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Relipidation of Phospholipid-Depleted Microsomal Particles with High Glucose 6-Phosphatase Activity

(enzyme enrichment/membrane effects/latency/diabetes/phenobarbital)

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ABSTRACT **Microsomal particles enriched up to 20-fold in glucose 6-phosphatase activity (as compared to crude microsomal fractions) were prepared from livers of phenobarbital-treated, normal, and diabetic rats by a method involving sucrose-density gradient centrifugation through a layer containing deoxycholate, followed by flotation of the delipidated particles in a phospholipid-detergent mixture. The flotation with phospholipid resulted in the extraction of additional protein, in a corresponding increase in specific activity, and in relipidation of the particles to their original phospholipid content. Detergents alone did not extract any additional protein. Relipidation caused a change in the properties of the microsomal membrane, as indicated by a 4-fold decrease in the K_m for glucose 6-phosphate and mannose 6-phosphate. The maximal rate was not affected. The crude homogenate of diabetic rat liver contained more latent enzyme than that of normal rats. The diabetic rats also yielded purified microsomal particles of specific glucose 6-phosphatase activity twice that of normal and five times that of phenobarbital-treated rats, indicating that some regulatory mechanism exists for the incorporation of different amounts of the enzyme into the endoplasmic reticulum.**

In a centrifugal method of purification described in a previous paper (1) use is made of the fact that microsomal particles, as they pass through a layer of sucrose containing deoxycholate, lose protein and phospholipid, but retain glucose 6-phosphatase which is recovered in the precipitate. Depending on the depth of the layer and the concentration of deoxycholate, up to 90% of the phospholipids could be removed. This resulted in loss of glucose 6-phosphatase activity, which could be fully restored by adding to the enzyme reaction mixture certain phospholipids, among which phosphatidylcholine was the most effective.

In the present paper, a flotation method for the relipidation of the depleted particles is described, which results in a further increase in specific activity and a decrease in the K_m value for substrate. The particles were prepared from the livers of normal rats and mice, from livers of rats treated with phenobarbital, and from diabetic rats. Some properties of the purified microsomal particles are described.

MATERIALS AND METHODS

The preparation of lyophilized M fraction from liver microsomes has been described (2). M is converted to M_2 as follows. In a 5-ml plastic centrifuge tube, the layers from the bottom up are: 2.0 ml of sucrose solution, density (d) 1.10 g/ml; 1.0 ml of sucrose solution, d 1.05, containing 0.4% deoxycholate; 1 ml of sucrose solution, d 1.04,—all of these buffered with 33 mM Na barbital, 1 mM ethylenediaminetetraacetate (EDTA) at pH 7.5—and finally 1–2 mg of M protein in 1 ml of sucrose solution, d 1.03, 0.1 M Tris·HCl, 1 mM EDTA, pH 10. After centrifugation for 2 hr at 50,000 rpm at 3° in the swinging bucket head of a Beckman L2-50 preparative ultracentrifuge, the precipitate is collected and stored frozen if not used immediately.[a] Lyophilized powders and precipitates were dispersed in solution by sonication with the Bronwill Biosonik II using the small probe tip.

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. The M_2 fractions were tested without and with added phospholipid (14 μg of egg phosphatidylcholine plus 34 μg of deoxycholate per test). All other fractions were tested without added phospholipids. Routinely, P_i was determined by the Fiske–Subbarow method (3). For the determination of lipid P the samples were ashed and analyzed by the Ames method (4).

Specific activity is given in μmoles of P_i formed per mg of protein per min at 30°. Protein was determined by the Lowry *et al.* method (5) with bovine serum albumin as standard. The occasional turbidity caused by added phospholipids was prevented by the addition of deoxycholate.

