containing receptors on the cell surface in a similar way to ricin (Nicolson and Blaustein, 1972). A similar situation is observed in the abrus seeds (Olsnes *et al.*, 1973[2]). It is an interesting possibility that the B chains of ricin and abrin represent monovalent lectins closely related to one of the constituent peptide chains of the agglutinin present in the seeds.

Acknowledgment

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Purification of Particulate Glucose-6-phosphatase†

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ABSTRACT: An ultracentrifugal method is described for the purification of glucose-6-phosphatase from a lyophilized microsomal fraction of rat liver. The method is based on a selective removal of protein and phospholipid by centrifuging the particles through successive layers of sucrose solution of increasing specific gravity, one layer containing buffer at pH 10 and another 0.4% deoxycholate at pH 7.5. The purified particles owing to the removal of phospholipids in the deoxycholate layer have an increased specific gravity and are recovered in the precipitate in a yield of up to 75% in terms of enzyme activity of the starting material and a six- to sevenfold increase in specific activity. Some properties of the purified particles are described. To date attempts to solubilize the enzyme with retention of activity have not been successful.

Glucose-6-phosphatase (EC 3.1.3.9), an enzyme bound to the endoplasmic reticulum of the liver and essential for blood glucose formation, has not so far been purified extensively, although the enzyme has been known for more than 30 years. Its association with the microsomal fraction in a liver homogenate and its dependence on phospholipids for activity have also been known for a long time (Hers *et al.*, 1951; Beaufay and de Duve, 1954). Two reasons are chiefly responsible for this situation—one is the instability of the enzyme and the other that solubilization of the enzyme with retention of activity has not been accomplished.

Our attempt has been to purify the enzyme as a particle by selective removal of other proteins and phospholipids. In a previous paper a chromatographic method for the separation of phospholipids from microsomal particles has been described (Garland and Cori, 1972). This method is briefly as follows. A lyophilized preparation (M_1), purified about 40-fold over a crude homogenate with respect to glucose-6-phosphatase activity, is dispersed by sonication in a small volume of pH 7.5 barbital buffer containing 0.4% deoxycholate. When applied to a column of Sepharose 4B equilibrated with the same buffer, the void volume contains most of the enzyme activity, whereas two-thirds of the protein inactive in the glucose-6-phosphatase test and more than 90% of the phospholipids appear in later fractions. The resulting enzyme preparation (M_2) required the addition of phospholipids for full activity and was purified about twofold over the starting material. Although the columns were run at 4°, some irreversible inactivation of the enzyme had occurred since the total recovery of activity in the different fractions rarely exceeded 50%. Deoxycholate even at 4° was found to inactivate the enzyme in a concentration- and time-dependent reaction. Concentrations of de-

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TABLE I: Buoyant Density Centrifugation of M_1.[a]

Fraction	% Protein	% Act.	Sp Act.	μmol of Lipid P/mg of Protein
1 + 2	25.5	35.4	1.68	1.93
3 + 4	16.2	19.9	1.50	1.84
5 + 6	16.3	15.0	1.12	1.71
7 + 8	13.0	12.1	1.14	1.56
9	6.8	6.4	1.14	1.43
10	22.2	11.2	0.62	0.76

[a] 1.1 mg of M_1 protein in 2.5 ml of a sucrose solution of sp gr 1.13 was overlaid with 2.5 ml of a sucrose solution of sp gr 1.10. Both sucrose solutions also contained 0.033 M barbital buffer–1 mM EDTA, pH 7.5. Centrifugation was for 17 hr at 40,000 rpm in the swinging bucket head at 3°. After centrifugation, 0.5-ml samples were removed from the top down and the precipitate was suspended in the last 0.5 ml. The M_1 protein had a sp act. of 1.2 μmol of P formed/mg of protein per min at 30° and contained 1.55 μmol of lipid P/mg of protein.

