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Distribution and Degradation of β-D-Glucose Oxidase intravenously injected into Rats and Mice

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The distribution and degradation of β-D-glucose oxidase (from *Penicillium amagasakiense*) intravenously injected into rats and mice are described in detail. The uptake of intravenously injected β-D-glucose oxidase (0.45 mg/kg) by various tissues of rats and mice was followed over a period of 60 hr by determining the enzyme activity with the polarographic oxygen electrode. The highest activity of the tracer enzyme injected into rats was found in the liver. Considerable amount was taken up by the spleen. The activity in the liver and spleen reached a maximum in 1—2 and 4—6 hr, respectively. Any activity was not found in other tissues 40 min after the injection. Activity in blood also disappeared in 40 min and that in the liver and spleen almost entirely disappeared in 60 hr. Only slight differences in the distribution of the enzyme were observed between rats and mice. β-D-Glucose oxidase in the liver of rats and mice was histochemically detected only in the Kupffer cells, but not in the parenchymal cells. The enzyme degradation studies *in vitro* suggested that the enzyme taken up by the Kupffer cells may be concertedly inactivated by the hydrolytic enzymes in lysosomes of the Kupffer cells and by the pH elevation (above 7.4) in the cell fluid.

Although the fates of some endogenous and exogenous proteins, *e.g.* albumin,[2,3)] horseradish peroxidase,[4,5)] hemoglobin,[6,7)] and ceruloplasmin,[8)] *etc.*, administered to higher animals have been studied, the fates of exogenous (foreign) proteins injected intravenously into higher animals have not so much been investigated notwithstanding the increase of enzyme therapeutics. β-D-Glucose oxidase (EC 1.1.3.4, β-D-glucose: oxygen oxidoreductase), one of the flavin enzymes, was chosen as a tracer protein to study the catabolism of foreign proteins from some reasons; 1) this enzyme is absent in higher animals, and 2) it is stable and almost non-dissociable,[9)] and 3) it can be assayed rapidly by the aid of a polarographic oxygen electrode. It was also interesting to investigate whether or not there are some differences in the distribution or degradation of β-D-glucose oxidase between rats and mice, because it had been demonstrated in our previous study[10)] that LD_{50} (8.50 mg/kg) of the injected enzyme for mice was about 10 times as large as that (0.80 mg/kg) for rats.

Experimental

Materials——Pure β-D-glucose oxidase from *Penicillium amagasakiense* was obtained from crude enzyme preparation (Nagase & Co. Ltd., Osaka) according to the method of Kusai, *et al.*[11,12)] and dissolved in 0.9%

1) Location: *Yagoto, Tenpaku-cho, Showa-ku, Nagoya.*

2) T. Freeman, A.H. Gordon, and J.H. Humphrey, *Brit. J. Exp. Pathol.*, **39**, 459 (1958).
3) J.L. Mego, F. Bertini, and J.D. McQueen, *J. Cell Biol.*, **32**, 699 (1967).
4) W. Straus, *J. Biophys. Biochem. Cytol.*, **4**, 541 (1958).
5) W. Straus, *J. Cell Biol.*, **20**, 497 (1964).
6) S. Kornfeld, B. Chipman, and E.B. Brown, *J. Lab. Clin. Med.*, **73**, 181 (1969).
7) S. Goldfisher, A.B. Novikoff, A. Albala, and L. Biempica, *J. Cell Biol.*, **44**, 513 (1970).
8) A.G. Morell, G. Gregoriadis, I.H. Scheinberg, J. Hickman, and G. Ashwell, *J. Biol. Chem.*, **246**, 1461 (1971).
9) J. Okuda, J. Nagamine, and K. Yagi, *Vitamins*, **37**, 623 (1968).
10) J. Okuda and I. Miwa, *Biochem. Pharmacol.*, **19**, 1777 (1970).
11) K. Kusai, *Ann. Rep. Scient. Works, Fac. Sci. Osaka Univ.*, **8**, 43 (1960).
12) K. Kusai, I. Sekuzu, B. Hagihara, K. Okunuki, S. Yamauchi, and M. Nakai, *Biochim. Biophys. Acta*, **40**, 555 (1960).