Deoxycholate was determined by the method of Mosbach *et al.* (6). Since sucrose interferes, aliquots of 0.01 ml of solutions containing sucrose were first precipitated with 0.1 ml of a mixture of four parts of 5% perchloric acid and one part of 50% trichloroacetic acid. The precipitate was washed once with 0.02 ml of the above mixture and dissolved in 0.07 ml of 0.1 M NaOH. The recovery of deoxycholate was 70%.

The source of bile salts, phospholipids, and glucose 6-phosphate has been given previously (2). Mannose 6-phosphate was obtained from the Sigma Chemical Co. The concentrations of the phosphorylated compounds in stock solutions were determined by ashing.

Sepharose 4B and Blue Dextran (for determination of void volume) were obtained from Pharmacia. Sepharose 4B columns (0.9 × 25 cm) were equilibrated with 33 mM Na barbital, pH 7.5. Freeze-dried M_2 samples were dispersed in 0.3–

Abbreviations: EDTA, ethylenediaminetetraacetate; d, density, g/ml. M, M_1, and M_2 are microsomal preparations containing increasing amounts of glucose 6-phosphatase activity per mg of protein.

[a] The amount of protein and phospholipid solubilized in the different fractions above the precipitate has been illustrated in Fig. 3 of ref. 1.

TABLE 1. *Enzyme activity of particulate glucose 6-phosphatase from the livers of phenobarbital-treated, normal, and diabetic rats*

Fraction	Phenobarbital	Normal*		Normal		Diabetic	
		−	+	−	+	−	+
Homogenate	0.024	0.05	0.07	0.045	0.06	0.045	0.11
Microsomes	0.08	0.16	0.30	0.16	0.28	0.26	0.55
Lyophilized M	0.33	0.63		0.68		1.61	
Delipidated M_2	1.40	3.11		3.50		7.03†	
Relipidated M_2		4.25		4.82		10.53†	

Enzyme activity is given in μmoles/mg of protein per min at 30° without (−) and with (+) 0.04% deoxycholate. The different fractions are described in the *text*.

* Mouse liver.

† Average of three experiments.

0.4 ml of the buffer solution by a 5-sec sonication with the Biosonik. Elution was with the solutions described in the legends of Figs. 1 and 2. Fractions of 1 ml were collected.

For the production of a proliferated hepatic endoplasmic reticulum, rats were injected intraperitoneally for 7 days with 10 mg of Na phenobarbital per 100 g. Diabetes was produced by intravenous injection of streptozotocin at 7.5 mg/100 g.[b] After 1 month, animals with fasting blood glucose of 300 mg/100 ml or higher were used for the preparation of lyophilized M.

RESULTS

The data in Table 1 show the following. Crude homogenates as well as microsomes, prepared as previously described, show latent enzyme activity which can be made evident by running an activity test in the presence of 0.04% deoxycholate or Triton X-100. By this criterion, livers of diabetic rats contain more latent enzyme than livers of normal rats. In the preparation of lyophilized M as previously described, the microsomes are exposed to 0.24% deoxycholate and this has the effect that no latent enzyme persists in later stages of the preparation. The problem of latency will be discussed later.

M is now directly converted to M_2 by the centrifugal method of purification (1), omitting an intermediate M_1 stage. It can be seen that the purified M_2 particles derived from the endoplasmic reticulum of rats with diabetes of one month duration show twice as much glucose 6-phosphatase activity per mg of protein as those of normal rats or mice, a difference which is also evident in the crude homogenate tested in the presence of deoxycholate but not in its absence. In other words, the extra amount of enzyme in the diabetic liver seems to be present mainly in the form of latent enzyme.

The marked hypertrophy of the endoplasmic reticulum that is brought about by phenobarbital treatment is associated with a decrease in the amount of glucose 6 phosphatase per mg of microsomal protein (Table 1). Thus, when the endoplasmic reticulum is laid down, various amounts of glucose 6-phosphatase become incorporated under different conditions. It is not known what regulatory factors are involved in this enzyme–membrane relationship.