TABLE II: Buoyant Density Centrifugation of M_2.[a]

	Specific Gravity				
	1.08	1.13	1.17	1.23	1.29
% protein	0.7 (1)	4.2 (2)	72.0 (3)	17.9 (2)	5.2 (1)
% activity	0.6 (1)	4.9 (2)	72.3 (3)	18.6 (2)	3.6 (1)

[a] 1.3 mg of M_2 protein containing 0.11 μmol of lipid P/mg of protein was dispersed in 0.65 ml of sucrose solution of sp gr 1.17. Sucrose solutions also contained 0.033 M barbital–1 mM EDTA, pH 7.5. In three plastic centrifuge tubes of 0.6-ml capacity the layering of three 0.2-ml samples was as follows from the bottom up: tube 1, sp gr 1.17, 1.13, 1.08, tube 2, sp gr 1.23, 1.17, 1.13; tube 3, sp gr 1.29, 1.23, 1.17. In each case, sp gr 1.17 contained the M_2 protein. The samples were spun for 23 hr at 40,000 rpm in the swinging bucket head at 3°. After centrifugation, two samples of 0.2-ml each were removed from the top down and the precipitate, if any, was suspended in the remaining 0.2 ml. The numbers in parentheses indicate the number of samples available at each specific gravity. Averages of per cent of protein and enzyme activity at each specific gravity are recorded. The activity was assayed in the presence of phosphatidylcholine as described under Materials and Methods.

oxycholate below 0.4% were less effective and the time required for column chromatography could not be shortened. It became clear that progress depended on developing different methods of separation. One such method which depends on ultracentrifugal separation of the particles is described in this paper.

Materials and Methods

Lyophilized microsomal fractions M, M_1, and M_2 were prepared from rat livers as previously described (Garland and Cori, 1972).[1] Analysis of inorganic P was by the Fiske–Subbarow (1925) or Ames (1966) method, depending on amounts available. The ashing of samples for lipid P determinations was carried out by the Ames method. Protein was determined by the method of Lowry *et al.* (1951). A standard curve for bovine serum albumin was run with each determination. Aliquots of solutions containing sucrose were generally small enough so that sucrose did not interfere. Glycerol (0.1 ml of 4%) gives a color in the protein method and accordingly shifts the standard curve upward.

The source of bile salts, phospholipids, and glucose 6-phosphate has been given previously. Glucose 6-phosphate was freed of inorganic phosphate as described previously.

All enzyme activity tests were for 20 min at 30° with 0.02 or 0.04 M glucose 6-phosphate, pH 6.8, in a volume of 0.5 ml.[2] The M_2 fractions were tested without and with added phospholipids (in each case 0.02 ml of 0.17% deoxycholate and 0.67 mg/ml of egg phosphatidylcholine per test). All other fractions were tested without added phospholipids. Specific activity is given in micromoles of P_i formed per milligram of protein per minute.

A Beckman L2-50 preparative ultracentrifuge with the swinging bucket head SW50 was used and was run at 3°. This head holds three centrifuge tubes of either 0.6- or 5-ml capacity. The latter were used for the preparation of M_2 fractions, while the former were used in some experiments on buoyant density centrifugation. Sonication was performed with a Bronwill Biosonik III using the small probe tip. Unless otherwise stated, lyophilized powders and precipitates were dispersed in solutions by sonication.

Results

As a preliminary the specific gravities of M_1 and M_2 particles in a sucrose gradient were determined.

Buoyant Density Centrifugation. The buoyant density of the bulk of the M_1 particles was found to be between 1.10 and 1.13. An experiment is shown in Table I. Fractions 1–4 in sucrose solution of sp gr 1.10 contained 42% of the protein and 55% of the enzyme activity. The buoyancy of the particles in the sucrose gradient appeared to be determined by their phospholipid content. The lighter fractions contained not only more phospholipid per unit of protein but also enzyme of higher specific activity than the original material, while the precipitate (fraction 10) was lower in both. Fractions 1 + 2 of Table I in 2.5 ml of sucrose solution of sp gr 1.10 were overlaid with 2.5 ml of sucrose solution of sp gr 1.08 and recentrifuged and analyzed in the same way as in Table I. The bulk of the protein and enzyme activity (over 75%) was distributed in fractions 5–8 of sp gr 1.10 and only about 10% had equilibrated with sucrose of sp gr 1.08. The precipitate contained only about 2% of the protein. As an indication that more homogeneous material had

[1] M is the fluffy layer obtained by centrifugation of a deoxycholate treated microsomal preparation. M_1, the first purified fraction, is prepared from M by treatment with pH 10 buffer. M_2 as indicated above is a delipidated particle prepared from M_1 either by column chromatography or by methods to be described below.

[2] With the more highly purified fractions 10–20 μg of protein per test were needed, when the Fiske–Subbarow (1925) method was used for analysis, but only 1/50th as much when the Ames method was used. In some cases, the specific activity was higher in the former than in the latter case. This is due to the fact that especially with M_2 fractions proportionality between activity and protein concentration breaks down at very low concentrations. Addition of 1 mg of bovine serum albumin to the reaction mixture exerts a protective action.

