Differentiation of phospholipases A in mitochondria and lysosomes of rat liver

MOSELEY WAITE, G. L. SCHERPHOF, F. M. G. BOSHOUWERS, and L. L. M. VAN DEENEN

The Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27103 and The State University of Utrecht, Utrecht, The Netherlands

ABSTRACT Highly purified mitochondria from rat liver contain a phospholipase A that catalyzes removal of 2-fatty acids, with a pH optimum above pH 8.0. Lysosomal preparations appeared to have two phospholipases A associated with them, one with a pH optimum at about pH 4.0, the second between pH 6.0 and 7.0.

Mitochondrial phospholipase A hydrolyzed exogenous phospholipid as fast as or faster than endogenous phospholipid. The difference in specific radioactivity of ^{14}C-ethanolamine-labeled endogenous mitochondrial phospholipid before and after incubation indicates that a fraction of mitochondrial phosphatidyl ethanolamine is hydrolyzed more rapidly than the mitochondrial phospholipids as a whole.

Acyl bond hydrolysis of exogenous and endogenous phospholipid by mitochondria was stimulated by free fatty acid, Ca^{++}, or in certain cases, monoacyl phospholipids or by treatments that disrupt the mitochondrial membrane. Of various fatty acids tested, lauric, myristic, oleic, and linoleic were most effective.

ADP and ATP inhibited mitochondrial phospholipase, probably because they compete for Ca^{++}. Mg^{++} also behaved as a competitive inhibitor; the effect was overcome by relatively little Ca^{++}.

SUPPLEMENTARY KEY WORDS pH optima · fatty acid stimulation · Ca^{++} requirement · inhibition by ATP, ADP, and Mg^{++} · endogenous vs. exogenous phospholipid hydrolysis · mitochondrial membrane

In earlier reports we described some biochemical characteristics of phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4) activities of rat liver (1, 2). Evidence was presented that the mitochondrial fraction primarily catalyzed hydrolysis of the 2-ester bond of phospholipids whereas the microsomal fraction mainly hydrolyzes the 1-ester bond. The composite fraction sedimenting between 4,500 *g* and 12,500 *g* gave rise mainly to fatty acid and little monoacyl GPE, which indicates that it contains a mixture of activities. No attempt was made at that time to characterize this fraction further or to determine the activity of the lysosomes. Bjørnstad (3, 4) demonstrated, in both mitochondrial and microsomal fractions, phospholipases that catalyze the hydrolysis of endogenously labeled phospholipids. He found that the mitochondrial activity was stimulated by the presence of Ca^{++}, and PE was hydrolyzed more rapidly than PC. Rossi et al. (5) partially purified the mitochondrial phospholipase A and described some of its characteristics. These few papers constitute the extent of the information on the mitochondrial phospholipase, despite the suggestion made several years ago (6, 7) of its existence and possible significance.

Recent reports (8–10) provide evidence that rat liver lysosomes have a high phospholipase A activity. Further, the membrane fraction from lysosomes can stimulate mitochondrial swelling and cause a decrease in oxidative phosphorylation (9). The reports of Mellors and Tappel (8), of Mellors, Tappel, Sawant, and Desai (9) and of Stoffel and Greten (10) differ in certain characteristics of the phospholipase ascribed to the lysosomes, namely in the requirement for Ca^{++} and the pH optimum, perhaps because of differences in methods of isolation and assay used by the two groups.

Two phospholipase A activities have been found in lysosomes of bovine adrenal medulla (11–13), one with a pH optimum at pH 4.2, and one with a pH optimum at pH 6.5. The enzyme with optimal activity at pH 4.2 was reported to be a phospholipase A_1 (catalyzing the removal of the 1-fatty acid) whereas the enzyme with an

Abbreviations: PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; GP, glycerophosphate; GPE, glycerophosphoryl ethanolamine; GPC, glycerophosphoryl choline.

optimum at pH 6.5 was found to be a phospholipase A_2. There was no appreciable lysophospholipase (EC 3.1.1.5) activity in the adrenal medulla, in contrast to the results of Mellors et al. (8, 9) and Stoffel and Greten (10) with rat liver lysosomes.

This paper reports that we have confirmed the existence of two rat liver lysosomal phospholipases A in addition to the mitochondrial phospholipase A. In addition, we have shown that certain compounds known to bring about mitochondrial swelling can influence the activity of the phospholipase A and have determined the relative hydrolytic activity of the mitochondrial phospholipase A on exogenous and endogenous phospholipids. A later report will quantitatively relate phospholipase A activity and mitochondrial swelling (13*a*).

MATERIALS

^{14}C-ethanolamine and ^{14}C-choline were purchased from the New England Nuclear Corp., Boston, Mass. ATP and ADP were products of Fluka, Buchs, Switzerland, and AMP of Pabst Laboratories, Milwaukee, Wis. Sodium deoxycholate was purchased from Merck and Co., Darmstadt, Germany. Oleic acid, obtained from Price Ltd., Bromborough, England, was shown by gas–liquid chromatography to be more than 95% pure. The monoacyl GPE and monoacyl GPC were prepared by the action of *Crotalus adamanteus* venom on the corresponding diacyl compound. 1-^{14}C-Linoleic acid with a specific activity of 47 μc/μmole (98% pure by gas–liquid chromatography) was obtained from the Radiochemical Centre, Amersham, England.

METHODS

2-[1′-^{14}C]-Linoleoyl PE and ^{32}P-PE were prepared as described before (2). The mitochondria isolated from rat liver homogenates as described earlier (2) are designated M-I. The mitochondria were further purified, yielding M-II, by means of a sucrose density gradient (14). Lysosomes were isolated by the method of Sawant, Shibko, Kumta, and Tappel (15). Rat liver mitochondria were radiolabeled essentially as described by Bjørnstad (4), except that male rats were used. In some experiments 20 μc of 1,2-^{14}C-ethanolamine (specific activity 2.8 mc/mmole) or 20 μc of ^{14}C-methyl-labeled choline (specific activity 4.5 mc/mmole) were used rather than the specified 10 μc of these compounds. About 2% of the injected ^{14}C-ethanolamine was incorporated into the liver mitochondrial phospholipids whereas about one-third of that percentage (0.6%) of injected ^{14}C-choline was incorporated. Chromatography showed that ^{14}C-ethanolamine was distributed among the various rat liver mitochondrial phospholipids as follows: PE, 84.4%; PC, 12.3%; monoacyl GPE, 2.5%, and monoacyl GPC, 0.6%.