NaCl. The pure enzyme thus obtained was almost completely homogeneous by ultracentrifugal analysis, and the Qo_2 (μl O_2 consumption/mg protein/hr) of the enzyme was 139500 under the condition reported by Kusai, *et al.*[12] D-Glucose, sodium azide, potassium cyanide, equine hemoglobin, sodium nitroprusside, and benzidine are commercial samples. Horseradish peroxidase (28 purpurogallin units/mg) was purchased from Sigma Chemical Co., St. Louis and dissolved in 0.01M sodium phosphate buffer (pH 7.0). Male Sprague-Dawley strain rats (150—200 g) and male dd strain mice (15—20 g) were used after fasting for at least 12 hr.

Preparation of Tissue Extracts——Into the tail vein of rats and mice, was injected the solution (0.1—0.3 ml) in 0.9% NaCl of pure β-D-glucose oxidase with the dosage of 0.45 mg/kg. After the injection, the animals were given food and water *ad libitum*. The animals were killed by decapitation at varying intervals, and the brain, heart, lung, stomach, liver, spleen, kidney, small intestine, skeletal muscle, adipose tissue, and blood were quickly removed, cooled in ice, and weighed. Each tissue obtained from three mice or one rat was finely minced with scissors and homogenized for 2 min in 8 volumes of ice-cold sodium acetate buffer (0.1M, pH 4.0) with a Polytron homogenizer (from Kinematica G.m.b.H., Luzern, Switzerland). Homogenates were centrifuged at $118000 \times g$ for 30 min at 4°. To every one ml of the supernatants, 30 μl of 1% KCN was added for the inhibition of catalase and the respiratory enzymes. The mixture was incubated for 30 min at 40° to denature hemoglobin and other proteins, and then centrifuged at $10000 \times g$ for 5 min if necessary. The tissue extracts thus obtained can be stored for at least several days at −15° without any loss of enzyme activity. These extracts were diluted if necessary and subjected to the assay of β-D-glucose oxidase. It was supposed for calculations of enzyme content in tissues that every tissue contains water corresponding to 60% of its wet weight.

Assay of β-D-Glucose Oxidase Activity——Since β-D-glucose oxidase is known to catalyze the following reaction in the absence of catalase, the enzyme activity can be determined by measuring the rate of oxygen consumption in the presence of an excess amount of D-glucose.

$$\beta\text{-D-glucose} + O_2 + H_2O \longrightarrow \text{D-gluconic acid} + H_2O_2$$

The assay was performed as follows: The samples were aerated at 30° to equilibrate with air. One ml of each sample was put into a cylindrical glass vial (2.0 cm in height, 1.4 cm in diameter) placed on a magnetic stirrer and controlled at 30° with a water circulating system, and the electrode of an oxygen analyzer (Model 777, Beckman Instruments Inc., California) connected with a recorder (EPR-2TC, Toa Electronics Ltd., Tokyo) was immersed into the solution. After adjusting the meter reading of the recorder at a proper position (about 8 ppm) under continuous stirring with a stainless steel-stirring bar, 0.1 ml of 20% D-glucose was added as an excess amount of substrate to measure the initial velocity of oxygen consumption. The β-D-glucose oxidase activity was estimated from a calibration curve obtained by use of the pure enzyme and tissue extracts.

Histochemical Studies——For histochemical studies, β-D-glucose oxidase was intravenously injected into rats and mice with the dosage of 2.2 mg/kg for rats and 4.5 mg/kg for mice, because clear color development was not observed with the dosage used for distribution studies (0.45 mg/kg). The livers of rats and mice were removed 2 hr after the administration of the enzyme and frozen in dry-ice. Frozen sections of the livers of approximately 15 μ thickness were placed on slide glass and stored to dryness at 10°. One drop of 0.1% horseradish peroxidase was dropped on the sections and blotted with filter paper. Immediately after the blotting, one drop of hydrogen peroxide free Wachstein and Meisel's medium[13] containing saturated benzidine and 600 mg of sodium nitroprusside in 100 ml of 25% ethanol was dropped, and then blotted. When the sections were incubated for 5 min at 37° with one drop of 1% D-glucose, β-D-glucose oxidase in the livers was visualized owing to the oxidation of benzidine by hydrogen peroxide to deep blue colored dye (benzidine blue) in the presence of peroxidase. The stained sections were embedded in Roulet's glycerogel.[14] The livers of rats and mice into which 0.2 ml of 0.9% NaCl was intravenously injected were used as controls. Following equations summarize this histochemical method for β-D-glucose oxidase.