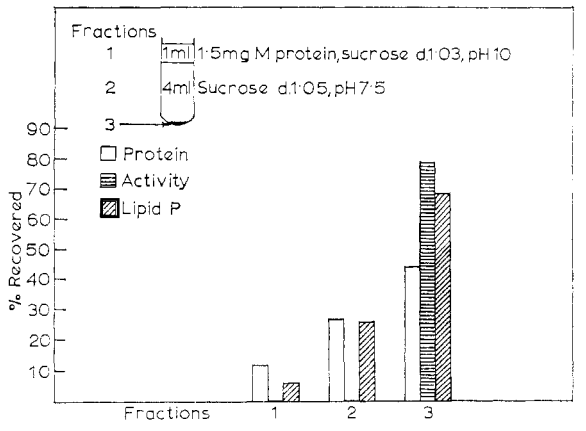

FIGURE 1: Conversion of M to M_1. Lyophilized M of sp act. 0.5 was dispersed in sucrose solution of sp gr 1.03 containing 0.01 M Tris–1 mM EDTA, pH 10, and was layered over a sucrose solution of sp gr 1.05 containing 0.033 M barbital–1 mM EDTA, pH 7.5. Centrifugation was for 2 hr at 50,000 rpm. Fractions were collected from the top down and the precipitate was dispersed in the barbital–EDTA buffer at pH 7.5.

been isolated by the first centrifugation, there was little variation of specific activity in the different fractions.

It seemed likely that removal of over 90% of phospholipid in the preparation of M_2 would result in particles of greater specific gravity. This was found to be the case (Table II). There was a rather sharp peak of protein and enzyme activity in sucrose solution of sp gr 1.17, with a little tailing at sp gr 1.23.

Preparation of M_1 Particles by Centrifugation. The preparation of lyophilized M fraction, stable for several months at −20°, and its conversion to M_1 has been described. M is converted to M_1 by dispersing it in sucrose solution of sp gr 1.03 containing 0.01 M Tris-HCl and 1 mM EDTA, pH 10 (measured at room temperature), and centrifuging down the residue. Protein of low specific activity remains in the supernatant fluid, so that a two- to threefold purification is achieved by this procedure. In a modification of this procedure the sucrose solution containing the M protein is layered on a sucrose solution of sp gr 1.05 containing 0.033 M barbital and 1 mM EDTA, pH 7.5. Since according to Table I the bulk of the M_1 particles has a sp gr of 1.10, they should appear in the precipitate after centrifugation. This was found to be the case. In the experiment in Figure 1 79% of the activity and 45% of the protein were recovered in the precipitate resulting in a doubling of the specific activity.

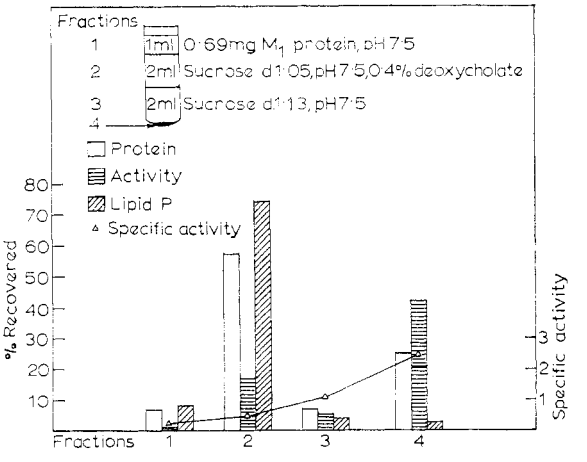

FIGURE 2: Conversion of M_1 to M_2. Lyophilized M_1 of sp act. 1.4 and containing 2.1 μmol of lipid P/mg of protein was dispersed in 0.033 M barbital–1 mM EDTA, pH 7.5. The same buffer was also present in the sucrose solutions of the botton and middle layers. Centrifugation was for 100 min at 50,000 rpm. The particles in the precipitate (fraction 4) contained 0.22 μmol of lipid P/mg of protein and required the addition of phosphatidylcholine for maximal activity. The bar indicates the per cent activity obtained without added phosphatidylcholine, while the total height of the column corresponds to activity with phosphatidylcholine. The sp act. of 2.4 is that obtained with added phosphatidylcholine.