The reaction mixtures were incubated at 37°C for 20 min in 0.125 M KCl with 0.01 M Tris buffer, pH 7.4, with a suspension of 0.010 μmole of 2-(1′-^{14}C)-linoleoyl PE unless otherwise specified. The substrate and fatty acids or monoacyl phospholipids were suspended by sonication of the dried compound in the same buffer as used in the reaction mixture. The extracted products of the hydrolysis of 2-[1′-^{14}C]-linoleoyl PE were separated as before (2). The incubation products (either from incubations using the endogenously labeled mitochondria or from mixed ^{14}C and ^{32}P incubations) were separated by paper chromatography according to Marinetti (16) or by thin-layer chromatography in chloroform–methanol–water–ammonia 70:30:2:3. Radioactivity of the monoacyl GPE and PE was determined as before (2).

Malate dehydrogenase (EC 1.1.1.37) was assayed by the procedure of Ochoa (17), fumarate (fumarate hypratase, EC 4.2.1.2) as described by Racker (18), acid phosphatase (EC 3.1.3.2) according to Gianetto and de Duve (19) with use of β-glycerophosphate, and ribonuclease (EC 2.7.7.16) by the procedure of de Duve et al. (20). Protein was determined by the procedure of Lowry, Rosebrough, Farr, and Randall (21) and phospholipid phosphorus by the method of Parker and Peterson (22).

RESULTS

Purification of Cell Fractions

Table 1 presents data on the specific activities of the phospholipases A at pH 4.5 and 8.8 in the original liver homogenate, in mitochondrial preparations at two stages of purification, and in lysosomal preparations. These activities were compared with two mitochondrial marker enzymes, malate dehydrogenase and fumarase, and two lysosomal marker enzymes, acid phosphatase and ribonuclease. The phospholipase A specific activity is lower in the mitochondria than in the homogenate; other fractions therefore must have a relatively high specific activity. The specific activity at pH 8.8 did not change when M-I was centrifuged on the sucrose density gradient to give M-II, which shows that this activity was not the result of lysosomal contamination. By contrast, there was a decrease in the specific activity at pH 4.5. The activities of the fumarase and malate dehydrogenase were the same in the M-I and M-II fractions whereas the acid phosphatase and ribonuclease activities decreased several fold, which demonstrates the removal of much of the lysosomal contamination. Glucose-6-phosphatase (EC 3.1.3.9) and NADPH-cytochrome *c*

TABLE 1 PHOSPHOLIPASES A AND MARKER ENZYME ACTIVITIES IN MITOCHONDRIAL AND LYSOSOMAL PREPARATIONS

	Specific Activity					
	PLA pH 8.8 (×1000)	PLA pH 4.5 (×1000)	MDH	Fumarase	AP	RNAase
	μmoles/mg protein					
Homog.	9.24	3.33	2.22	1.33	0.211	0.453
M-I	5.82	2.86	6.28	2.04	0.132	0.298
M-II	5.21	1.68	5.42	2.23	0.019	0.084
F_4	31.8	77.0	0.64	0.52	2.73	3.94

	Ratios of Activities (×1000)								
	PLA 8.8/ PLA 4.5	PLA 8.8/ MDH	PLA 8.8/ Fumarase	PLA 8.8/ AP	PLA 8.8/ RNAase	PLA 4.5/ MDH	PLA 4.5/ Fumarase	PLA 4.5/ AP	PLA 4.5/ RNAase
Homog.	2.77	4.16	6.94	43.8	20.4	1.5	2.50	15.8	7.35
M-I	2.03	0.93	2.85	44.1	19.5	0.45	1.40	21.7	9.60
M-II	3.10	0.96	2.33	274	62.0	0.31	0.75	88.4	20.0
F_4	0.41	49.7	61.1	11.6	8.1	120	148	28.2	19.5

Phospholipases A were assayed by use of added 2-[1′-^{14}C]-linoleoyl PE, as described under *Methods*. Incubations at pH 4.5 were in 0.08 M sodium acetate buffer with 5.0 mM EDTA, those at pH 8.8 were in 0.08 M sodium glycylglycine buffer, with 5.0 mM $CaCl_2$ and various levels of protein (0.10–2.0 mg).

Abbreviations: PLA, phospholipase A; MDH, malate dehydrogenase; AP, acid phosphatase; RNAase, ribonuclease. Homog., homogenate; M-I, mitochondrial preparation; M-II, purer mitochondrial preparation; F_4, lysosomal preparation.

oxidoreductase, two microsomal marker enzymes, were not present in measurable amounts in M-II. Phospholipase A active at pH 4.5 tends to remain more closely associated with the mitochondria than does the acid phosphatase or ribonuclease, but not as closely as pH 8.8-active phospholipase A, malate dehydrogenase, or fumarase.

Lysosomes in the fraction designated F_4 had an acid phosphatase and ribonuclease activity about 10 times that of the homogenate, and a phospholipase A activity at pH 4.5 about 20 times that of the homogenate. The phospholipase A activity at pH 8.8 increased less than the activity of these lysosomal enzymes, a fact indicating that this enzyme is not as closely associated with the lysosomes. The specific activity of the mitochondrial marker enzymes in F_4 was quite low when compared with either the homogenate or mitochondrial fractions; evidently much of the mitochondrial contamination had been removed from the lysosomes.

Ratios of these activities are also shown in Table 1. The main feature of these data is the fact that the ratio of mitochondrial phospholipase A at pH 8.8 to either malate dehydrogenase or fumarase remained constant as the mitochondria were purified, whereas the ratio of the phospholipase A activity at pH 8.8 to acid phosphatase or ribonuclease increased considerably. The phospholipase A activity at pH 4.5 exhibited the opposite characteristic during this purification, that is, its ratio to the mitochondrial marker enzymes decreased nearly twofold. Also, there was an eightfold difference in the ratio of the phospholipase activities at pH 4.5 and 8.8 between the M-II and F_4.

pH Optima of the F_4 and M-II Phospholipases

Fig. 1 presents the results of a study in which F_4 was assayed at various pH's in the presence of either $CaCl_2$ or EDTA. In the presence of EDTA (open symbols), maximal activity was at pH 4.0. This activity was inhibited by glycylglycine buffer[1] (compare curves designated Δ and ○ at 5.0). In the presence of $CaCl_2$ (closed designations) maximal activity was at pH 6.5–7.0, indicating the presence of a second enzyme in the preparation. The enzyme operating at pH 4.0 was inhibited by $CaCl_2$ (compare curves designated Δ and ▼). The activity at pH 6.0–7.0 was completely inhibited by EDTA and at pH 5.0 was higher in the presence of glycylglycine than of sodium acetate. In some preparations the level of activity at pH 7.0 was lower, an observation which suggests that this enzyme might be in a subcellular particle different from that containing the activity at pH 4.0.

