[Chem. Pharm. Bull. 16(10)1942—1947 (1968)]

UDC 577.156.025

# Studies on Glutamic Oxalacetic Transaminase in the Rat. II.[1] Selective Denaturation of Isoenzymes by Cellulose Acetate Treatment[2]

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(Received February 2, 1968)

A new technique of mild denaturation of glutamic oxalacetic transaminase on the surface of cellulose acetate film was introduced as an application for an assay procedure of enzyme activity in crude materials. This treatment modified the enzymatic character of mitochondrial enzyme in the presence of phospholipids in crude preparations after which the isoenzymes were estimated quantitatively using a concentration of substrate adequate for the enzyme in the soluble fraction which was a minor component in rat liver.

In our previous report,[1] a new technique was introduced to determine the isoenzyme activity of glutamic oxalacetic transaminase (GOT) in liver homogenates. This technique involved modification of the enzymatic character by mild denaturation on cellulose acetate film. In this paper, the details of this treatment were studied to demonstrate the quantitation of the assay procedure of GOT activity in homogenates.

## Methods

**Materials**——Male rats of the Wister Strain were used. Liver homogenates were prepared with nine volumes of water, and treatment with DEAE-cellulose was carried out batchwise using DEAE-cellulose buffered with 0.1 M phosphate buffer, pH 7.0. Isotonic preparations of isoenzyme materials were prepared as follows: 10% isotonic homogenates were prepared with 0.25 M sucrose solution and fractionated by centrifugation according to the literature.[4] Mitochondrial fractions were resuspended in water and mitochondrial enzyme (m-GOT) was extracted from particles by five times of repeated lyophilization in dry ice-acetone. The soluble fraction was prepared by further centrifugation at $105{,}000 \times g$ for 60 min using supernatant fluid free of mitochondria.

**Assay Method for Enzyme Activity**——Ten $\mu$l of enzyme solution was treated with about $1 \times 2$ cm of cellulose acetate film. This film was introduced as an enzyme solution into a reaction mixture containing 1.0 ml of 0.1 M phosphate buffer, pH 7.4, 0.5 ml of 0.02 M *l*-aspartic acid and $\alpha$-ketoglutaric acid adjusted to pH 7.4 with 0.1 M phosphate buffer, respectively. After incubation for 30 min at 37.5°, the enzyme reaction was stopped by addition of 1.0 ml of 10% trichloroacetic acid. Two ml of this mixture was pipetted into another test tube, followed by addition of 0.4 ml of 1 N NaOH and 1.0 ml of 0.1 M phosphate buffer, pH 7.4. This tube was heated at 60° for 60 min to convert oxalacetate produced in the reaction to pyruvate. After cooling to room temperature, 0.5 ml of 0.05% $NADH_2$ solution and 0.2 ml of lactic dehydrogenase solution were added and the decrease in optical density at 340 m$\mu$ was followed.

In this procedure, a blank test was employed in the system either without amino acid as substrate or by adding trichloroacetic acid to the system before addition of enzyme. As a comparison with the cellulose acetate treatment, 10 $\mu$l of enzyme solution was added into the reaction mixture without any pretreatment, and the reaction was proceeded as above.

**Assay Method for Enzyme Activity by Malic Dehydrogenase System**——This method was employed to determine the activity which was calculated according to the initial velocity of this enzyme reaction. The enzyme activity was determined by the progressive decrease in optical density at 340 m$\mu$ after addition

1) Part I: Y. Ogawa, Y. Kometani, and Y. Baba, *Chem. Pharm. Bull.* (Tokyo), **16**, 1937 (1968).
2) This paper was presented at the 18th Sym. Enzyme Chem. (Tokyo), Sapporo, Aug. 26, 1966: Abstracts, p. 310.
3) Location: *Fukushima-ku, Osaka.*
4) G.H. Hogeboom, W.C. Schineder, and G.E. Palade, *J. Biol. Chem.*, **172**, 619 (1948).

of 10 $\mu$l of enzyme solution or cellulose acetate film, which was treated with 10 $\mu$l of enzyme solution, into the reaction mixture to which the following components were added: 0.4 ml of water, 0.5 ml of 0.05% $NADH_2$ solution and 0.1 ml of a diluted commercial preparation of malic dehydrogenase solution. Concomitance of glutamic dehydrogenase activity was deducted by the blank which contained no amino acid as substrate. The activity per unit of GOT was defined as the amount of enzyme to produce $1 \times 10^{-6}$ M of oxalacetate per min in this system.

