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Studies on Glutamic Oxalacetic Transaminase in the Rat. I. New Assay Procedure for Enzyme Activity in Crude Preparations

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Although the effects of various agents on glutamic oxalacetic transaminase (GOT) in tissue have been studied, the presence of isoenzymes, which have different characters, showed the lack for quantitativity in many of assay methods and the possibility of misleading results. In this report, the assay procedure for this enzyme activity in liver homogenate of rats was modified respect to the existence of isoenzymes. One procedure is a modified manometric method consisting of a coupled-system with chemical decarboxylation of oxalacetate to eliminate the inhibitory action of mitochondrial enzyme (m-GOT), and the other is a new type of spectrophotometric procedure. The latter could also be employed for the approximate calculation of isoenzyme levels in homogenates in combination with electrophoretic procedures for isoenzymes ratio with cellulose acetate film.

Although the clinical significance of this enzyme has been previously reported,[2-4] the presence of isoenzymes was only recently confirmed in the extracts from tissues[5] and an interest is being manifested in the clinical area.

This investigation was undertaken to study the biological variance of these isoenzymes in rat liver. However, we could not employ assay procedures for estimation of isoenzyme levels in crude preparations, because little attention has been devoted to the basic properties of these isoenzymes. As a consequence, some of the convenient procedures for assay of total activity and isoenzyme levels in crude preparations, were investigated in this paper.

Methods

Materials——In this experiment, male rats of the Wister strain were used. Serum was prepared within an hour after decapitation at room temperature. Liver was immediately homogenized with nine volumes of water and stored in the refrigerator.

Manometric Assay Procedure——Warburg flasks with two side arms were used for the determination of enzyme activity. Into the main compartment of each flask, 1.0 ml of 0.04 M *l*-aspartate adjusted to pH 6.4 with 0.1 M phosphate buffer, 1.0 ml of 0.04 M nickel chloride solution adjusted to pH 7.4 with 0.025 M Tris-buffer and 1.0 ml of diluted homogenate were pipetted. The first side vessel received 0.4 ml of 0.1 M α-ketoglutaric acid adjusted to pH 6.4 with 0.1 M phosphate buffer, and the second side vessel received 0.4 ml of aniline citrate solution. After temperature equilibrium had been reached at 37.5°, α-ketoglutarate was added into the main compartment and the mixture incubated for 15 min. The reaction was stopped with addition of aniline citrate and the manometer was read after temperature equilibrium had been achieved. This reading showed the output of CO_2 from the oxalacetate produced in this reaction in addition to the blank readings which can be deducted from the system by adding aniline citrate at the same time substrate was added.

Spectrophotometric Assay Procedure——This procedure is composed of three parts: Enzyme reaction, conversion fo oxalacetate produced by the reaction to pyruvate, and spectrophotometric assay of pyruvate by coupling with $NADH_2$-linked lactic dehydrogenase.

1) Location: *Fukushima-ku, Osaka.*
2) S.J. LaDue, F. Wróblewski, and A. Karmen, *Science*, **120**, 497 (1954).
3) F. Wróblewski and S.J. LaDue, *Ann. Int. Med.*, **43**, 345 (1955).
4) H. Wada and Y. Morino, *Clin. All-round* (Osaka), **13**, 758 (1964).
5) W.J. Jenkins, D.A. Yphantis, and I.W. Sizer, *J. Biol. Chem.*, **234**, 51 (1959).

Into a test tube were pipetted 0.5 ml of 0.02 M *l*-aspartic acid and α-ketoglutaric acid adjusted to pH 7.4 with 0.1 M phosphate buffer, 0.1 M phosphate buffer pH 7.4 and enzyme solution to make a total volume of 2.0 ml. After incubation for 30 min at 37.5°, the reaction was stopped by the addition of 1.0 ml of 10% trichloroacetic acid. The mixture was centrifuged for 10 min at 3000 rpm if a precipitate was produced, and then 2.0 ml of supernatant fluid was pipetted into another test tube. Four-tenths ml of 1N NaOH and 1.0 ml of 0.1 M phosphate buffer pH 7.4 were added. After heating at 60° for 60 min, in order to decompose oxalacetate produced within the reaction to pyruvate, and cooling to room temperature, 0.5 ml of 0.05% $NADH_2$ solution and 0.2 ml of a diluted commercial preparation of lactic dehydrogenase were added and the decrease in optical density at 340 mμ was followed. As a blank test in this procedure, trichloroacetic acid was added before addition of enzyme to the reaction mixture.