The M-II fraction had the greatest activity at pH 8.8 (Fig. 1) with an attenuated activity between pH 7.0 and 7.5. The activity at both pH 7.0 and 8.8 was sensitive to EDTA (not shown in the figure). This profile is quite different from that obtained using F_4. The activity at neutral pH relative to that at acid pH is much higher in M-II than F_4. A similar curve was obtained when ^{14}C-ethanolamine-labeled M-II was used to examine the hydrolysis of endogenous phospholipids.

Preliminary experiments on the positional specificity of the phospholipases, in which we used a mixed-labeled

[1] Glycylglycine and Tris gave the same results in these experiments. In order to get a wider range of pH, glycylglycine was chosen for these experiments.

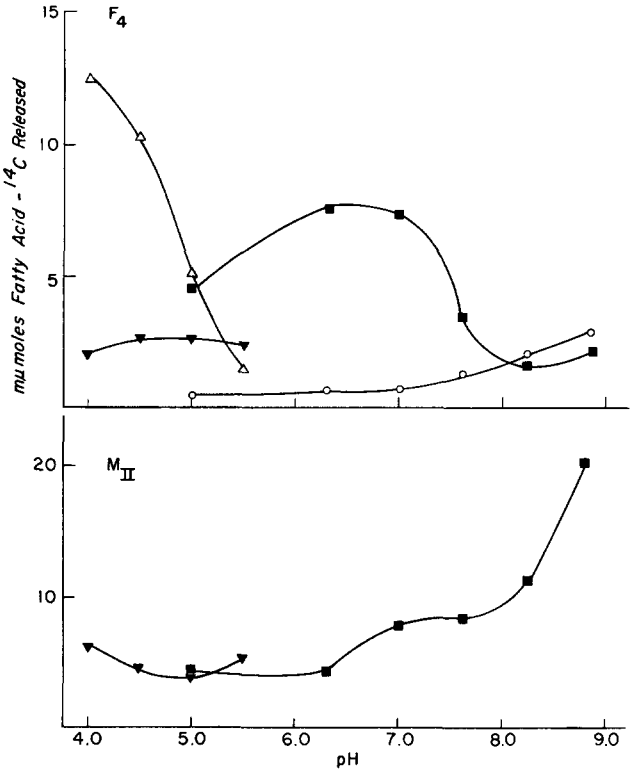

FIG. 1. Effect of pH on phospholipase A activity on exogenous 2-[1′-^{14}C]- linoleoyl PE (100 mμmoles). The incubation mixture with F_4 (lysosome fraction) contained 0.10 mg of protein in the following: Δ, 0.10 M acetate buffer with 5.0 mM EDTA; ▼, 0.10 M acetate buffer with 5.0 mM $CaCl_2$; ■, 0.10 M glycylglycine buffer with 5.0 mM $CaCl_2$; ○, 0.10 M sodium glycylglycine buffer with 5.0 mM EDTA. The incubation mixture with M-II (purified mitochondria) contained 1.5 mg of protein in the following: ▼, 0.10 M acetate buffer with 5.0 mM $CaCl_2$; ■, 0.10 M glycylglycine buffer with 5.0 mM $CaCl_2$. The other conditions were as described under Methods.

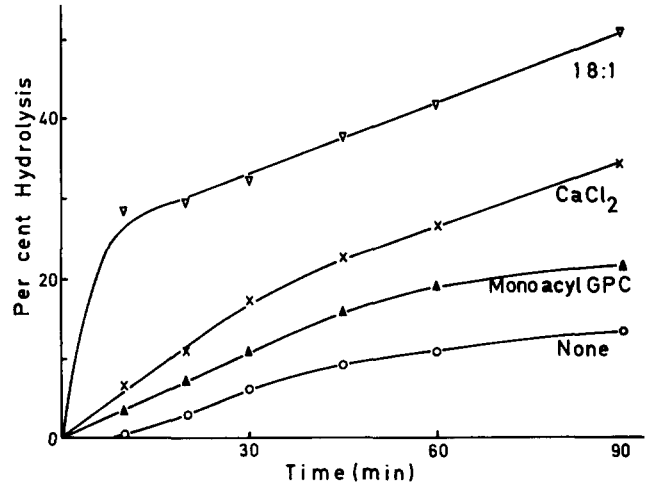

FIG. 2. Stimulation of phospholipase A activity on exogenous ^{14}C-PE. Incubations contained 9.0 mg of M-I (mitochondrial) protein plus the indicated additions, in a total volume of 1.0 ml. Oleic acid was 0.4 mM, monoacyl GPC was 0.2 mM, and $CaCl_2$ was 1.0 mM. Percent hydrolysis is the amount of ^{14}C-fatty acid released divided by the total recovered ^{14}C times 100.

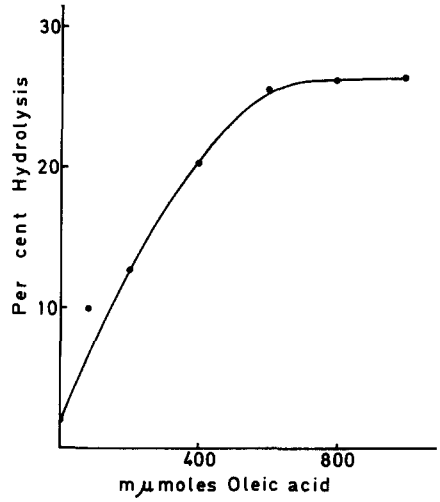

FIG. 3. Oleic acid stimulation of phospholipase A activity on exogenous ^{14}C-PE. Conditions and calculations were the same as those in Fig. 2 except that the incubation time was 20 min.