## Results

### Denaturation with Cellulose Acetate Treatment

Although the same denaturation could be observed by treatment on filter paper, the effect on enzymatic character was not as constant as that observed with cellulose acetate film. The degree of denaturation was found to be defined by the following three factors: pH value of enzyme mixture, duration of time that the enzyme solution is in contact with the film and content of phospholipids in the enzyme mixture.

As seen in Fig. 1, the mildest effect was observed when the enzyme mixture had an alkaline pH and this effect reached a constant level within 30 min after spread of the enzyme on the film as shown in Fig. 2. This inactivation based on denaturation on film was observed to be more remarkable in partially purified materials, and this high inactivation could be protected to maintain the homogenate level by addition of a lipid suspension into the enzyme solution as seen in Table I.

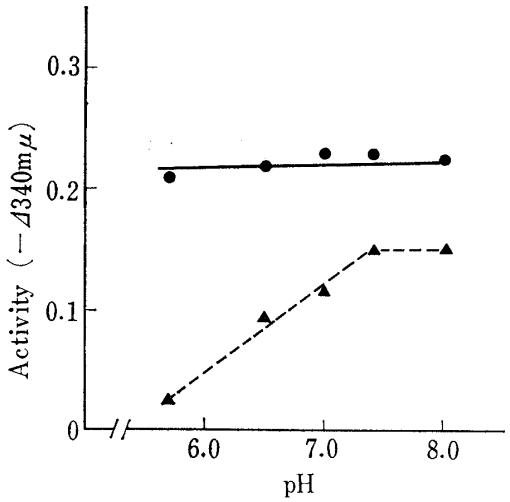


Fig. 1. Effect of pH on Inactivation of GOT Activity by Treatment with Cellulose Acetate

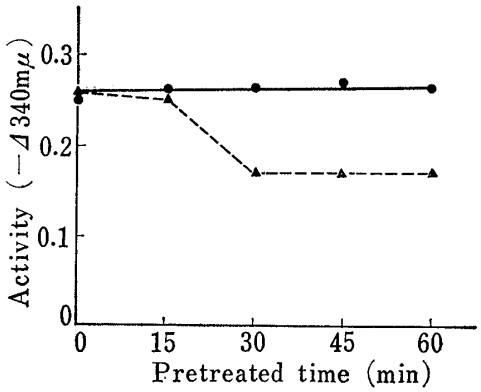


Fig. 2. Inactivation of GOT Activity by Treatment with Cellulose Acetate

Table I. Effect of Lipids on GOT Activity inactivated by Treatment with Cellulose Acetate

| | Material | GOT activity ($-\Delta$ 340 m$\mu$) | |
|---|---|---|---|
| | | Without treatment | With treatment |
| I | homogenate | 0.227 | 0.213 |
| II | extract from DEAE-cellulose | 0.233 | 0.154 |
| III | II+lipids | 0.236 | 0.210 |

## Characteristic Effect on m-GOT by Cellulose Acetate Treatment

For the purpose of investigation of the selective effect of this treatment on isoenzymes, mitochondrial extract and supernatant fluid in the presence of lipids were respectively treated with cellulose acetate film after dilution with 0.1 M phosphate buffer, pH 7.4, and their concentration curves were studied. Fig. 3 shows that m-GOT did not react quantitatively in this assay condition, and that the amounts of reaction product could be estimated quantitatively only with materials treated by cellulose acetate film. Those of the enzyme in the soluble fraction (s-GOT) on the contrary, could be estimated quantitatively without cellulose acetate treatment. Furthermore, this treatment could be used to simultaneously extract m-GOT from mitochondria as shown in Fig. 4 which facilitated the quantitative assay of m-GOT.

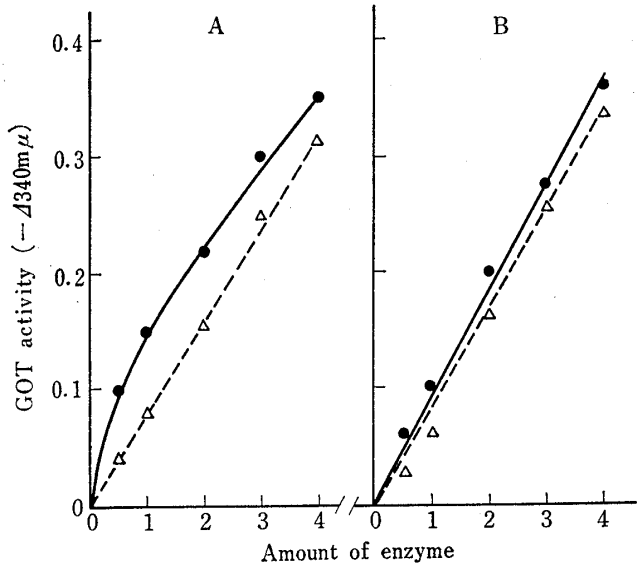


Fig. 3. Effect of Treatment with Cellulose Acetate on GOT Isoenzymes in Rat Liver