Solubility of M_2 Protein. The purification of glucose 6-phosphatase achieved so far (Table 1) is based on differential extraction of other proteins from the microsomal particles. This raised the question whether the limit of this method had been reached. Table 2 shows that neither 0.5 M KCl alone nor 1% glycodeoxycholate[c] alone extracts much protein from the M_2 particles, but that both together extract about $^2/_3$ of the protein. However, no activity was recovered in the supernatant fraction and the specific activity in the precipitate decreased to $^1/_2$ the original value.

In the last experiment in Table 2 the sonicated mixture was immediately diluted 10-fold before centrifugation in order to see whether any reaggregation of particles occurred. This did not seem to be the case. Although in this case the inactivating effect of deoxycholate (2) was minimized by dilution, the specific activity in the precipitate was not increased.

Addition of 0.5 M KCl to the 0.4% deoxycholate layer in the standard M_2 preparation did not achieve further purification of the enzyme.

Column Chromatography of M_2 Particles. An unexpected finding that made further development possible is illustrated in Figs. 1 and 2. When an M_2 preparation is eluted from a Sepharose 4B column with 33 mM barbital, 1 mM EDTA, pH 7.5, practically all of the protein and activity appears in the void volume fraction. Fig. 1 shows that this is so, even if 0.4% deoxycholate is present in the buffer used for elution. However, if in addition to deoxycholate there is also present phosphatidylcholine in the elution fluid (Fig. 2), about 50% of the protein becomes solubilized and appears as a peak after the void volume fraction. An increase in the specific activity of the M_2 particles recovered from the void volume fraction indicates that proteins other than the enzyme were solubilized.[d]

In order to make use of these observations for preparative purposes and to study them in greater detail, a method of flotation has been developed which is described in the next section.

[b] We wish to thank the Upjohn Co., courtesy of Dr. W. E. Dulin, for a generous supply of streptozotocin.

[c] Glycodeoxycholate was used instead of deoxycholate because the latter forms a gel during centrifugation with 0.5 M KCl at 3°. The maximum relative centrifugal force of 98,600 × g for 2 hr was enough to satisfy the operational definition of soluble protein usually adopted, namely, not sedimentable in one hour at 144,000 × g.

[d] It has been noted previously that, when protein is extracted from the particles during preparation of M_2, it is generally accompanied by a relatively constant amount of phospholipid in the different fractions, about 1 μmole or more of phospholipid per mg of protein (1). The solubilization of protein by added phospholipid as in Fig. 2 could be due to phospholipid exchange and elution of protein attached to phospholipid by hydrophobic interaction.

TABLE 2. *Effect of KCl and glycodeoxycholate (GDOC) on solubility of M_2 protein*

Mixture	% Protein recovered after centrifugation		
	Supernatant	Precipitate	Total
0.5 M KCl	2	82	84
1% GDOC	19	70	89
1% GDOC + 0.5 M KCl	67	25	92
1% GDOC + 0.5 M KCl*	48	33	81

M_2 in 0.02 M barbital, pH 9.5, was sonicated in the cold in the mixtures shown in column 1. Aliquots were centrifuged for 2 hr at 30,000 rpm at 3° in the SW 50 swinging bucket head. Protein was determined in the original mixture and in the supernatant and precipitate after centrifugation.

* Entire mixture diluted 10-fold before centrifugation.

Flotation and Relipidation of M_2 Particles. The relipidation of M_2 particles (Table 1) by a method involving flotation is illustrated in Fig. 3. M_2 particles containing 0.3 μmole of residual lipid P per mg of protein have a specific gravity of about 1.17, whereas microsomal particles containing 1.3–1.5 μmoles of lipid P per mg of protein have a specific gravity of about 1.10 (1). Consequently, if relipidation of the M_2 particles occurs in the experiment in Fig. 3, they should float up during centrifugation and there should be no precipitate. This is found to be the case. It can be seen that fractions 2 and 3 with sucrose solution of d 1.10 to 1.12 contain the bulk of protein and enzyme activity. These peak fractions also contain enzyme of higher specific activity than the starting material, owing to the fact that about 37% of protein of low specific activity is retained in other fractions. Deoxycholate did not float up with the phospholipid, most of it remaining in the 1 ml bottom fraction.[e]

