amount of carbonate-free sodium hydroxide. Each determination required one blank tube containing all the constituents in the reaction tube, with deproteinizing agent added at zero time, which was treated similarly as the reaction tube. The density was measured in a 1 cm. cell at 310 mμ in a Unicam spectrophotometer Model S. P. 500 and the amount of anthranilic acid formed was read from a calibration curve. Normal serum, anti-enzyme, etc. where added, have been mentioned with the appropriate experiment concerned.

Glutamic Acid Decarboxylase

Extraction of the enzyme. The technique for growing *E. Coli* has been that of Gale (1940). The washed suspensions of *E. Coli* were observed to

decarboxylate glutamic acid but there was no glutamic acid decarboxylase activity in the acetone dried powder of these cells. The cells harvested from 10 l. medium were then forced through the Hughes press (Hughes, 1951) which had been cooled by putting it overnight in a deep freeze (—20°C). The crushed material was suspended in 40 ml. 0·025 M phosphate buffer pH 7·0 which had been previously cooled. After making it homogeneous the insoluble material was removed by centrifugation which was carried out at 4°C. The cell-free enzyme extract was markedly active in decarboxylating glutamic acid. It was stored in the deep freeze and there was no marked decay in the enzymic activity for one month.

Measurement of the enyzmic activity. The CO_2 liberated by the action of the enzyme of glutamic acid was measured in warburg manometers. Warburg flasks containing 0·25 ml. crude enzyme preparation, 1 ml. phthalate buffer pH 4·87, 0·5 ml. M/15 glutamic acid and suitable amount of normal serum or anti-enzyme where necessary in the total volume of 3 ml. were incubated at 30°C for 15 minutes for equilibration of temperature. The manometric readings were taken at the end of ten minutes, after the addition of the substrate.

Method for Immunizing Rabbits Against Kynureninase And Glutamic Acid Decarboxylase

Kynureninase. 0·1 ml. enzyme solution was injected into the marginal vein of the ear. The injections were given every fifth day until a total of 10 injections were given ; the dose of every alternate injection was increased by 0·1 ml.

Glutamic acid decarboxylase. The injection of 0·1 ml. of the crude enzyme preparation resulted in the death of a rabbit. Therefore the enzyme was diluted hundred times and 0·1 ml. of the diluted enzyme preparation was injected intravenously and the dose of each subsequent injection was increased by 0·1 ml., the injections being given every fifth day. A total of 12 injections were given.

Tryptophanase. Anti-tryptophanase was obtained from Prof. F.C. Happold.

Collection of Serum

The rabbits were bled from the marginal vein on the 10th day after the last injection of the enzyme. The sera were separated and stored in the deep freeze. Serum was also collected from a normal rabbit and stored similarly.

Buffers

The following buffers were used : phosphate (Sorenson, 1909); phthalate (Clark and Lubs, 1916).

Chemicals

DL—kynurenine, Roche Product Ltd., Ammonium nitrate, May & Baker., Glutamic acid, Light & Co., Anthranilic acid, Reynold & Branson. Anthranilic acid was recrystallized several times from hot water until the sample has a constant m.p., at 145°C. All other chemicals are commercial products A. R. grade.

RESULTS

The effect of normal serum, anti-kynureninase, anti-tryptophanase and anti-glutamic acid decarboxylase on kynureninase activity

Normal serum and anti-kynureninase. It was noticed in one of the preliminary experiments that normal serum inhibited the activity of kynureninase. Therefore, two rabbits were selected—one for normal serum and the other to be immunized against kynureninase. The degree of inhibition by both the sera before immunizing the rabbit was similar. Fig. 1 shows the effect of varying the concentration of normal serum and anti-kynureninase on the enzymic activity. There was no turbidity in the solution containing the enzyme and normal serum but marked turbidity was noticed in the solution containing the enzyme and anti-kynureninase.