A: mitochondria fraction
B: supernatunt fraction
●——● without treatment
▲------▲ with treatment

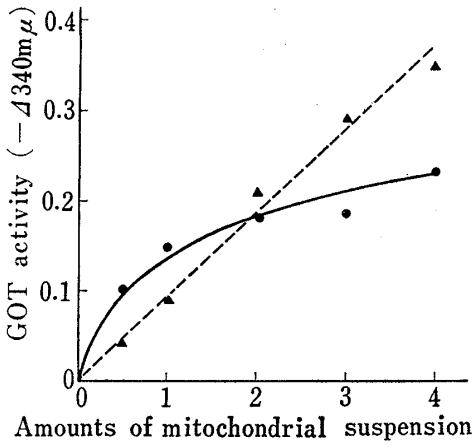


Fig. 4. Effect of Treatment with Cellulose Acetate on Mitochondrial Suspension

●——● without treatment
▲------▲ with treatment

## Role of Phospholipids in Cellulose Acetate Treatment

A partial protective effect to inactivation by this treatment was found in the presence of a lipid suspension in the reaction mixture. In mitochondrial extracts the type of lipid effective

Table II. Protective Action to Inactivation of Partially Purified m-GOT by Cellulose Acetate Treatment

| Compounds | Relative activity[a] | Compounds | Relative activity[a] |
|---|---|---|---|
| Exp. 1 | | Exp. 2 | |
| None | 0.54 | None | 0.32 |
| Acetone extracts from rat liver, 0.125% | 0.62 | Phospholipids $1\times10^{-3}$ M | 0.58 |
| Ether extracts from rat liver, 0.125% | 0.84 | Pyridoxal phosphate $1\times10^{-3}$ M | 0.60 |
| Purified lecithin from rat liver, 0.125% | 0.93 | Oxalacetate $1\times10^{-3}$ M | 0.52 |
| Cerebrosider from rat brain, 0.125% | 0.58 | Aspartate $1\times10^{-3}$ M | 0.56 |
| Commercial triolein, 0.125% | 0.63 | α-Ketoglutarate $1\times10^{-3}$ M | 0.36 |
| Commercial oleic acid, 0.125% | 0.65 | α-Ketoglutarate $5\times10^{-3}$ M | 0.32 |
| Commercial palmitic acid, 0.125% | 0.62 | Glutamate $1\times10^{-3}$ M | 0.32 |
| Boione albumin, 0.125% | 0.95 | Glutamate $5\times10^{-3}$ M | 0.34 |

a) amounts of oxalacetate produced by the enzyme treated with cellulose acetate to that by the enzyme without treatment

was studied and found to be phospholipids as seen in Table II. This protective effect was shown not only with phospholipids but also with pyridoxal phosphate, aspartate, oxalacetate and albumin. However, the effect with albumin was different from that of phospholipids in respect to the concentration curve of the enzyme reaction as shown in Fig. 5.

On the other hand, pyridoxal phosphate or substrate analogs were not observed to prevent inactivation with adenosine triphosphate as seen in Table III, while the presence of adenine nucleotide or semicarbazide which are well known as inhibitor to this enzyme, caused inactivation by this treatment only in the mitochondrial extract.