Direct evidence for the binding of phosphatidylcholine to the M_2 particles during flotation is shown in Table 3. In these experiments different M_2 preparations were floated by centrifugation in a sucrose–phosphatidylcholine–deoxycholate mixture as in Fig. 3. The peak fraction containing active enzyme in sucrose solution of d about 1.11 was diluted 5-fold with 33 mM barbital, 1 mM EDTA, pH 7.5, and recentrifuged. In each case, the recentrifuged M_2 particles carried with them an amount of phospholipid that was independent of the amount added and that corresponded roughly to that present in the microsomal M fraction before delipidation. When deoxycholate was omitted from the flotation mixture, there seemed to be less phospholipid bound to the M_2 particles than when it was present. The recentrifuged M_2 particles were fully active enzymatically without added phospholipids.

As a further check on the state of the membrane after relipidation of the M_2 particles, the K_m was determined (Table 4). Double reciprocal plots were linear for a range of substrate concentrations of 0.5–6 mM. It can be seen that relipidation caused a marked decrease in the K_m for glucose 6-phosphate as well as mannose 6-phosphate without much effect on V_{max}.[f] A possible interpretation is that, as additional phospholipid molecules are inserted in the membrane between the enzyme molecules, the enzyme assumes a more favorable configuration of the active site, resulting in increased affinity for substrate.

[e] In this experiment, the molar ratio deoxycholate/phosphatidylcholine in the mixture used for flotation was 8.5/3.1. In other experiments, ratios of 8.5/1.7 and 4.25/1.5 gave results very similar to those obtained with the 8.5/3.1 ratio.

[f] It has been shown previously that the mere addition of phospholipid to the enzyme reaction mixture does not change the K_m of M_2 preparations (1).

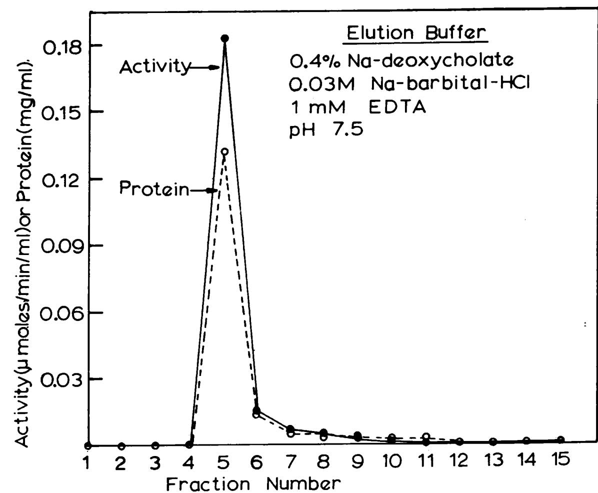

FIG. 1. Elution of M_2 protein on Sepharose 4B column with 0.4% deoxycholate, 0.033 M barbital, 1 mM EDTA, pH 7.5, at 4°. Glucose 6-phosphatase activity and protein are eluted in the void volume.

Latency. Arion *et al.* (7) have shown that the glucose 6-phosphatase activity of rat liver microsomes before disruption with detergents or with ammonia was specific for glucose 6-phosphate and that after such disruption a variety of other phosphorylated compounds, including mannose 6-phosphate, were hydrolyzed. An experiment which confirms Arion *et al.* is shown in Table 5. It can be seen in the column numbered 1 that mannose 6-phosphate, in contrast to glucose 6-phosphate, is hardly split in the absence of detergents. With detergents, latent enzyme activity is detected, amounting to about 49% of total activity in the case of glucose 6-phosphate and 91% in the case of mannose 6-phosphate. Stetten and Burnett (8) had shown previously that addition of ammonia to produce a high pH abolished latency with glucose 6-phosphate as substrate in a time-dependent reaction. The same effect is seen