PE (1, 2), showed that the activity of the M-II at pH 8.8 was mainly at the 2-position of PE and gave rise to 1-acyl GPE. Of this, about 20% was further hydrolyzed to free fatty acid and GPE. The F_4 fraction at pH 4.5 gave rise to both 1-acyl GPE and 2-acyl GPE. The amount of fatty acid released was about twice the (molar) amount of accumulated monoacyl GPE. At pH 7.0, however, the activity of both F_4 and M-II was exclusively at the 2-position.

Stimulation of Mitochondrial Phospholipase A Activity

Figure 2 shows the hydrolytic activity of M-I at pH 7.4 on exogenous PE as a function of time, with and without various stimulators. Without addition there was no activity for the first 10–20 min. Addition of oleic acid, Ca^{++}, or monoacyl GPC eliminated this lag period and increased the over-all extent of hydrolysis. Monoacyl GPE and monoacyl GP stimulated to nearly the same extent as the monoacyl GPC. Disruption of the M-I by freezing and thawing or by sonication also increased the hydrolytic activity, freezing and thawing being the more effective treatment.

Further investigations were made on the concentration and the type of fatty acid causing stimulation. 3 Fig. shows the stimulatory effect of increasing amounts of oleic acid on hydrolysis of exogenous PE up to about 600 mμmoles/ml of reaction mixture containing 9.0 mg of protein. This relationship was dependent on protein concentration, i.e., the greater the protein concentration, the greater the amount of fatty acid required to give an equivalent stimulation. Fig. 4 indicates the effect of different fatty acids on the stimulation of phospholipase A. All acids examined gave stimulation. The pattern of

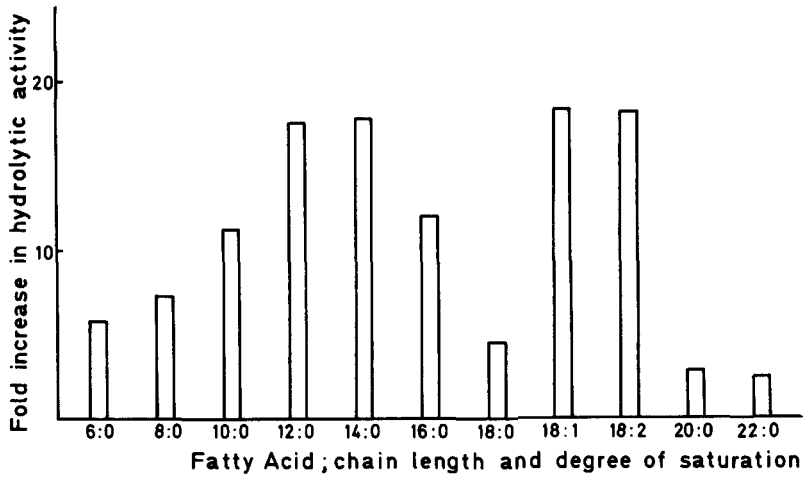

FIG. 4. Fatty acid stimulation of phospholipase A activity on exogenous ^{14}C-PE. Sonicates of the various fatty acids (1 mg/ml) were 0.4 mM in the incubation mixture. The control with no fatty acid added had a 1.8% hydrolysis. Other conditions were the same as for Fig. 2 except that the incubation time was 20 min.

stimulation is similar to that found by Zborowski and Wojtczak, who studied mitochondrial swelling (23), and by Epstein and Shapiro (24), who used phospholipase A from intestinal mucosa. Both 20:0 and 22:0 gave us very poor sonicates (as judged by their cloudiness); such differences in the physical form of the various sonicates may well affect their stimulatory behavior.

As a preliminary to determination of the level of Ca^{++} required to give maximal activity, attempts were made to remove endogenous Ca^{++} by dialysis of frozen and thawed M-I. Dialysis against 200 volumes of water, changed three times over a 24 hr period, lowered the activity on exogenous PE only a few per cent. The enzyme was therefore "titrated" with EDTA and to the EDTA-inhibited enzyme were added increasing amounts of Ca^{++}. 0.25 mM EDTA was found to be the minimum concentration giving complete inhibition with several 10 mg preparations of frozen and thawed M-I. As shown in Fig. 5, 0.5 mM Ca^{++} gave maximal stimulation of frozen and thawed M-I containing 0.25 mM EDTA. This extent of hydrolysis is similar to the level in a control incubation containing 1.0 mM Ca^{++} without EDTA. Intact M-I required a higher Ca^{++} concentration than frozen and thawed M-I before activity could be detected and 1 mM was necessary for maximal activity.

Inhibition of Phospholipase A Activity

Certain compounds that have been shown to prevent mitochondrial swelling were examined for possible effects on phospholipase A activity. High concentrations of Mg^{++}, an ion shown to have such activity (25), inhibited hydrolysis of exogenous PE (Fig. 6 A). Increasing amounts of Ca^{++} added to M-I in the presence of 10 mM Mg^{++} countered this inhibition (Fig. 6 B). A concentration of Ca^{++} about equal to one-half that of

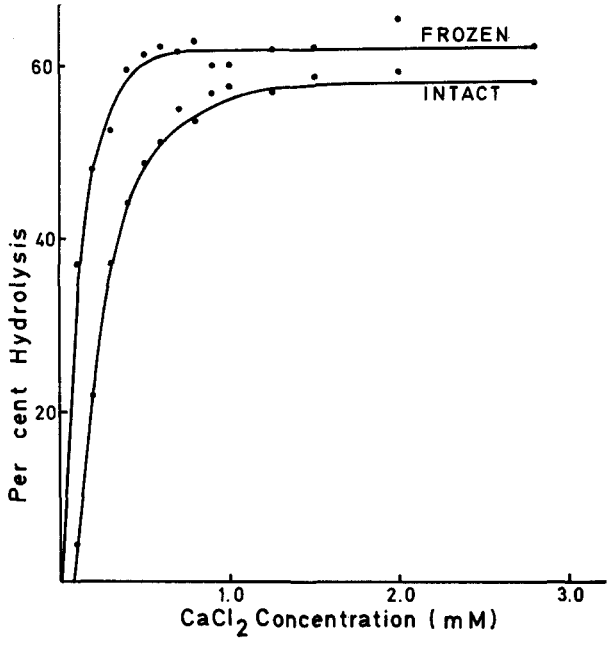

FIG. 5. Calcium dependence of phospholipase A activity on exogenous ^{14}C-PE. 10.0 mg of protein was incubated for 20 min as in Fig. 2, except that $CaCl_2$ and the substrate were both added after 5 min incubation of the M-I with 0.25 mM EDTA.