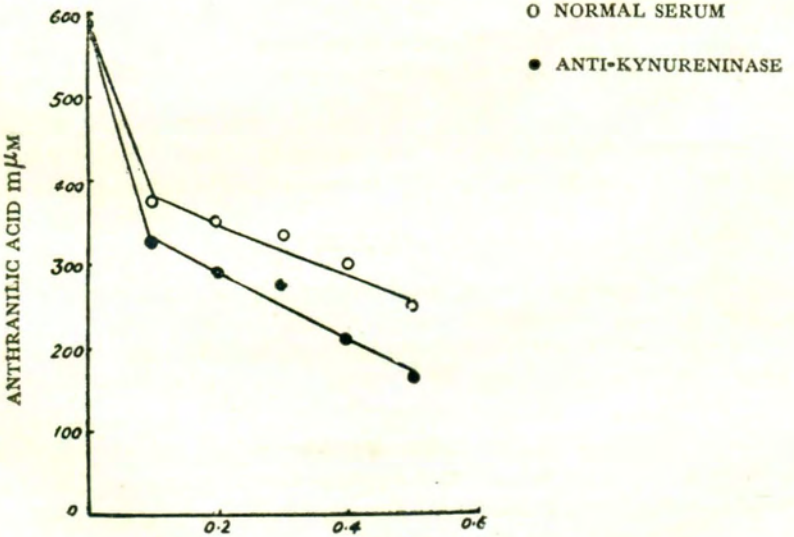

Fig. 1.

Jakoby and Bonner (1953b) state that kynureninase is inhibited by amino acids, Normal serum was dialyzed for 4 hr. against 4 1. distilled water and the precipitate which appeared during dialysis was removed by centrifugation. The dialyzed and normal serum inhibited the enzymic activity to the same extent, which shows that the factor responsible for inhibition does not appear to be dialyzable.

The following experiments were done in order to investigate whether or not the protein present in serum inhibited the kynureninase activity. Normal serum (0.5 ml) was deproteinized with 20 ml. ethanol. The precipitated protein was removed by centrifugation and dissolved in 10 ml. 0.1 M phosphate buffer pH 7.0. All the precipitated protein did not go into the solution. The supernatant was then dialyzed against 4 1. distilled water for 1 hr. in order to get rid of traces of ethyl alcohol. All operations were carried out in the cold room (4-5°C). Table 1 shows the effect of normal serum and the serum protein solution on the enzymic activity. It appears that the serum protein is inhibitory.

TABLE 1.

Effect of normal serum and protein solution on kynureninase activity.

Expt. No.	Additions	Activity (% of original)
1.	None	100
2.	0. 5 ml. protein solution	50
3.	0. 5 ml. normal serum	42.5

Table 2. relating activity to anti-enzymes shows that normal serum and anti-tryptophanase inhibit the enzymic activity by 56.2 and 61.0% respectively, but anti-glutamic acid decarboxylase inhibits only by 12.9%.

TABLE 2.

Effect of normal serum, anti-tryptophanase and anti-glutamic acid decarboxylase on kynureninase activity.

Expt. No.	Additions	Activity (% of original)
1.	None	100
2.	0.5 ml. normal serum	43.8
3.	0.5 ml. anti-tryptophanase	39.0
4.	0.5 ml. anti-glutamic acid decarboxylase	87.1

Effect of glutamic acid decarboxylase. In one of the experiments there was suggestion that glutamic acid decarboxylase may activate kynureninase, which was corroborated in the following experiments. The kynureninase activity was determined in a total volume of 2.6 ml. comprising 0.25 ml.

kynureninase 0.5 ml. of 0.1 M phosphate buffer pH 7.7, 0.5 mg. DL-kynurenine, 10 μg pyridoxal phosphate, and in the presence and absence of 0.5 ml. glutamic acid decarboxylase (Table 3).

TABLE 3.

Effect of glutamic acid decarboxylase on the kynureninase activity.

Expt. No.	Additions	Activity (% of original)
1.	None	100
2.	0.5 ml. glutamic acid decarboxylase	190

The activation of kynureninase by glutamic acid decarboxylase may be due to one of the following causes—(1) that the glutamic acid decarboxylase contains kynureninase or (2) glutamic acid decarboxylase activates kynureninase in a non-specific manner. However, there was no evidence for the presence of kynureninase in glutamic acid decarboxylase preparation.

Effect of normal surum, anti-glutamic acid decarboxylase, anti-tryptophanase and anti-kynureninase on glumatic acid decarboxylase activity

pH optimum of glutamic acid decarboxylase. The curve relating activity to pH shows an optimum pH at 4.87 (Fig. 2).

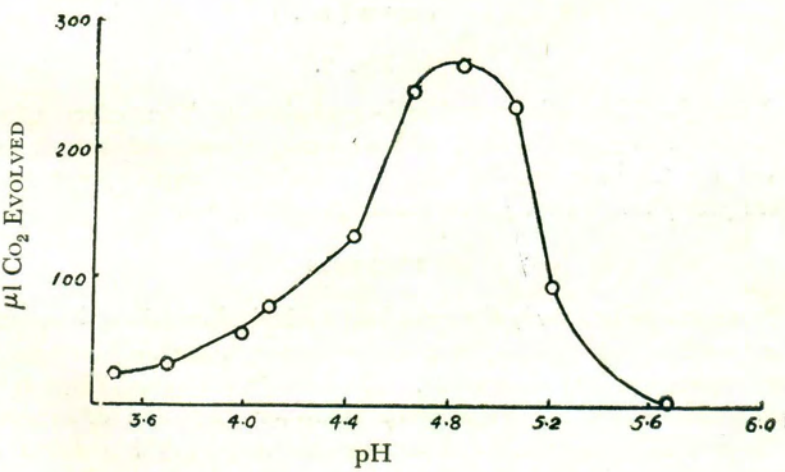

Fig. 2.