TABLE III: Effect of Deoxycholate on Sedimentation of M_2 Particles.[a]

% Deoxycholate	Depth of Middle Layer (ml)	% Act. Recovd in Ppt	μmol of P/mg of Protein in Ppt
None	2	4.2	1.41
0.2	2	8.8	0.93
0.4	2	41.6	0.23
0.4	1	54.5	0.39
0.6	1	31.7	0.21
0.8	1	31.7	0.20

[a] The layering in a centrifuge tube of 5-ml capacity was as follows: bottom, 2 or 3 ml of sucrose solution (sp gr 1.10); middle, 1 or 2 ml of sucrose solution (sp gr 1.05) containing varying concentrations of deoxycholate; top, 1 ml of M_1 in 0.033 M barbital–1 mM EDTA, pH 7.5, containing 0.7–1.0 mg of protein. The same barbital buffer was also present in the sucrose solutions. Centrifugation was for 2 hr at 50,000 rpm in the swinging bucket head. The supernatant fluid was poured off and the precipitate dispersed in the above barbital buffer.

Preparation of M_2 Particles by Centrifugation. This is based on the following considerations. If M_1 particles having a sp gr of about 1.10 were layered over a sucrose solution of sp gr 1.05 containing deoxycholate, they would pass through this layer during centrifugation and lose their phospholipids. This would create M_2 particles of sp gr 1.17 which would pass through an underlying sucrose solution of sp gr 1.10 and appear in the precipitate. An experiment of this type is shown in Figure 2. It can be seen that the precipitate (fraction 4) contained a considerable part of the original enzyme activity and that the addition of phospholipids was required for maximal activity. By analysis, the precipitate contained 0.22 μmol of lipid P/mg of protein, while the original M_1 particles contained 2.1 μmol/mg of protein. Thus, most of the phospholipids were retained in fraction 2 which also contained a considerable amount of protein of low enzyme activity. As a result the specific activity of the enzyme in the particles was increased nearly twofold by this procedure.

In order to find optimal conditions for purification of the enzyme on the particles, the effect of a number of variations was explored (Table III). It can be seen that not enough phospholipid was removed during passage of the particles through a layer of 0.2% deoxycholate to change their buoyancy. Consequently, little activity was recovered in the precipitate. With 2 ml of 0.4% deoxycholate more phospholipid was removed than with 1 ml, but the recovery of activity in the precipitate was better with 1 ml, owing to shorter contacts with deoxycholate. Concentrations of deoxycholate greater than 0.4% caused considerable enzyme inactivation

TABLE IV: Reproducibility and Stability of M_2 Preparations.[a]

Prepn	Sp Act.	μmol of Lipid P/mg of Protein	Remarks
1	4.0	0.37	As prepared
	3.7		Lyophilized, tested after 1 day
2	4.4	0.39	As prepared (expt in Figure 3)
	3.6		Lyophilized, tested after 11 days
3	3.8	0.33	Lyophilized, tested after 1 day
	4.0		Lyophilized, tested after 5 days
4	3.6	0.27	As prepared
	3.4		Frozen[b] in 4% glycerol, tested after 18 days
	1.9		Frozen[b] without glycerol, tested after 19 days

[a] The lyophilized M powder used as starting material, the amount used (about 1.5 mg of protein per tube), and the method of preparation of M_2 (fraction 5) were the same as in Figure 3. Of three tubes of identical make-up centrifuged simultaneously, one was used for immediate analysis while the others were used for tests of stability under various conditions. [b] Samples also contained 0.033 M barbital buffer–1 mM EDTA, pH 7.5.

