The values were corrected for blanks which were incubated with each substrate under identical conditions except that the enzyme was inactivated by heating for 10 min at 100° prior to the incubation. In addition, the flasks containing phosphatidyl choline and phosphatidyl ethanolamine were examined for the production of phosphorylcholine and phosphorylethanolamine. Under conditions of the incubation, no phosphorylcholine or phosphorylethanolamine was produced from these two phosphatides when examined by the method of DAWSON[12]. This finding would indicate a rather marked specificity for the enzyme(s).

From the reported result as well as those previously published by SMITH *et al.* it would appear that the enzyme(s) has at least two requirements for activity. First of all the presence of at least one acyl group on glycerophosphate is necessary since α-glycerophosphate is not a suitable substrate. Secondly, it would appear that a monosubstituted phosphate is required since phosphatidyl choline and phosphatidyl ethanolamine are both inactive.

The demonstration of the presence of rather specific phosphatidate phosphatase in the intestinal mucosa provides additional evidence for the occurrence of the suggested sequence of reactions involving phosphatidic acids in the intestinal absorption of fatty acids[13].

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## Determination of glycerol in phosphatides

Our laboratory's need for a simple, yet sensitive and accurate method for determination of glycerol in phospholipids prompted the development of the method reported here. The method is based on a complete, single-stage acid hydrolysis of phosphatides under such conditions that no destruction of glycerol takes place. The free glycerol is subsequently determined by $HIO_4$ oxidation and spectrophotometric

estimation of the resulting formaldehyde[1]. Our procedure is essentially an extension of the observations reported by HANAHAN *et al.*[2,3] and by HÜBSCHER AND CLARK[4].

Suitable conditions for the hydrolysis of phosphatides were found by using glycerophosphate and free glycerol as model compounds. The following procedure finally evolved. The sample containing 0.2–1.0 $\mu$mole of glycerol is heated in a sealed tube with 5 ml of 2 *N* aq. HCl at 125° for 48 h. The acid hydrolysate is shaken with 2 ml of chloroform. After centrifugation, two samples of 2 ml are withdrawn from the aqueous layer for glycerol assay. For oxidation 0.1 ml of 10 *N* $H_2SO_4$ and 0.5 ml of 0.1 *M* $NaIO_4$ are added and the mixture is incubated at room temperature for 5 min, after which the reaction is stopped by adding 0.5 ml of 10% $NaHSO_3$. For estimation of the formaldehyde a sample of 0.5 ml is withdrawn from the oxidation mixture into a clean tube and 5 ml of 0.18% chromotropic acid in 20 *N* $H_2SO_4$ are added. The mixture is incubated in an oven at 100° for 135 min for maximum color development. After cooling the tube to 25° the color is read in a 2-cm cuvette in a Zeiss PMQ II spectrophotometer at 570 m$\mu$ against a reagent blank.

The glycerol content in unknown samples is obtained from absorbancies of known standards of free glycerol and glycerophosphate*, which are simultaneously run through the whole procedure.

A sample of 1.00 $\mu$mole of pure glycerol taken through the whole procedure yields an absorbancy of 0.865 $\pm$ 0.020 (standard deviation). A linear relationship between the absorbancy and the amount of free as well as lipid-bound glycerol in the sample was confirmed.

The sensitivity of the method can easily be increased by using smaller volumes of acid in the hydrolysis, or by doubling the amount of the reactants in the final color reaction, when the colors can be read in 5-cm cuvettes.

Confirming the findings of HANAHAN[5] we found that only 2% of free glycerol is destroyed during the hydrolysis. Both $\alpha$- and $\beta$-glycerophosphate gave very nearly quantitative glycerol yields (95–96%) after the hydrolysis. The contaminants most likely to be encountered in the water-soluble fraction of lipid hydrolysates: choline, ethanolamine, serine, *m*-inositol, glucose, and galactose, after heating under the conditions of our hydrolysis, gave only insignificant (0–2%) molar yields of "apparent glycerol".

The results of replicate glycerol assays in pure lipids** are given in Table I.

Table I shows that the procedure described yields satisfactory glycerol values in simple glycerides and in the common glycerophosphatides. The ether phosphatide does not give any glycerol response, which means that the ether linkages between glycerol and long-chain alcohols are stable under the conditions of our hydrolysis. Sphingomyelin also does not yield any "apparent glycerol" as the liberated sphingosine is extracted into the chloroform from the acidic water phase[9].

In addition to results shown in Table I the procedure described has yielded correct analyses also in all lipid mixtures tested.

So far we have no direct proof of the correctness of the procedure in analysis of

* Glycerol standards were obtained by determining pycnometrically the glycerol content in two commercial samples of highest quality. Glycerophosphate standards were obtained by drying high-quality commercial samples to constant weight in high vacuum at 50–60°. The samples used had a theoretical P content.

** The purity of lipid samples was ascertained by paper chromatography[6,7] and by determination of phosphorus and acyl ester groupings[8].

TABLE I

RESULTS OF GLYCEROL ASSAYS IN PURE LIPIDS

| *Lipid** | *Size of samples* | | | *Found*** glycerol content (μmole)* | *Yield (%)* |
|---|---|---|---|---|---|
| | *Weight** (μmole)* | *P content (μatom)* | *Calculated glycerol content (μmole)* | | |
| 1-Monopalmitin | 0.500 | | 0.500 | 0.504 | 101 |
| 1-Mono-olein | 0.497 | | 0.497 | 0.506 | 102 |
| 1,3-Dipalmitin | 0.500 | | 0.500 | 0.505 | 101 |
| Trilinolein | 0.353 | | 0.353 | 0.345 | 98 |
| Phosphatidyl choline | | 0.234 | 0.234 | 0.230 | 98 |
| Lysophosphatidyl choline | | 0.574 | 0.574 | 0.548 | 95 |
| Phosphatidyl ethanolamine | | 0.302 | 0.302 | 0.289 | 96 |
| Lysophosphatidyl ethanolamine | | 0.532 | 0.532 | 0.524 | 98 |
| Phosphatidyl serine | | 0.241 | 0.241 | 0.222 | 92 |
| Phosphatidyl inositol | | 0.448 | 0.448 | 0.462 | 103 |
| "Ether phosphatide"§ | | 0.496 | 0.496 | 0.025 | 5 |
| Sphingomyelin | | 0.488 | 0.000 | 0.011 | — |

* The origin of the lipid samples has been described elsewhere[8].

** The samples were dried to constant weight at 50–60° at 0.05 torr.

*** The glycerol values found in the simple glycerides are based on absorbancies of simultaneously run standards of free glycerol. The glycerol values in the phosphatides are based on glycerophosphate standards.

§ Stearyl ether of 2-stearoyl glycerylphosphoryl ethanolamine.

the plasmalogens, but this seems rather certain because of the acid lability of the linkage between glycerol and the long-chain aldehydes.

The existing and suggested methods for estimation of glycerol in phosphatides[10–12] are rather complicated in comparison to the procedure reported here. We think that the possibility of serial glycerol analysis of large numbers of chromatographic fractions will be helpful, for instance in studies on the little-known polyglycerophosphatides.

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