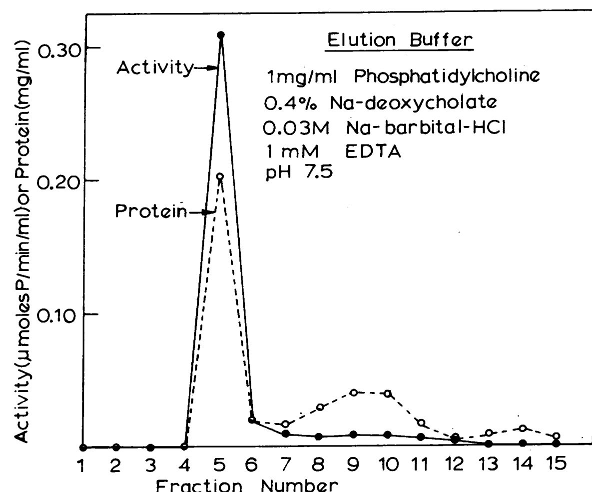

FIG. 2. Elution of M_2 protein when 1 mg/ml of phosphatidylcholine is included in the elution fluid. The other conditions are the same as for Fig. 1. In this case 50% of the protein but no enzyme activity is eluted after the void volume.

TABLE 3. *Flotation-recentrifugation of M_2*

Source of M_2	Lipid P added with flotation mixture, μmole/mg of protein	Lipid P in peak fraction, μmole/mg of protein	Lipid P in precipitate after recentrifugation, μmole/mg of protein	Specific activity in precipitate, μmole/mg of protein per min at 30°
Normal liver	4.4	2.54	1.04	—
Diabetic liver*	13.8	2.01	0.65	5.5
Diabetic liver	27.3	2.75	1.25	7.1
Diabetic liver	46.9	7.83	0.93	8.6

Different M_2 preparations containing 0.29 to 0.32 μmole of lipid P per mg of protein were floated by centrifugation in a buffered sucrose (d 1.14)-phosphatidylcholine-0.35% deoxycholate mixture, as in Fig. 3. A peak fraction corresponding to fraction 3 + 1/2 of 2 in Fig. 3 was collected, diluted 5-fold with buffer, and recentrifuged for 2 hr at 50,000 rpm. The well packed and drained precipitate was analyzed for protein, lipid P, and activity.

* No deoxycholate in flotation mixture.

with mannose 6-phosphate in Table 5; that is, the activity without detergents rises progressively with the length of time of preincubation at high pH, without there being much change in total activity.[g]

The M_2 particles before or after relipidation with phosphatidylcholine do not show any latency; that is, glucose 6-phosphate and mannose 6-phosphate are split at nearly equal rates and addition of detergents does not increase activity with either substrate. In one experiment relipidation was with a mixture of phospholipids and cholesterol resembling that found in microsomes.[h] The specific activity of the relipidated M_2 increased in the same manner as in a parallel run with phosphatidylcholine alone, but latency was not reestablished.

DISCUSSION

Although the marked proliferation of the hepatic endoplasmic reticulum that results from the treatment of rats with phenobarbital is primarily related to drug metabolism, it was of interest to find out how much glucose 6-phosphatase is incorporated into the newly formed endoplasmic reticulum. By contrast, severe insulin-deficient diabetes is associated with increased gluconeogenesis for which glucose 6-phosphatase is a key enzyme, and an increase in the activity of this enzyme in liver homogenates has repeatedly been reported (9). (For a review see ref. 10.)