Electrophoretic Separation of the Active Zone of Isoenzymes on Cellulose Acetate Film——One ml of diluted homogenate was solubilized with 1.0 ml of 1% desoxycholate dissolved in 0.1 M phosphate buffer, pH 7.4. Ten μl of this mixture was immediately fractionated by electrophoresis with cellulose acetate film.

For electrophoresis, 9×5 cm of "OXOID" cellulose acetate film was used and two samples were spotted on 1×0.1 cm of filter paper at an origin 3.5 cm from the anode side and at intervals of 1.0 cm according to the literature.[6] Electrophoresis was carried out for 90 min at 200 V in 0.02 M phosphate buffer, pH 7.4. Immediately after electrophoresis, each of the films was cut into several pieces, 0.5×2.5 cm, and were placed in 1.0 ml of 0.1 M phosphate buffer, pH 7.4, followed by the addition of substrate, to determine their activities according to the spectrophotometric procedure.

Results

Total Activity in Liver Homogenate

In the manometric assay procedure, modification consisted of the addition of nickel chloride into the reaction mixture. This modification aimed to correct a defect in previously established procedure[7] by referring to the enzymic characteristic of m-GOT, reported in a recent investigation.[8] The effect of nickel chloride could be recognized by the establishment of lineality in the concentration curve as shown in Fig. 1. In our modified method, the enzyme reaction was carried out in a more acidic medium than the optimum pH for this enzyme reaction, because the decarboxylation of oxalacetate by nickel ion could not take place in an alkaline medium. The optimum pH in this coupled system was pH 6.4 as shown in Fig. 2. Another important factor was the concentration of nickel ion in the medium, which must be kept below the concentration of *l*-aspartic acid to prevent the inactivation of enzyme. In this method, the time course was shown quantitatively as seen in Fig. 3, and the concentration curve was proportional to the amounts of enzyme below the output of 60 μl of CO_2 as shown in Fig. 4.

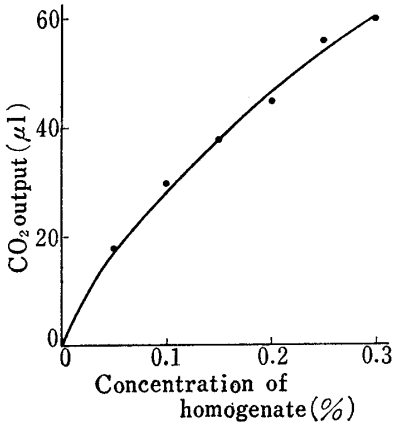

Fig. 1. Relation between CO_2 Output and Concentration of Rat Liver Homogenate in Manometric Assay[7]

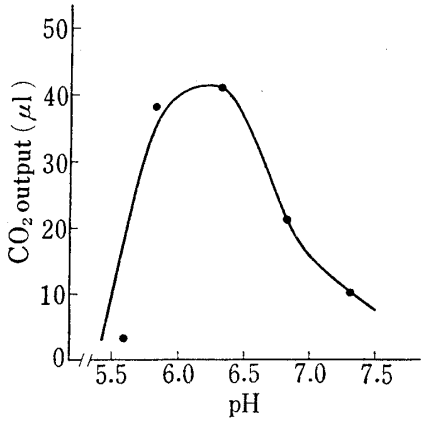

Fig. 2. Influence of pH on Decarboxylation of Oxalacetic produced by GOT in Medium Containing Nickel Ion

6) Y. Ogawa, *Japan Clin.*, **21**, 2419 (1963).
7) D.E. Green, L.F. Leloir, and V. Nocito, *J. Biol. Chem.*, **161**, 559 (1954).
8) H. Wada and Y. Morino, *Vitamins and Hormones*, **22**, 411 (1964).

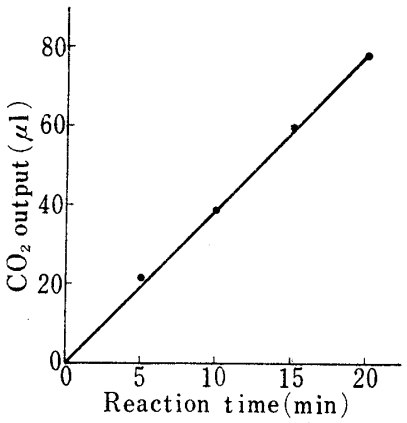

Fig. 3. Progress Curve in Modified Manometric Method

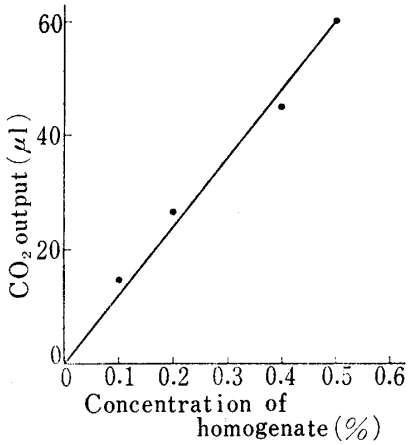

Fig. 4. Relation between CO_2 Output and Concentration of Homogenate in Madified Manometric Method