Mg^{++} was sufficient to overcome the inhibition of the phospholipase activity of the intact M-I; with frozen and thawed M-I a concentration of Ca^{++} nearly equal to that of the Mg^{++} was required.

Figure 7 shows the effect[2] of ATP and ADP on the release of ^{14}C-fatty acid from exogenous PE. 0.1 mM ATP or ADP markedly inhibited the M-I phospholipase activity;

[2] Frozen and thawed M-I were used in this experiment to minimize complications arising from the poor permeability of the mitochondrial membrane to ATP.

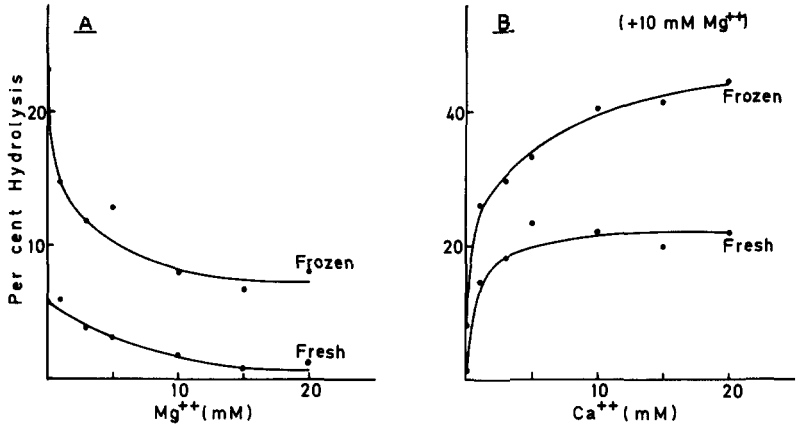

FIG. 6. Mg^{++} inhibition of phospholipase A activity on exogenous ^{14}C-PE. *A*, Conditions were as described in Fig. 2 except for the indicated amounts of $MgSO_4$. In this experiment 5 mg of M-I protein (fresh or frozen and thawed) was used per incubation. *B*, Conditions were the same as for part *A*, except that $MgSO_4$ was 10 mM throughout and the indicated amounts of $CaCl_2$ were added.

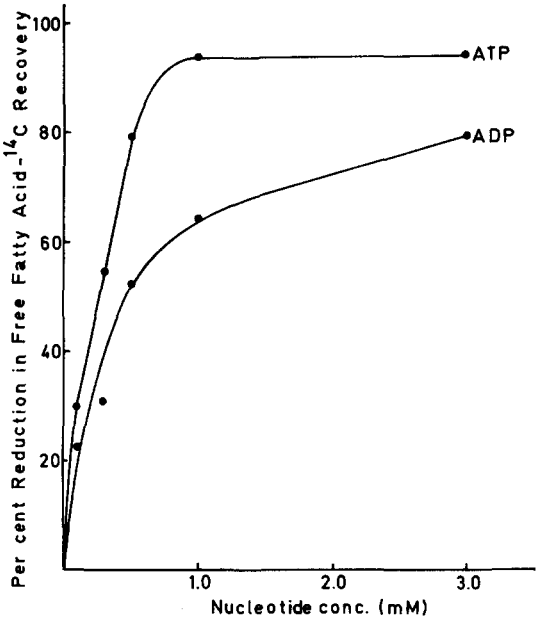

FIG. 7. ATP and ADP inhibition of phospholipase A activity on exogenous ^{14}C-PE. Conditions were as described in Fig. 2 except that the M-I protein concentration was 2.7 mg/ml and the M-I were frozen and thawed. Without addition of nucleotide, a 17.7% hydrolysis was observed.

the effect leveled off at 1.0 mM ATP. At all levels tested (up to 10 mM), ADP inhibited less than ATP. In this experiment AMP and inorganic phosphate at similar levels did not inhibit the liberation of the fatty acid.

To demonstrate that the apparent decrease in the amount of ^{14}C-linoleic acid hydrolyzed from the PE was not the result of a concomitant reacylation, we studied incorporation of ^{14}C-linoleic acid in the presence and absence of added ATP with or without *p*-chloromercuribenzoate. Incubation without added ATP showed no acylation whereas addition of ATP caused up to 27% of the added linoleic acid to be incorporated into PE and PC. *p*-Chloromercuribenzoate inhibited this reacylation [see Lands and Hart (26)] without affecting the ATP inhibition of phospholipase activity; thus the observed ATP inhibition of the phospholipase is not the result of a reacylation.

Hydrolysis of Endogenous and Exogenous Phospholipids Compared

Earlier, Scherphof and van Deenen (27) reported that endogenous mitochondrial phospholipids were hydrolyzed much more slowly than exogenous phospholipid. Since Bjørnstad (4) reported on the contrary that endogenous phospholipids were preferentially hydrolyzed, we decided to reinvestigate the question with a double isotope technique. Simultaneous hydrolysis of endogenous and exogenous substrates by phospholipase A at pH 7.4 was examined by incubating ^{14}C-ethanolamine-labeled M-I in the presence of various amounts of exogenous, ^{32}P-labeled PE (Table 2). The level of radioactivity in endogenous ^{14}C-PC was too low to allow determination of hydrolysis. Therefore, PC hydrolysis was estimated on the basis that PC is hydrolyzed at about one-half the rate of PE, as found by Bjørnstad (4) and by us (1, 2). Comparison of the hydrolysis and endogenous and exogenous phospholipid shows that both are readily hydrolyzed, although the exogenous appears to be somewhat preferred (Table 2). This indicates that, to be active, the system does not have an absolute requirement for a particular physical form of the phospholipid.