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\beta\text{-D-glucose}} \text{D-gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{benzidine} \xrightarrow{\text{peroxidase}} \text{benzidine blue} + 2H_2O$$

Preparation and Assay of Rat Liver Cathepsin D——Rat liver cathepsin D was prepared by extracting from the acetone-dried powder of rat liver with 0.05M sodium acetate buffer (pH 3.5) according to the method[15] of Ikezawa, *et al.* for preparing hog liver cathepsin D. Assay of cathepsin D was also done according to the method[15] of Ikezawa, *et al.* as follows:

A mixture of enzyme sample (1 ml) and 1% solution (3 ml) in 0.2M sodium acetate buffer (pH 3.2) of acid-

13) M. Wachstein and E. Meisel, *J. Histochem. Cytochem.*, **12**, 538 (1964).
14) F. Roulet, "Methoden der Pathologischen Histologie," Springer Verlag, Wien, 1948, p. 162.
15) H. Ikezawa and A. Makino, personal communication.

denatured equine hemoglobin was incubated at 37° for 30 min. After the incubation, 2 ml of 9% trichloroacetic acid was added and incubated for a further 30 min. The precipitate was filtered off and the E_{280} of the filtrate was measured. One unit of enzyme activity was the quantity that would have produced an increase in extinction (E_{280}) of 1.0 unit in the assay.

Preparation of Rat Liver Lysosomes——Rat liver lysosomes were prepared by the method of Ragab, *et al.*[16] Cathepsin D in lysosomes was assayed by the method of Ikezawa, *et al.*[15]

Result and Discussion

Distribution and *in Vivo* Degradation of β-D-Glucose Oxidase injected into Rats and Mice

The enzyme activity in various tissues of rats and mice injected with β-D-glucose oxidase was assayed at varying intervals after intravenous injection and expressed as percent of the injected dose (Table I).

Table I. Tissues Distribution of β-D-Glucose Oxidase intravenously injected into Rats and Mice

Minutes after injection	Rat				Mouse			
	5	20	40	60	5	20	40	60
	Percent of injected dose							
Brain	0.1	0	0	0	0.2	0.1	0	0
Heart	0.3	0	0	0	0.3	0.1	0	0
Lung	0.2	0.1	0	0	0.1	0	0	0
Stomach	0	0	0	0	0	0	0	0
Liver	51.5	70.0	81.5	78.0	20.5	73.1	73.3	82.0
Spleen	2.5	4.3	3.3	2.9	1.5	6.0	6.8	6.4
Kidney	0.1	0	0	0	0.3	0.2	0	0
Small intestine	0	0	0	0	0	0	0	0
Skeletal muscle	0	0	0	0	0	0	0	0
Adipose tissue	0	0	0	0	0	0	0	0
Blood	37.3	4.0	0	0	56.3	10.8	5.0	0

All data presented here are mean values obtained from 2 experiments. See the text for further details.

The enzyme injected into rats was removed from the circulation within 20 min, while it took about 60 min in the case of mice. The enzyme was largely taken up by the liver and slightly by the spleen in both cases of rats and mice. A trace amount of the enzyme found in the brain, heart, lung, and kidney only at the early stage may be derived from blood contained in those tissues. While the maximum distribution (80—85% of the injected dose) of the enzyme activity in the liver of rats was reached in 1—2 hr as well as in the case of mice, the enzyme activity in the spleen reached a first peak about 30 min after administration and then a second peak in 3—6 hr (Fig. 1 and 2). The first peak may be due to the enzyme activity in blood contained in the spleen and the second peak to that released little by little from the liver with time. Most of the enzyme activity in the liver and spleen disappeared in 60 hr.

The distribution pattern of β-D-glucose oxidase is distinctly different from that[4] of horseradish peroxidase injected into rats as a foreign protein. It had been reported by Straus[4] that the liver contained 29%, the kidney 21%, and the spleen 2.8% of the injected horseradish peroxidase 1 hr after intravenous administration (6.25 mg/kg) at which time the maximum concentration of the enzyme was observed in most of tissues, and that approximately 20% of the injected enzyme was excreted in the urine during the first 6 hr. The reason for which the difference occurs is remained unsolved yet, but it is an important problem for the further development of enzyme therapeutics.