Effect of anti-glutamic acid decarboxylase. Fig. 3 shows the effects of varying the concentration of normal serum and anti-glutamic acid decarboxylase. Anti-glutamic acid decarboxylase gave marked precipitation with glutamic acid decarboxylase whilst the normal serum did not. The 0.1, 0.2, and 0.3 ml.

of the normal serum activated the enzyme to 88.0, 63.1 and 24.8% respectively, whilst 0.1 ml. of anti-glutamic acid decarboxylase activated the enzyme to 41% and 0.2 and 0.3 ml. of anti-glutamic acid decarboxylase inhibited the enzyme by 27.8 and 72.7%.

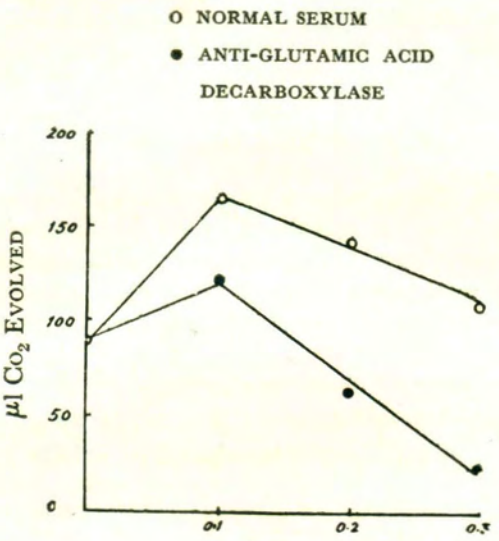

Fig. 3.

Effect of anti-kynureninase and anti - tryptophanase. The effect of varying concentrations of normal serum, and anti-enzymes showed that all of them affected the enzymic activity similarly; although anti-tryptophanase gave marked precipitation with glutamic acid decarboxylase.

DISCUSSION

In an attempt to study the cross immunological reactions between some of the pyridoxal phosphate-linked enzymes and their anti-enzymes, some interesting observations were made. Kynureninase was inhibited by serum protein of normal serum. What is the cause of inhibition of kynureninase activity by the serum protein ? A categorical answer to this question cannot be made, but certain plausible mechanism of inhibition may be suggested. The interactions between proteins are well known (Cohn *et al.*, 1951). The trypsin activity was inhibited by soyabean globulin (Sevag, 1954). Therefore the possibility that the interaction between kynureninase and serum protein in a manner which will affect the active catalytic centres of kynureninase has to be borne in mind.

There was no marked difference between normal serum and anti-kynureninase in their inhibitory capacity. Some enzymes are known which when injected into animals are unable to produce their respective antibodies. Cohn and Monod (1951) observed that the antiserum against galactosidase of *E. Coli* showed no inhibitory action on the enzyme activity.

The pH optimum of glutamic acid decarboxylase appears to vary slightly in different species of bacteria. The pH optimum of glutamic acid decarboxylase from *E. Coli* was at 4.87, whilst Gale (1945) observed the optimum pH of the enzyme from Cl. welchii at 4.25.

Evidence has been presented for the formation of anti-enzyme to glutamic acid decarboxylase. Anti-tryptophanase also gave marked precipitation with glutamic acid decarboxylase, but did not inhibit the enzyme activity, which further corroborates the suggestion that specific antigenic property of a protein depends on its potential catalytic centres. The formation of precipitate between anti-tryptophanase and the glutamic acid decarboxylase was due to the fact that tryptophanase was also obtained from *E. Coli*. There was no evidence for cross immunological reactions between pyridoxal phosphate-linked enzymes and their anti-enzymes.

SUMMARY

1. Normal serum inhibited kynureninase activity and the inhibitory factor was present in the protein fraction of blood.

2. Glutamic acid decarboxylase activated kynureninase markedly.

3. Glutamic acid decarboxylase was activated by normal serum at lower concentrations, but higher concentrations were inhibitory.

4. The production of anti-enzyme to glutamic acid decarboxylase has been demonstrated.

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