The spectrophotometric method could not be employed for estimation of total activity in liver homogenate, but quantitation was established by application of a new technique designated as the "cellulose acetate treatment" as shown in Fig. 5. The standard procedure was defined as follows: 1.0 ml of diluted homogenate was mixed with 1.0 ml of 0.1 M phosphate buffer, pH 7.4, containing 2 μmole of phospholipids which was prepared from ether extracts of rat liver. Ten μl of this mixture was spread on about 1×2 cm of cellulose acetate film and kept for exactly 30 min at room temperature, and then this film was placed in a reaction mixture as the enzyme material. Enzyme activity was defined in this procedure as follows: 1 unit is the enzyme amount which decreased the optical density 0.1 at 340 mμ.

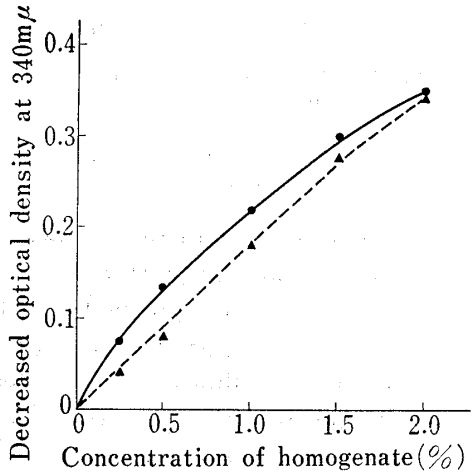

Fig. 5. Effect of Cellulose Acetate Treatment on Micro Assay of GOT Activity in Rat Liver Homogenate

Electrophoretic Separation of Isoenzymes in Liver Homogenate

Two active zones could be separated by electrophoresis and each of the active zones could be identified at the same position as the enzyme in the soluble fraction (s–GOT) and m–GOT as seen in Fig. 6. In this procedure, we could not employ the same methods of denaturation on m–GOT as on the crude preparation, because the materials were highly purified on the surface of the cellulose acetate film.

While the activities were not exactly quantitative for m–GOT, as shown in Fig. 7, the *m*/s values which were calculated from the summation of two active zones were within possible assay ranges.

Calculation of Isoenzyme Activity in the Homogenate

Further studies were undertaken to calculate each of the isoenzyme activities in the homogenate. The relationship of the enzyme activities was studied by two assay systems which were different in principle.

The assay procedure for total activity was based on the selective modification of m–GOT by cellulose acetate treatment for quantitative estimation of both isoenzymes. On the other hand, the m–GOT activity in the electrophoretic isoenzyme ratio was defined by the summation of several points within the possible assay range in the spectrophotometric procedure, which