On the basis of these facts, the standard procedure for cellulose acetate treatment was defined as follows: 1.0 ml of diluted enzyme solution was mixed with 1.0 ml of 0.1 M phosphate

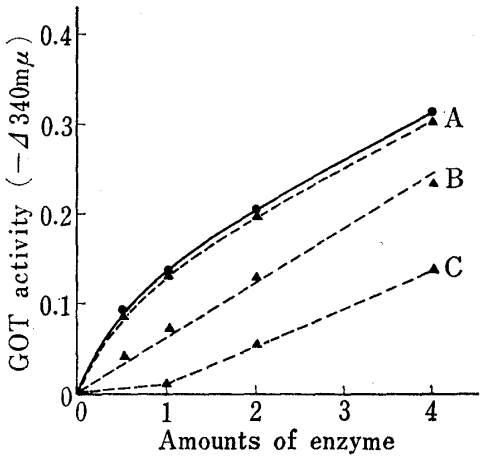


Fig. 5. Protective Effect of Albumin or Phospholipids on Inactivation of m-GOT by Treatment with Cellulose Acetate

A: added 0.125% of bovine albumin
B: added $1\times10^{-3}$ M of phospholipids
C: none
●——● without treatment
▲-----▲ with treatment

Table III. Inactivation of m-GOT Activity by ATP on Cellulose Acetate Treatment and Protective Action of Phospholipids in This Inactivation

| Compounds | Relative activity |
|---|---|
| None | 0.37 |
| ATP $1\times10^{-2}$ M | 0.17 |
| ATP $1\times10^{-2}$ M + phospholipids $2\times10^{-3}$ M | 0.60 |
| ATP $1\times10^{-2}$ M + pyridoxalphosphate $2\times10^{-3}$ M | 0.27 |
| ATP $1\times10^{-2}$ M + oxalacetate $2\times10^{-3}$ M | 0.32 |
| ATP $1\times10^{-2}$ M + $\alpha$-ketoglutarate $2\times10^{-3}$ M | 0.12 |

buffer, pH 7.4, containing 2.0 μmole of phospholipids extracted from rat liver. Ten μl of this mixture was spread on about $1\times2$ cm of cellulose acetate film and this was employed as enzyme material after keeping exactly 30 min at room temperature.

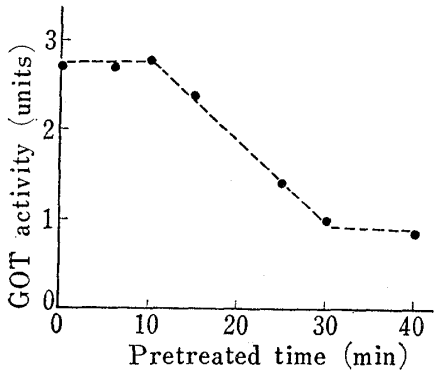


Fig. 6. Inactivation of m-GOT Activity by Treatment with Cellulose Acetate in Assay Method with Malic Dehydrogenase System

## Effects of Cellulose Acetate Treatment on the Character of m-GOT

First, the effect on the initial velocity of this enzyme reaction was studied using an assay procedure of a coupled system with $NADH_2$-linked malic dehydrogenase. Fig. 6 corresponds to the results in our assay procedure for the effective time after spread of enzyme on the film, but the concentration curve with this assay procedure demonstrated, as seen in Fig. 7, that the initial velocity of the enzyme after treatment was no longer proportional to the enzyme amounts in this assay system.

Further investigation was undertaken to investigate the kinetics involved in our assay procedure.

5) J.R. Florini and C.S. Destling, *Biochem. Biophys. Acta*, **25**, 575 (1957).

The initial velocity was calculated by progress curves for each concentration of substrate in this study and Fig. 8 was plotted according to Florini's method.[5]

As a result of this analysis, it is suggested that the affinities to both substrates were increased by cellulose acetate treatment in spite of the decrease in maximum initial velociy. On the contrary, the degree of inhibition by oxalacetate was also emphasized in the enzyme treated with cellulose acetate, as shown in Fig. 9.

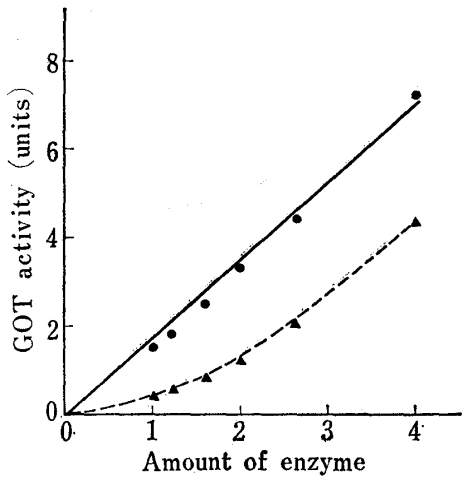


Fig. 7. Effect of Treatment with Cellulose Acetate on Concentration Curve of m-GOT in Aassay Method with Malic Dehydrogenase System