In several experiments, the ^{14}C and phosphorus content of PE and PC were compared before and after incubation of the mitochondria. As can be seen in Table 3, the specific radioactivity of the PE and PC decreased after incubation. PC, which was hydrolyzed more slowly than PE (2, 4), showed a slower change in specific

TABLE 2 COMPARATIVE HYDROLYSIS OF ENDOGENOUS ^{14}C-ETHANOLAMINE-LABELED PE, AND ADDED ^{32}P-PE BY M-I

Exogenous PE Added	Substrate Hydrolyzed					
	Exogenous PE		Endogenous PE (A)		Endogenous PC (B)	PE + PC* (A + B)
mμmoles	%	*mμmoles*	%	*mμmoles*	*mμmoles*	*mμmoles*
15	73	11	16	47	33	80
70	34	24	16	47	33	80
190	27	51	9	30	21	51
610	19	115	10	33	23	56

Clear dispersions of ^{32}P-PE were prepared as follows: 130 mμmoles (160,000 dpm) of ^{32}P-labeled PE plus various amounts of unlabeled substrate (0, 0.8, 2.4, and 8.0 μmoles) were sonicated in 2.0 ml of isotonic Tris-KCl in the presence of 0.2 mg of Triton X-100 [a nonionic detergent, mixture of *p,t*-octyl poly(phenoxyethoxy) ethanols]. 0.15 ml of the dispersions was incubated with an amount of ^{14}C-ethanolamine-labeled mitochondria corresponding to 6 mg of protein (containing 15,000 dpm of ^{14}C-PE) in the presence of 1.0 mM $CaCl_2$; the total volume was 1.0 ml. After incubation the reaction mixture was extracted and the labeled PE and monoacyl GPE were separated.

* Calculated from the extent of hydrolysis of PE and PC in the presence of $CaCl_2$ based on the observation that the ratio of hydrolytic activity on PE and PC is 2:1 in favor of the PE, and that these preparations contained 102 mμmoles of PC and 73 mμmoles of PE per mg of protein.

TABLE 3 CHANGE IN SPECIFIC RADIOACTIVITY OF ^{14}C-PE AND ^{14}C-PC DURING INCUBATION OF ^{14}C-ETHANOLAMINE-LABELED M-I

	Phosphorus in Fraction	Radioactivity in Fraction	Specific Activity	% Change in Specific Activity*
	μg	*dpm*	*dpm/μg P*	
PE–Before Incubation	8.4	8120	967	
After Incubation	5.3	4070	768	−21
PC–Before Incubation	23.7	1800	76	
After Incubation	17.3	1100	64	−16

Incubations were for 80 min using 5.5 mg of mitochondrial protein in 1.0 mM $CaCl_2$. After separation by thin-layer chromatography and elution from the silicic acid the ^{14}C and phosphorus content of phospholipid of each fraction was determined.

* Calculated as the specific activity after incubation, divided by the specific activity before incubation, multiplied by 100, subtracted from 100%.

activity. The decrease in specific activity was time-dependent and was reproducibly the same for a given percentage of hydrolysis.

Studies on the stimulation of mitochondrial phospholipase A activity were repeated with mitochondria prepared from rats that had been injected with ^{14}C-ethanolamine. When labeled M-I was incubated for various lengths of time, a low but significant amount of ^{14}C-PE disappeared, with a concomitant increase in the level of monoacyl GPE-^{14}C (Fig. 8). As with the exogenous substrate, Ca^{++} and especially oleic acid stimulated the phospholipase activity. Contrary to the effect on the hydrolysis of exogenous PE, added monoacyl phospholipids inhibited hydrolysis of endogenous PE at the longer incubation times. These various compounds behaved in the same fashion with ^{14}C-choline-labeled M-I and with M-II preparations.

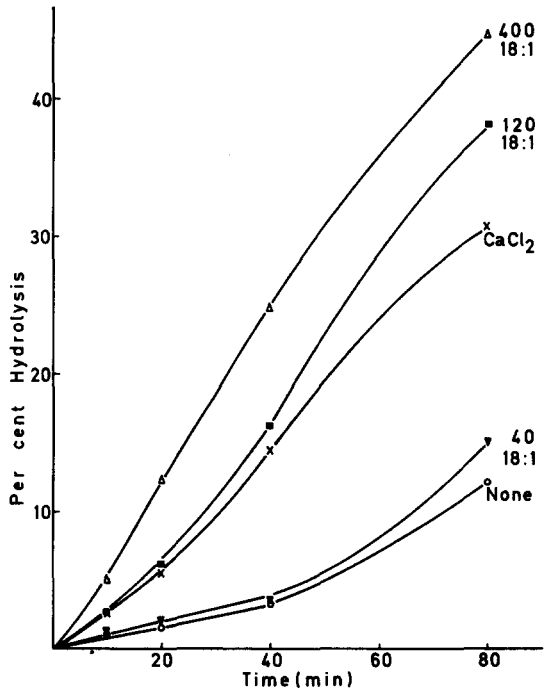

FIG. 8. Effect of $CaCl_2$ and oleic acid on hydrolysis of ^{14}C-ethanolamine-labeled endogenous phospholipid. ^{14}C-ethanolamine-labeled M-I containing 10.0 mg of protein was incubated in the presence of the additions indicated. $CaCl_2$ was 1.0 mM; the concentration of oleic acid is expressed in mμmoles per sample. The percent hydrolysis was the amount of ^{14}C in the monoacyl GPE fraction, divided by the amount of ^{14}C in the monoacyl GPE plus PE fractions, times 100.

Comparison of Mitochondrial with Lysosomal Hydrolysis of Mitochondrial Phospholipid

To ascertain the role of the mitochondrial and lysosomal phospholipase A in the hydrolysis of endogenous mitochondrial phospholipid, we injected rats with ^{14}C-ethanolamine. The isolated labeled M-I, M-II, and F_4 fractions were then incubated alone or in combination, and the extent of hydrolysis was determined. The results of such an experiment are presented in Table 4. During purification of the mitochondria there was a considerable loss of activity at both pH 4.5 and at 7.0 but only about a 40% loss at pH 8.8. By comparison, there was a five- to sixfold decrease in the specific activity of the acid phosphatase during this purification, whereas the specific activity of the malate dehydrogenase and fumarase remained constant. The amount of monoacyl GPE-^{14}C formed when the M-II and F_4 were incubated at pH 8.8 in combination (1160 dpm) was similar to the

TABLE 4 HYDROLYSIS OF MEMBRANE ^{14}C-ETHANOLAMINE-LABELED PE BY MITOCHONDRIAL AND LYSOSOMAL PHOSPHOLIPASES

Fraction	Monoacyl GPE-^{14}C Formed					
	At pH 4.5		At pH 7.0		At pH 8.8	
	dpm	%	*dpm*	%	*dpm*	%
A						
M-I	1930	16.1	900	7.5	2070	17.2
M-II	259	3.3	278	3.5	991	12.5
F_4	448	40.7	334	30.4	282	25.6
M-II + F_4	766	8.5	671	7.5	1160	12.9
B						
Sum of M-II and F_4	707		612		1270	
% sum of M-II and F_4		7.9		6.8		14.1