16) H. Ragab, C. Beck, C. Dillard, and A.L. Tappel, *Biochim. Biophys. Acta*, **146**, 501 (1967).

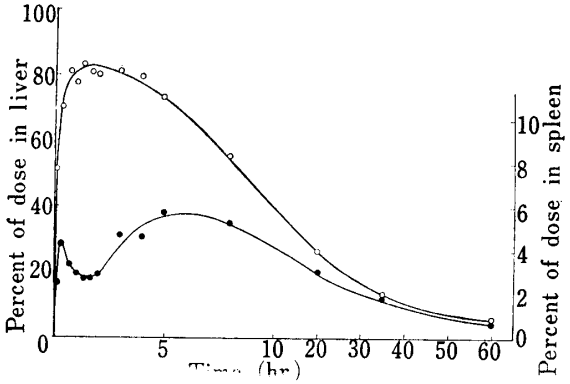

Fig. 1. Percent of the Injected β-D-Glucose Oxidase in the Liver and Spleen of Rats at Various Times after Intravenous Injection (0.45 mg/kg)

—○—: liver; —●—: spleen

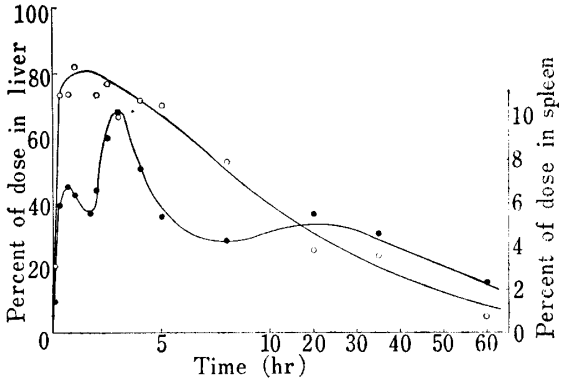

Fig. 2. Percent of the Injected β-D-Glucose Oxidase in the Liver and Spleen of Mice at Various Times after Intravenous Injection (0.45 mg/kg)

—○—: liver; —●—: spleen

Since any marked difference in the patterns of the distribution or *in vivo* degradation of β-D-glucose oxidase was not observed between rats and mice, it would be supposed that the difference[10] of LD_{50} of the injected β-D-glucose oxidase for rats (0.80 mg/kg) and mice(8.50 mg/kg) would not depend on the difference in the distribution or degradation of the enzyme. Therefore, this distribution study would rather support the conception that the difference would depend on the different susceptibility of the central nervous system of rats and mice to the simultaneous depletion of oxygen and D-glucose in blood as suggested in our previous paper.[10]

Histochemical Studies

It is well known that intravenously injected native plasma proteins, *e.g.* albumin,[2] ceruloplasmin,[8] and so on, are almost entirely transferred from blood plasma into the liver and taken up only by parenchymal cells (hepatocytes) and also that in contrast, denatured plasma proteins are deposited only in liver reticuloendothelial cells (Kupffer cells),[17] but relatively little is known of the localization of the injected native foreign proteins in the liver.[5,18]

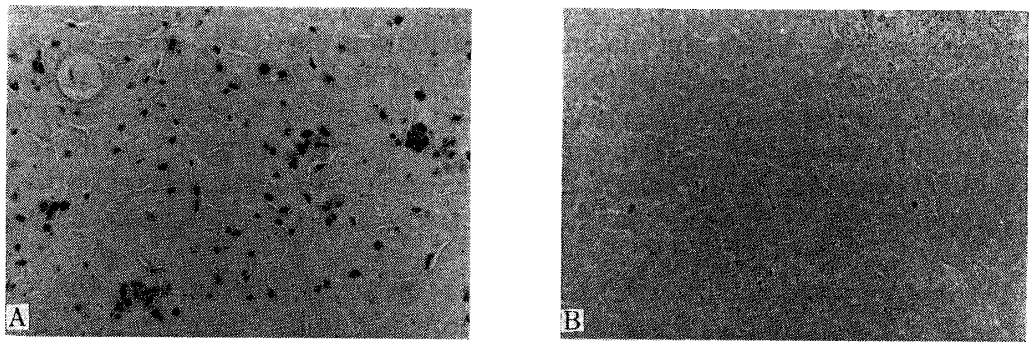

Fig. 3. Photographs showing the Localization in Mouse Liver of intravenously injected β-D-Glucose Oxidase

A: Section of mouse liver showing positive staining reaction for β-D-glucose oxidase 2 hr after the intravenous injection of the enzyme (4.5 mg/kg). Reaction product is present only in Kupffer cells as dark spots. A round-shaped blood vessel is seen on the left-upper side. Section of rat liver is also stained as in the case of mouse liver ×200.