The problem encountered here is latent enzyme activity, detectable in homogenates and untreated microsomes by running an activity test in the presence of detergents. The question is what happens to the latent enzyme when microsomal particles are purified. Thus, the step from crude homogenate to microsomes involves an about 4- to 5-fold enrichment of glucose 6-phosphatase activity, and a further 17- to 30-fold enrichment can be achieved by methods described in this paper, the two numbers being based on the specific activity of microsomes with and without detergents, respectively (see Table 1). It was found that, once the microsomes had been exposed to detergents, no latent enzyme activity remained, although the detergents were removed in subsequent steps of purification. Since homogenates and microsomes of diabetic liver show considerably more latent enzyme activity than normal liver,[i] one would expect this to be reflected in the final product, provided the latent enzyme is a real entity and fractionates in the same way as the active enzyme. The result was that the purified microsomal particles from diabetic liver contained glucose 6-phosphatase with a specific activity twice that of normal and five times that of phenobarbital-treated rats. Thus, glucose 6-phosphatase that is firmly bound to the endoplasmic reticulum becomes incorporated in various amounts under different conditions, but what regulates the process or indeed in what manner the enzyme becomes attached is not known at the present time.

It was thought that progress with these and related problems required the development of reproducible purification procedures. The principal steps, starting with a stable lyophilized microsomal preparation, are sucrose-density gradient centrifugation through a layer containing deoxycholate, recovery of the now delipidated particles in the precipitate, and flotation of the particles in a phospholipid-detergent mixture. The latter step results in extraction of protein of low specific activity (about 40% of total) and in relipidation of the particles to near their original phospholipid content. Detergents alone do not extract any more protein at this stage of purification. The need for phospholipid is attributed to a competition of added phospholipid for hydrophobically bound protein. The relipidation has a marked effect on the properties of the microsomal membrane, as shown by the fact that the K_m for glucose 6-phosphate and mannose 6-phosphate decreases about 4-fold. V_{max} was not affected by relipidation. Presumably, the insertion of phospholipid molecules in the membrane allows the enzyme to assume a more favorable conformation for substrate binding. However, latent enzyme activity such as is present in microsomes before treatment with detergents is not reestablished.

Up to 70% of the proteins present in the glucose 6-phosphatase-enriched particles could be solubilized in a mixture of 1% glycodeoxycholate–0.5 M KCl at pH 9.5 at 3°, where

[g] In a similar experiment, microsomes washed once with barbital buffer were used. The samples were incubated for 20 min at 30° (a) at pH 10.9 with ammonia and (b) at pH 6.8 with neutralized ammonia, and were centrifuged for 2 hr at 105,000 × g at 3°. Protein determinations showed that the supernatant of (a) contained 27.4% and that of (b) 13.7% of the total protein present. (a) also contained in the supernatant 1.64 μmoles of ashed P as compared to 1.22 μmoles for (b). So far it has not been possible to connect the loss of latency at pH 10 with the extraction of a specific protein or phospholipid from the microsomal particles.

[h] 60% phosphatidylcholine, 15% phosphatidylethanolamine, 10% phosphatidylserine, and 5% each of phosphatidyl inositide, sphingomyelin, and cholesterol.

[i] This was first shown in Table 4 of a paper by Segal and Washko (11). These authors also reported that the K_m for glucose 6-phosphate was 4.2 mM in diabetic rats as compared to 1.8 mM for normal.

TABLE 4. *K_m determinations with M_2 before and after relipidation*

Preparation	Glucose 6-*P*		Mannose 6-*P*	
	K_m	V_{max}	K_m	V_{max}
M_2	3.2	7.0	3.45	6.4
Relipidated M_2	0.85	6.7	0.82	6.5

The M_2 preparation from the liver of a diabetic rat was relipidated and recentrifuged as in Table 3 and was tested without the addition of phospholipid. The reaction mixture contained 0.02 M citrate, 1 mM EDTA, and substrate at pH 6.8. K_m is given in mM and V_{max} in μmole/mg of protein per min at 30°.

either alone had little effect, but no enzyme activity was found in the supernatant after centrifugation (2 hr at 30,000 rpm) and there was no increase in the specific activity of glucose 6-phosphatase in the precipitate.