The assay mixtures contained either 1.08 mg of F_4 or 4.86 mg of M-I or M-II protein in either 0.080 M sodium acetate or 0.080 M sodium glycylglycine, pH 7.0 or pH 8.8 with 5.0 mM $CaCl_2$. The F_4 was frozen and thawed three times prior to the incubation to disrupt the lysosomal membrane. The level of radioactivity in the fractions used was 1100 dpm in the F_4, 12,000 dpm in the M-I, and 7900 dpm in the M-II. The sum of M-II + F_4 dpm used to obtain the theoretical hydrolysis of M-II and F_4 together (*B* part) was 9000 dpm. All values presented in the table have been corrected for zero time controls. Part *A* shows observed results; Part *B* data were calculated from part *A*.

sum of the monoacyl GPE-^{14}C formed when the two fractions were incubated separately (1270 dpm) even though the concentration of lysosomes in the reaction mixture increased fivefold upon the addition of F_4 to the M-II used.

DISCUSSION

The detection of two phospholipases, with different pH maxima (Fig. 1), in lysosomes confirms the observations of both Mellors et al. (8, 9) and Stoffel and Greten (10). The reason that each group found only one of these activities is probably that $CaCl_2$ or EDTA was added during preparation or assay. The results presented here (Fig. 1) show that the acid-active enzyme is inhibited by Ca^{++} and therefore would not have been observed by Stoffel and Greten (10), since their assay solutions contained $CaCl_2$, whereas the enzyme with activity at the neutral pH would not have been observed by Mellors and Tappel (8) because their lysosomes were prepared in EDTA, which inhibits the activity at neutral pH.

At acid pH the lysosomal preparations used in these experiments exhibited both phospholipase A_1 and A_2 activity. Further hydrolysis of monoacyl GPE might be due to a lysophospholipase or to the combined activities of phospholipase A_1 and A_2. In theory such a combination of activities could account for the lysophospholipase activity reported by Mellors et al. (8, 9) and Stoffel and Greten (10), since Winkler, Smith, Dubois, and van den Bosch (12) questioned the presence of a lysophospholipase in lysosomes, at least in the adrenal medulla.

Possibly the phospholipase A activity of both M-II and F_4 at neutral pH (Fig. 1) is due to a single common enzyme, since this property of the two particulate fractions has many common characteristics. However, the distribution of this activity does not parallel the major activity found in either the lysosomes (acid-active) or the mitochondria (alkaline-active). If the neutral activity were due to a single enzyme, it would probably be found preferentially in one fraction or the other and would parallel the other enzyme found with that fraction. Further work will be necessary to clarify this point.

The lysosomal fraction, added to ^{14}C-ethanolamine-labeled M-II, catalyzed only limited hydrolysis of mitochondrial phospholipid (Table 4). The amount of monoacyl GPE-^{14}C formed when F_4 and M-II were incubated together was nearly the same as the sum of the amounts of monoacyl GPE-^{14}C formed when the two fractions were incubated separately (Table 4). Lysosomal phospholipase is possibly saturated by the phospholipids in the F_4 fraction; studies will have to be done with isolated lysosomal phospholipases to settle this point. M-II lost considerable activity on endogenous phospholipid at neutral pH's as a result of gradient centrifugation, partly because of the removal of contaminating lysosomes. This observation is difficult to reconcile with the observation that lysosomal enzymes, when added to the M-II, hydrolyzed little endogenous phospholipid. Possibly it resulted from the removal, during gradient centrifugation, of mitochondrial fragments that might be more susceptible to lysosomal phospholipase A hydrolysis than are intact mitochondria. Mellors et al. (9) found that alterations in mitochondrial structure and function were attributable to adsorption of the hydrolysis products of lysosomal membrane phospholipase A, rather than to a direct hydrolysis of the mitochondrial membrane by the lysosomal phospholipase. This supports our conclusion that lysosomal phospholipase A hydrolyzes mitochondrial phospholipid at a low rate.

Mitochondrial phospholipase A catalyzed the hydrolysis of exogenous phospholipid about as fast as or somewhat faster than that of endogenous phospholipid (Table 2). Earlier (27), hydrolysis of endogenous phospholipid was not found, probably because the lipids were detected on silicic acid thin-layer plates with I_2 vapor, a less sensitive method than the measurement of ^{14}C-labeled monoacyl phospholipid used here. These results agree with those of Bjørnstad (4), although we found greater activity on exogenous phospholipid than he reported, possibly because of differences in the method of substrate preparation.

Never more than 75% of the exogenous phospholipid was hydrolyzed, even though sufficient enzyme was present for complete hydrolysis. Possibly the substrate micelle has critical structural characteristics that are lost when a certain percentage of the substrate is hydrolyzed. Bangham and Dawson (28), studying the hydrolysis of PC catalyzed by a partially purified phospholipase C, made similar observations, and concluded that the critical surface charge requirements of the micelle for enzymatic attack were no longer fulfilled.

The change in the specific radioactivity of the endogenous phospholipid, as measured by ^{14}C and phosphorus content (Table 3), indicates that not all of a phospholipid is metabolically equivalent. This is in agreement with the recent studies on phospholipid turnover in rats by Bailey, Taylor, and Bartley (29), who found that at least two phospholipid pools exist in liver mitochondria. This might be related to differences in the molecular species (the type of acyl groups present) or in the submitochondrial location (inner vs. outer membrane) of the phospholipid.

Earlier reports (2) showed that almost no phospholipase A activity could be detected for about the first 20 min of an incubation of intact mitochondria. Later it was found that $CaCl_2$, fatty acid, and, to a limited extent, monoacyl GPC or monoacyl GPE could eliminate this lag period and give an over-all stimulation of the activity on added phospholipid. Furthermore, treatments such as freezing and thawing increased hydrolytic activity. It was concluded that such stimulation could largely be accounted for by an alteration of the mitochondrial membrane that allows a better exposure of the enzyme to the substrate. This is borne out by the finding that higher levels of Ca^{++} are required to stimulate phospholipase A activity in intact mitochondria than in those that had been frozen and thawed. In model systems, such Ca^{++}–phospholipid interactions have been shown to cause changes in the physical structure of the phospholipid (30–32). The stimulatory effect of fatty acid also is thought to result from an increased exposure of the phospholipid to the enzyme. When fatty acid was added to phospholipase A that had been solubilized by freezing and thawing, with the membrane fraction removed, hydrolysis was inhibited; this indicated that the stimulation by the fatty acid is not via a direct effect on the phospholipase A.