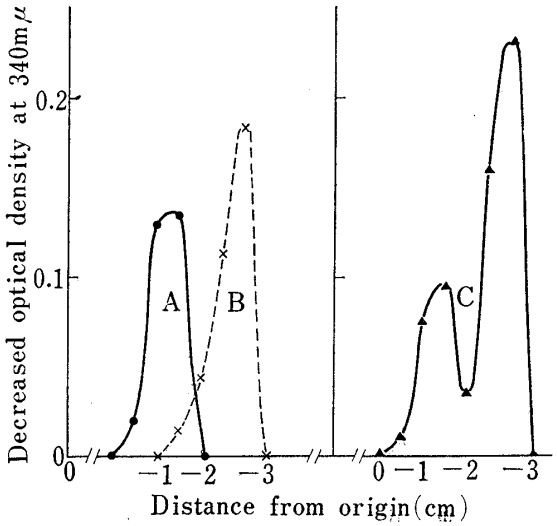

Fig. 6. Electrophoretic Separation of GOT Isoenzymes in Rat Liver Extracts

A: supernatant fraction
B: mitochondria fraction
C: homogenate

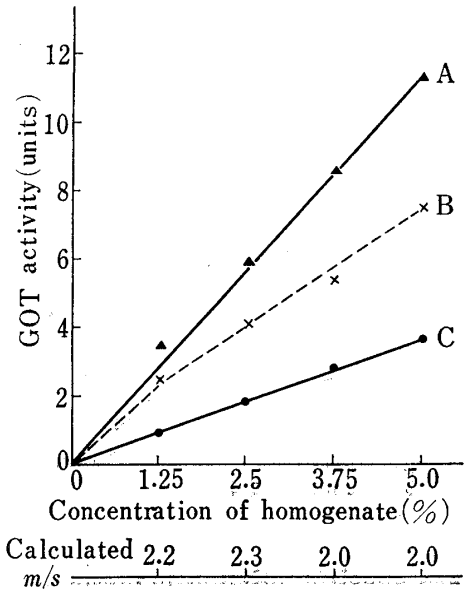

Fig. 7. Quantitativity and Isoenzyme Ratio on Electrophoretic Procedure

A: total activity
B: m-GOT
C: s-GOT

was not exactly quantitative owing to product inhibition. The experimental ratio of this difference was calculated from several points of enzyme amounts within the possible assay range with a partially purified m-GOT preparation, and the mean value by the procedure without cellulose acetate treatment was found to show a 1.7-fold increase of reaction products over that of the cellulose acetate treated. In homogenates, the isoenzyme ratio was shown to be 2.0-fold over this procedure by the system coupled with $NADH_2$-linked malic dehydrogenase, in which the activity of m-GOT could be estimated quantitatively without product inhibition. However, total activity in this system was estimated as 3.5-fold that of our procedure when the two assay procedures were compared using s-GOT as standard material. This would prove the fact that the m-GOT activity in the assay procedure for isoenzyme ratio was expressed as approximately the mean value of the relation to that of total activity even in this case. On the basis of these studies, the following equilibrium was established for the purpose of approximate estimation of isoenzyme levels in liver homogenate.

Equilibrium calculation

$$x+y=A$$

$$1.7\,y/x=B$$

A : total activity in homogenate (units/g liver)
B : isoenzyme ratio
x : s-GOT activity (units/g liver)
y : m-GOT activity (units/g liver)

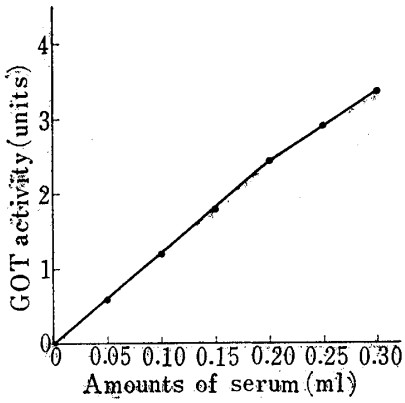

Fig. 8. Concentration Curve of GOT Activity in Rat Serum

Enzyme Activity in Serum

Though many excellent procedures[9,10] have been developed for the assay of serum GOT, our spectrophotometric procedure can also be employed as a convenient method. In this method, the activity of serum

9) A. Karmen, *J. Clin. Invest.*, **34**, 131 (1955).
10) N. Katsunuma and Y. Nishii, *Vitamin* (Kyoto), **31**, 72 (1965).

GOT could be determined with less than 0.2 ml of serum from intact rats as seen in Fig. 8. No pretreatment was necessary for serum in this procedure, for the isoenzymic character of serum was identified to be like that of s–GOT in tissue.

Discussion

In the assay procedure for total GOT activities in liver homogenate, two types of modifications were employed. The manometric method was modified by the application of coupled system with chemical decarboxylation of oxalacetate which is well known to play an inhibitory action in this enzyme reaction. Although this procedure employed chemical decarboxylation instead of enzymatic elimination of oxalacetate as in Karmen's method,[9] this is the most economical procedure only in the case where total activity is estimated.

On the other hand, the spectrophotometric procedure can be applied to even small amounts of materials and their isoenzyme levels can be investigated in combination with the electrophoretic procedure. In this procedure, cellulose acetate treatment is a new technique for the quantitative modification of m–GOT which will be described in detail in a future report. Consequently, tissue homogenates can be directly estimated for enzyme activity without any pretreatment or extraction from intracellular particles or intact cells.

The one failure in this program is the fact that only approximate values could be calculated for the isoenzyme levels in homogenates for use as an experimental ratio in the relation of simultaneous equations. However, the error in this approximation is so small in comparison with the biological variance that we could employ this program as a primary study of the estimation of isoenzyme activity.

When applying the same procedure to serum GOT activity, this can be employed more conveniently than Karmen's method[9] in animal experiments where many determinations are required.