B: Section of the liver of control mouse showing negative staining for β-D-glucose oxidase ×200.

17) B. Benacerraf, G. Biozzi, B.N. Halpern, C. Stiffel, and D. Mouton, *Brit. J. Exp. Pathol.*, **38**, 35 (1957).
18) R.C. Graham and R.W. Kellermeyer, *J. Histochem. Cytochem.*, **16**, 275 (1968).

Histochemical studies on the β-D-glucose oxidase taken up by the liver after intravenous injection into rats (2.2 mg/kg) and mice (4.5 mg/kg) indicated that the enzyme enters exclusively Kupffer cells and scarcely hepatocytes in both cases of rats and mice (Fig. 3). Although it has been demonstrated that native foreign proteins intravenously injected, *e.g.* horseradish peroxidase (120 mg/kg) into rats[5] and bovine lactoperoxidase (113 mg/kg) into mice,[18] are taken up prominently by Kupffer cells and also considerably by hepatocytes, the uptake of these enzymes by hepatocytes may be due to the large doses which were unavoidable for histochemical detection.

However, it has also been reported[7] that hemoglobin, although not a foreign protein, was barely demonstrable in rat liver parenchymal cells even after the intravenous injection of massive amounts of the protein (4 g/kg) and that it was taken up only by Kupffer cells at the lower doses of injected protein (150 mg/kg). So, further investigations are required to know what kind of intravenously injected native proteins enters preferentially Kupffer cells (or hepatocytes).

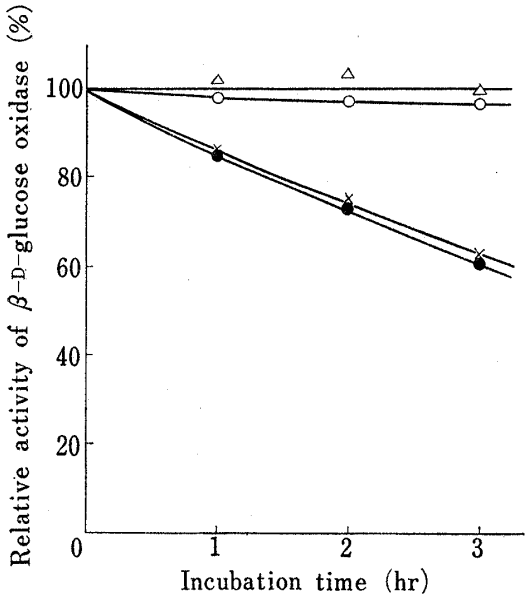

Fig. 4. Degradative Action of Cathepsin D and Lysosomes of Rat Liver on β-D-Glucose Oxidase

β-D-Glucose oxidase (10 μg) was dissolved in 10 ml of 0.1M sodium acetate buffer (pH 4.0) containing 30 units of rat liver cathepsin D or in 10 ml of the suspension in 0.25M sucrose containing 1mM, pH 7.0 EDTA buffer of rat liver lysosomes (cathepsin D activity: 11 units) and incubated at 37° over a period of 3 hr. One ml of the reaction mixture was pipetted out at the indicated times and the β-D-glucose oxidase activity was analyzed.
—△—: in cathepsin D solution
—○—: in lysosomes suspension
The liver lysosomes isolated from 5 rats that had been injected 90 min previously with β-D-glucose oxidase (0.45 mg/kg) were suspended in 5 ml of 0.25M sucrose containing 1mM, pH 7.0 EDTA buffer. The suspension (2.5 ml) was added to 12.5 ml of the same sucrose solution or to 12.5 ml of 0.1M sodium phosphate buffer(pH 7.0) and incubated at 37°. Two ml of the reaction mixture was pipetted out at the indicated times, sonicated for 3 min at 20kc and 60W in a sonic vibration generator (Model KMS-250, Kubota Seisakusho Co. Ltd., Tokyo), and subjected to the assay of β-D-glucose oxidase activity.
—×—: in sucrose solution;
—●—: in phosphate buffer