The inhibition of phospholipase A by ATP and ADP might suggest that the prevention of swelling by ATP found by Carafoli and Lehninger (33) is due in part to the ATP inhibition of phospholipase A. Addition of an excess of Ca^{++} overcame the nucleotide inhibition (Fig. 7), which demonstrates that nucleotide binding of the Ca^{++} caused the inhibition in our experiments.

Rasmussen and Tenenhouse, (34) have recently proposed that Ca^{++} might act as an integrator of metabolic sequences involved with membranes and that conversion of ATP to 3′,5′-AMP could be the reaction controlling the amount of free Ca^{++} able to interact with membranes. On the basis of the work presented here we suggest that, in the case of the liver mitochondrion, the phospholipase A could be one enzyme mediating the change in membrane permeability that occurs when Ca^{++} is released; the change presumably is brought about by hydrolysis of membrane phospholipid.

The inhibitory effect of Mg^{++} on the phospholipase A (Fig. 6) is probably produced by the replacement of the required Ca^{++} by Mg^{++}. Such an interpretation is in keeping with that of Lipsett and Corwin (25), who found that either citrate or $MgSO_4$ is capable of inhibiting the swelling phenomenon, and attributed this to a reduction in the availability of Ca^{++} to the mitochondria.

The authors wish to thank Mr. A. J. Aarsman and Miss Patricia Sisson for expert technical assistance in various experiments.

This work was supported by grants from the American Heart Association (66-789 and 67-844) sponsored in part by the North Carolina Heart Association, and from the National Institutes of Health (AM 11799).

Manuscript received 9 October 1968; accepted 10 March 1969.

References

1. Scherphof, G. L., M. Waite, and L. L. M. van Deenen. 1966. *Biochim. Biophys. Acta.* **125:** 406.
2. Waite, M., and L. L. M. van Deenen. 1967. *Biochim. Biophys. Acta.* **137:** 498.
3. Bjørnstad, P. 1965. *Biochim. Biophys. Acta.* **116:** 500.
4. Bjørnstad, P. 1966. *J. Lipid Res.* **7:** 612.
5. Rossi, C. R., L. Sartorelli, L. Tato, L. Baretta, and N. Siliprandi. 1965. *Biochim. Biophys. Acta.* **98:** 207.
6. Lehninger, A. L., and L. F. Remmert. 1959. *J. Biol. Chem.* **234:** 2459.
7. Wojtczak, L., and A. L. Lehninger. 1961. *Biochim. Biophys. Acta.* **51:** 442.
8. Mellors, A., and A. L. Tappel. 1967. *J. Lipid Res.* **8:** 479.
9. Mellors, A., A. L. Tappel, P. L. Sawant, and I. D. Desai. 1967. *Biochim. Biophys. Acta.* **143:** 299.
10. Stoffel, W., and H. Greten. 1967. *Hoppe-Seyler's Z. Physiol. Chem.* **348:** 1145.
11. Blaschko, H., A. D. Smith, H. Winkler, H. van den Bosch, and L. L. M. van Deenen. 1967. *Biochem. J.* **103:** 30*c*.
12. Winkler, H., A. D. Smith, F. Dubois, and H. van den Bosch. 1967. *Biochem. J.* **105:** 38*c*.
13. Smith, A. D., and H. Winkler. 1968. *Biochem. J.* **108:** 867.

13a. Waite, M., L. L. M. van Deenen, T. J. C. Ruigrok, and P. F. Elbers. 1969. *J. Lipid Res.* In press.

14. Parsons, D., G. R. Williams, W. Thompson, D. F. Wilson, and B. Chance. *In* Mitochondrial Structure and Compartmentation. E. Quagliarello, S. Papa, E. C. Slater, and J. M. Tager, editors. Adriatica Editrice, Bari, Italy. 74.
15. Sawant, P. L., S. Shibko, U. S. Kumta, and A. L. Tappel. 1964. *Biochim. Biophys. Acta.* **85:** 82.
16. Marinetti, G. V. 1962. *J. Lipid Res.* **3:** 1.

17. Ochoa, S. 1955. *In* Methods of Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press, Inc., New York. **1:** 739.
18. Racker, E. 1950. *Biochim. Biophys. Acta.* **4:** 20.
19. Gianetto, R., and C. de Duve. 1955. *Biochem. J.* **59:** 433.
20. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. 1955. *Biochem. J.* **60:** 604.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* **193:** 265.
22. Parker, F., and N. F. Peterson. 1965. *J. Lipid Res.* **6:** 455.
23. Zborowski, J., and L. Wojtczak. 1963. *Biochim. Biophys. Acta.* **70:** 596.
24. Epstein, B., and B. Shapiro. 1959. *Biochem. J.* **71:** 615.
25. Lipsett, M. N., and L. M. Corwin. 1959. *J. Biol. Chem.* **234:** 2448.
26. Lands, W. E. M., and P. Hart. 1965. *J. Biol. Chem.* **240:** 1905.
27. Scherphof, G. L., and L. L. M. van Deenen. 1965. *Biochim. Biophys. Acta.* **98:** 204.
28. Bangham, A. D., and R. M. C. Dawson. 1962. *Biochim. Biophys. Acta.* **59:** 103.
29. Bailey, E., C. B. Taylor, and W. Bartley. 1967. *Biochem. J.* **104:** 1026.
30. Shah, D. O., and J. H. Schulman. 1967. *J. Lipid Res.* **8:** 227.
31. Papahadjopoulos, D., and A. D. Bangham. 1966. *Biochim. Biophys. Acta.* **127:** 185.
32. Rendi, R. 1967. *Biochim. Biophys. Acta.* **135:** 333.
33. Carafoli, E., and A. L. Lehninger. 1964. *Biochem. Biophys. Res. Commun.* **16:** 66.
34. Rasmussen, H., and A. Tenenhouse. 1968. *Proc. Nat. Acad. Sci. U. S. A.* **59:** 1364.