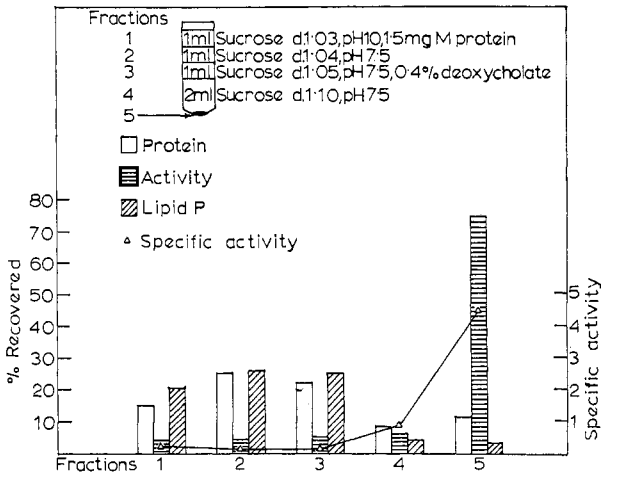

FIGURE 3: Conversion of M to M_2. The lyophilized M had a sp act. of 0.6 and contained 1.2 μmol of lipid P/mg of protein. Layers 1 and 3 are equivalent to those in Figure 1, while layers 3 and 4 correspond to layers 2 and 3 in Figure 2. Centrifugation was for 2 hr at 50,000 rpm. The protein in fraction 5 contained 0.39 μmol of lipid P/mg and there was no effect of added phosphatidylcholine on activity.

as shown by poor recovery of activity in the precipitate. As a compromise between incomplete removal of phospholipids and enzyme inactivation, the conditions used in experiment 4 were chosen for further work.

Combined Procedure for Conversion of M to M_2 Particles. The conversion of M to M_2 in one operation is shown in Figure 3. In this experiment the summated fractions gave a recovery of protein, activity, and phospholipid of 80, 93, and 83%, respectively. Thus, there appeared to be little if any irreversible inactivation of the enzyme. The protein in fractions 1–3 had very low specific activity and was accompanied by phospholipid, giving it buoyancy in the sucrose solution of sp gr 1.05. The precipitate contained 75% of the original activity of the M fraction, but only 10.5% of the protein, resulting in a sevenfold increase in specific activity. The phospholipid content of the M_2 fraction was 0.39 μmol of P/mg of protein. A second passage of M_2 particles through a 1-ml layer containing 0.8% deoxycholate reduced the phospholipid content to 0.13 μmol of P/mg of protein, but did not result in the solubilization of significant amounts of protein.[3] The substitution of 0.3 or 0.5% deoxycholate or 0.4% Triton X-100 for 0.4% deoxycholate gave M_2 particles of lower specific activity. When 0.4 or 0.6% cholate was substituted for deoxycholate, phospholipid and protein were incompletely removed, so that little purification was effected.

In one experiment the order of layering shown in Figure 3 was reversed, that is, the particles during centrifugation passed first through the layer containing 0.4% deoxycholate and then through the layer containing pH 10 buffer. A centrifugation with the usual sequence of layering was carried out simultaneously. The results showed that irrespective of the order, each of the two treatments produced their separate effects in solubilizing protein from the particles. The resulting M_2 preparations also showed nearly the same specific activity.

Reproducibility was quite good when the same M powder was used as starting material and when similar amounts were processed in the different preparations (Table IV). The stability of different M_2 preparations, either as lyophilized powder or frozen in 4% glycerol, was satisfactory in most cases. In several trials activity was completely preserved for up to 2 weeks when the centrifuged M_2 particles were kept as such at −20° in a stoppered tube. In general, the M_2 preparations are more sensitive to inactivating influences than M_1, which in turn is more sensitive than M.

Some Properties of the M_2 Fraction. A phospholipid content of the M_2 particles below 0.4 μmol/mg of protein requires the addition of phospholipid for full enzymatic activity (Garland and Cori, 1972). The K_m for glucose 6-phosphate was determined over a 12-fold range of concentrations with an M_2 preparation that was doubled in its activity by the addition of phosphatidylcholine. With added phosphatidylcholine the K_m (determined from double reciprocal plots) was 2.0 mM. A similar M_2 preparation without added phosphatidylcholine gave a K_m value of 2.1 mM. These K_m values are not substantially different from those obtained with M_1 particles which have a much higher phospholipid content.[4] It thus appears that the effect of phospholipid on activity is not mediated through changes in the affinity of the enzyme for its substrate.

The pH–activity curve of M_2 with added phosphatidylcholine is fairly flat between pH 6.2 and 7.0; that is, at these two pH values the activity was 94 and 96% of maximum, respectively. The curve then falls off rather steeply on the alkaline side and somewhat less so on the acid side. To give a few more reference points, the activity was 63, 72, and 43% of

[3] Complete removal of phospholipids has not been accomplished so far. The nature of this tightly bound residual phosphate is being determined. An M_2 preparation containing less than 0.1 μmol of phospholipid/mg of protein was extracted with chloroform–methanol. Thin layer chromatography of the extract on silica gel showed the presence of the usual phospholipids found in liver microsomes except for the absence of lysolecithin.