●—● without treatment
▲---▲ with treatment

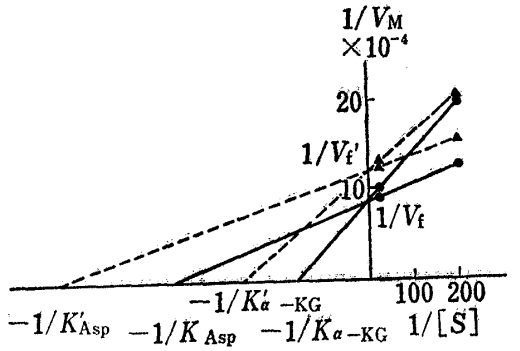


Fig. 8. Effect of Treatment with Cellulose Acetate on Approximate $K_m$ and Maximum Velocity

$V_f$: maximum velocity
$K_{Asp}$: $K_m$ for aspartic acid
$K_{\alpha-KG}$: $K_m$ for α-ketoglutarate
[S]: concentration of substrate
$V_M$: maximum velocity in constant concentration of other substrate

●—● without treatment
▲------▲ with treatment

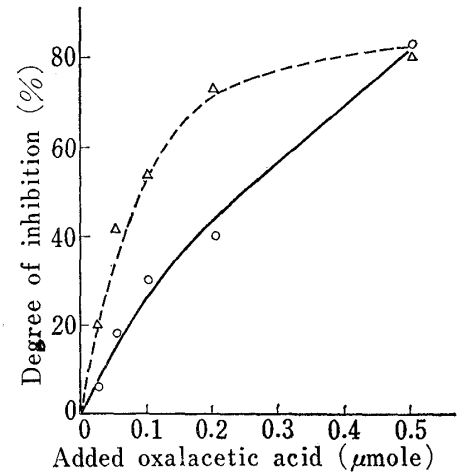


Fig. 9. Effect of Treatment with Cellulose Acetate on Inhibition by Oxalacetic Acid to m-GOT

●—● without treatment
▲------▲ with treatment

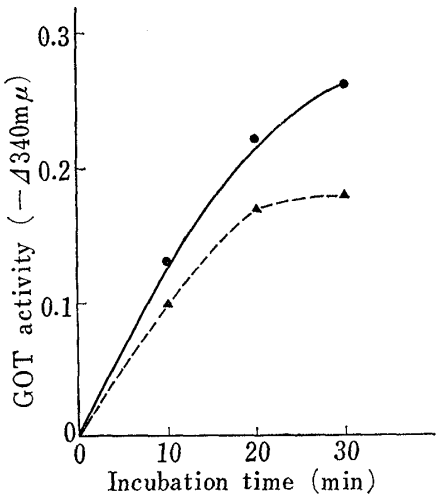


Fig. 10. Effect of Treatment with Cellulose Acetate on Progress Curve of m-GOT Activity

●—● without treatment
▲------▲ with treatment

## Basis for Quantitative Estimation of m-GOT or Total GOT by Cellulose Acetate Treatment

This enzyme reaction was also defined by the concentration of two substrates, α-ketoglutarate and aspartate.

Our employed assay conditions are favorable for s-GOT rather than for m-GOT in regard to the affinity for each substrate. However, cellulose acetate treatment plays a role in modifying the character of m-GOT and changing the affinity for the substrates without any effects on

s–GOT in the presence of phospholipids. Consequently, maximum amounts of reaction could be shown within the defined incubation period, 30 min, as seen in Fig. 10 with either equal concentrations of the two substrates or high a concentration of aspartate.

## Discussion

A very simple technique was applied for the selective modification of isoenzymes in crude preparations. Cellulose acetate film could be easily employed to evaporate water in materials readily on the surface of the film and quantitatively denaturate the character of the enzyme.

Concerning the quantitation in this assay procedure for m–GOT or total GOT activity, it seemed reasonable to assume that m–GOT activity was shown at the maximum amounts in the enzyme reaction corresponding to the enzyme amounts, and s–GOT activity was expressed in the 30 min reaction and was proportional to the initial velocity. Consequently, we could employ this treatment as a method of assay for this enzyme, especially for mixed materials of isoenzymes. However, this procedure must not be employed for purified materials which no longer have the protective effect of phospholipids to prevent inactivation, for example, extracts from precipitation with ammonium sulfate. With these considerations, it might be concluded that our developed assay procedure can be employed as a convenient assay system only in the investigation of crude preparations.

In addition, investigation of the mechanism in this treatment by studies of reaction velocity of this enzyme and the relationship of phospholipids to the structure of this enzyme were pursued.