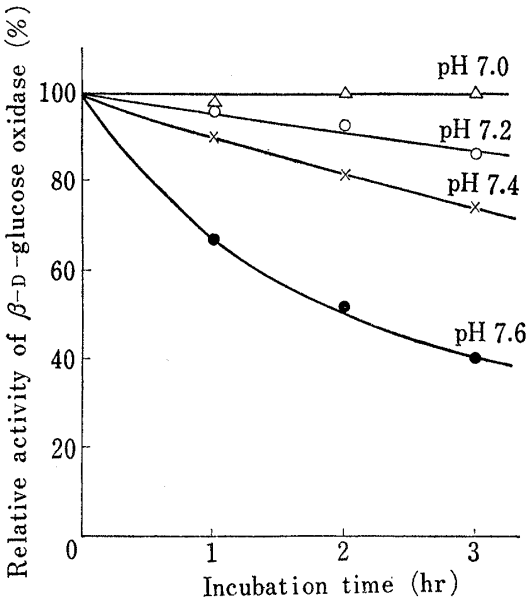

Fig. 5. pH Effect on Degradation of β-D-Glucose Oxidase

β-D-Glucose oxidase was dissolved in 0.1M sodium phosphate buffers of various pH values to be 1.0 μg/ml and incubated at 37°. One ml of each solution was pipetted out at the indicated times and subjected to the assay of β-D-glucose oxidase activity.

In Vitro Degradation of β-D-Glucose Oxidase

Since it is suggested that proteins taken up by the liver are digested by hydrolytic enzymes contained in lysosomes of the liver cells,[19,20] β-D-glucose oxidase was incubated at 37° with

19) J.L. Mego and J.D. McQueen, *Biochim. Biophys. Acta*, **100**, 136 (1965).
20) A.L. Tappel, "Lysosomes in Biology and Pathology," Vol. 2, ed. by J.T. Dingle and H.B. Fell, North-Holland Publishing Company, Amsterdam, 1969, p. 228.

rat liver lysosomes or cathepsin D, which is thought to primarily act as a proteolytic enzyme in lysosomes, but any lowering of β-D-glucose oxidase activity was not found in both cases as shown in Fig. 4. On the other hand, when the liver lysosomes isolated from rats that had been injected 90 min previously with β-D-glucose oxidase (0.45 mg/kg) were suspended in 0.25M sucrose containing 1 mM, pH 7.0 EDTA buffer or in 0.1M sodium phosphate buffer (pH 7.0) and then incubated at 37°, about 30% loss of β-D-glucose oxidase activity was observed after 3 hours' incubation in each case (Fig. 4). These data imply that the *in vitro* uptake of β-D-glucose oxidase by lysosomes is almost negligible, the rate of the inactivation of β-D-glucose oxidase taken up by rat liver lysosomes is about the same as that observed in the distribution studies, and the concerted action of hydrolytic enzymes, *e.g.* glycosidases and proteases, in lysosomes may be necessary for the inactivation of β-D-glucose oxidase, a glycoprotein,[21] as suggested by Mahadevan, *et al.*[22] for the degradation of fetuin, a serum glycoprotein.

Next, the influence of pH was tested on the stability of β-D-glucose oxidase, and it was found that the enzyme is rather rapidly inactivated if the pH deviates from 7.4 toward an alkaline side (Fig. 5). Therefore, the small changes of pH in Kupffer cells by which β-D-glucose oxidase was taken up would also give a potent influence upon the *in vivo* inactivation of the enzyme.

It may be given as a conclusion that the enzyme taken up by the liver is concertedly inactivated by the hydrolytic enzymes in lysosomes of Kupffer cells and the pH elevation (above 7.4) in the cells.

21) S. Nakamura and S. Fujiki, *J. Biochem.*, **63**, 51 (1968).
22) S. Mahadevan, C.J. Dillard, and A.L. Tappel, *Arch. Biochem. Biophys.*, **129**, 525 (1969).