[4] It should be noted that both M_1 and M_2 had been exposed to deoxycholate during their preparation. Treatment of a sucrose homogenate of rat liver with 0.2% deoxycholate has been reported to decrease the average K_m for glucose 6-phosphate from 2.2 to 1.7 mM and to increase V_{max} about 1.2-fold (Hanson and Nordlie, 1970). The more purified M_1 and M_2 fractions are never stimulated only inhibited by deoxycholate.

maximum at pH 5.0, 7.5, and 8.0, respectively. The pH–activity curve for M_1 is very similar.

The enzyme is inhibited by *p*-chloromercuribenzoate, suggesting that SH groups are essential for its activity. Thus, exposure of an M_1 preparation for 10 min at 30° to 2×10^{-5} M *p*-chloromercuribenzoate resulted in 59% inhibition, whereas addition of the mercurial at the start of the activity measurements gave only 11% inhibition, from which it can be seen that the substrate (20 mM glucose 6-phosphate) exerts a protective action. In a similar experiment with 2×10^{-4} M of the mercurial, the inhibition of enzyme activity reached 90%. Addition of 10 mM cysteine or dithiothreitol after exposure to the mercurial resulted in only a slight reversal of the inhibition. M_2 preparations were inhibited in a similar manner as the M_1 preparations and the substrate exerted a protective action against the inhibition. No protein was solubilized from M_2 preparations after exposure to the mercurial.

Glucose-6-phosphatase in the M_1 and M_2 particles is accessible to proteolytic enzymes such as trypsin, chymotrypsin, papain, and a fungal protease from *Streptomyces griseus*. In each case the enzyme can be protected from inactivation by its substrate. Attempts to make use of these observations in the purification of the enzyme were so far unsuccessful. Two examples may be cited. An M_2 preparation of sp act. 3.74 was treated for 20 min at 30° with 50 μg of a fungal protease in the presence of 0.04 M glucose 6-phosphate and was then centrifuged for 2 hr at 50,000 rpm. The precipitate contained 69.5% of the protein with a specific activity of 3.75. That protein had been solubilized could also be shown by analysis of the supernatant fluid with the Lowry method[5] and by column chromatography on Sephadex G-200. In another experiment 48% of the protein was solubilized again without any change in the specific activity of the protein recovered in the precipitate. In both cases the solubilized protein did not show any glucose-6-phosphatase activity. This result is unusual, for if there was a loss of protein from each particle, there should have been an increase in specific activity in the precipitate. It would appear that unless a method for the solubilization of active enzyme can be found, little additional purification will be possible.

Discussion

The vesicles formed from the endoplasmic reticulum by homogenization contain tightly bound glucose-6-phosphatase besides many other proteins, some of which can be solubilized selectively, thereby increasing the specific activity of the enzyme on the particles. The chief problem is how to avoid irreversible inactivation of a very labile enzyme, because a particle carrying both active and inactive enzyme molecules cannot be separated into active and inactive fractions. The specific activity of the final product will therefore depend on the activity of the starting material and the success in removing other proteins without inactivation of glucose-6-phosphatase. Two procedures have been found successful. The first one is extraction of the particles with alkaline sucrose solution. This removes loosely bound proteins and probably also proteins trapped within the lumen of the vesicles. The second procedure consists in extraction of both protein and phospholipid with a solution containing 0.4% deoxycholate. Since deoxycholate inactivates the enzyme irreversibly, even at 3°, speed is essential. The present method combines these two procedures in one operation and is rapid enough so that up to 75% of the enzyme activity can be recovered in the final product with a six- to sevenfold increase in specific activity over the starting material. The starting material is a purified microsomal fraction (M fraction) enriched about 18-fold over the crude homogenate.[6] It seems probable that further progress in the purification of the enzyme will depend on finding a method for its solubilization.

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[5] From this analysis one calculates a greater loss of protein in the precipitate than has actually been found, presumably because the peptides give a stronger biuret color than the original proteins.

[6] The degree of purification depends on how one interprets the effect of detergents on K_m and V_{max} (*cf.* footnote 4). In crude homogenates, the detergents through their effect on the phospholipid membrane could increase the accessibility of substrate to enzyme as reflected in the changes in V_{max} and K_m or they could bring out some latent enzyme activity. In the latter case the purification would be less by a factor determined by the increase in V_{max} by detergents, it being assumed that no latent enzyme remains in subsequent fractions after one treatment with detergent.