The possibility exists that glucose 6-phosphatase is an integral part of the protein matrix of the hepatic endoplasmic reticulum, which would explain the difficulty in solubilizing the enzyme. This idea is also suggested by the observation that in a mutant strain of mice with glucose 6-phosphatase deficiency there are structural abnormalities of the endoplasmic reticulum in liver and kidney but not in other organs (12). Further support comes from the observation that it is not possible to purify each microsomal preparation to the same level of specific activity. Thus, in the last purification step in Table 1, the increase in specific activity is percentagewise the same, irrespective of the preceding level of specific activity, probably because in each case the maximal amount of extraneous protein has been extracted. In other words, the final purification of each preparation is about the same, even if the specific activity varies from one preparation to another.

TABLE 5. *Effect of detergents and pH on the latency of glucose 6-phosphatase*

Activity tests with (+) or without (−) 0.04% deoxycholate	Treatment prior to activity tests			
	1	2	3	4
Glucose 6-*P* (+)	425	370		
Glucose 6-*P* (−)	218	288		
Difference	207	82		
Mannose 6-*P* (+)	301	306	266	320
Mannose 6-*P* (−)	27	110	242	30
Difference	274	196	24	290

A mouse liver microsomal pellet, prepared as previously described (2), was resuspended in 33 mM barbital, 1 mM EDTA, pH 7.5. Aliquots prior to activity tests were incubated at 30° as follows: 1, no treatment; 2, 10 min at pH 10 with ammonia; 3, 20 min at pH 10 with ammonia; 4, 20 min at pH 6.8 with neutralized ammonia. The reaction mixture for activity contained 0.04 *M* glucose 6-*P* or mannose 6-*P*, with the pH adjusted to 6.8 in each case. The numbers are readings in the Klett colorimeter in the determination of P_i by the Fiske-Subbarow method.

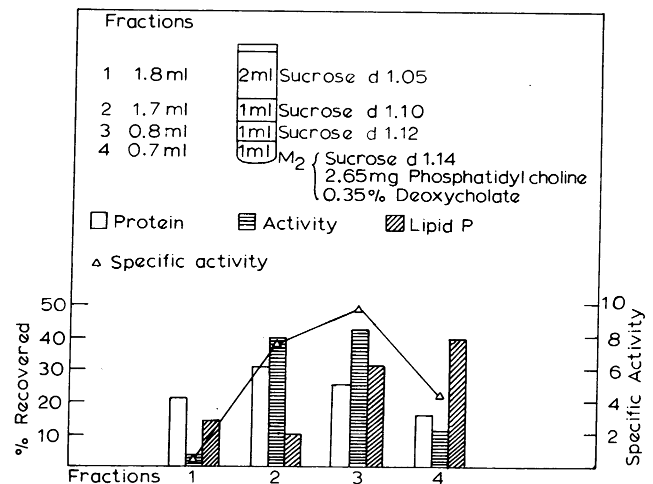

FIG. 3. Flotation of M_2 particles prepared from liver of diabetic rats. The bottom layer (1 ml) contained 0.4 mg of M_2 protein of specific activity 7.0 sonicated in the solution shown. All solutions were buffered with 33 mM barbital, 1 mM EDTA, pH 7.5. Centrifugation was for 2 hr at 50,000 rpm in the SW 50 swinging bucket head at 3°. After centrifugation, fractions were collected from the top down, as indicated in upper left. Percent recovered in the different fractions adds up to 92.1, 95.6, and 94% of protein, activity, and phospholipid, respectively.

As a preliminary to work on the detection of the enzyme by acrylamide gel electrophoresis, labeling experiments have been carried out with ^{14}C-labeled *p*-hydroxymercuribenzoate and ^{32}P-labeled inorganic pyrophosphate. This will be reported in a subsequent paper (C. F. Cori, R. C. Garland, and H. W. Chang, manuscript in preparation).

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