

2. Lehman, R. L. and Wallace, R. In Pullman, B. *Electronic Aspects of Biochemistry*, Academic Press, New York 1964, p. 43.
3. Henriksen, T. and Pihl, A. *Nature* **185** (1960) 307.
4. Prydz, S. and Henriksen, T. *Acta Chem. Scand.* **15** (1961) 791.
5. Rotblat, J. and Simmons, J. A. *Phys. Med. Biol.* **7** (1963) 489.
6. Müller, A., Schambra, P. E. and Pietsch, E. *Intern. J. Radiation Biol.* **7** (1963) 587.
7. Müller, A. *Intern. J. Radiation Biol.* **6** (1963) 137.
8. Köhnlein, W. and Müller, A. *Intern. J. Radiation Biol.* **8** (1964) 141.
9. Henriksen, T., Sanner, T. and Pihl, A. *Radiation Res.* **18** (1963) 147.

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Effect of Phospholipases A, C, and D on the Iodide-Complexing Phospholipid of Thyroid

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An iodide-complexing factor has been previously demonstrated by one of the authors to occur in the lecithin fraction of thyroid phospholipids.^{1,2} This specific factor resisted all attempts to separate it from lecithin by conventional methods employing precipitation, extraction by different solvents, countercurrent distribution, or chromatographic separation. None of the other lipid fractions showed any affinity towards iodide. From these observations it appeared obvious that the iodide-complexing factor is a lecithin. To prove this the lecithin fraction was subjected to the action of phospholipases A, C, and D, in order to elucidate whether also the enzymatic criteria for lecithin are met by the iodide-complexing factor.

Lecithin was obtained by homogenizing human thyroid tissue* in chloroform containing 1% methanol by a Waring Blender type

* Obtained from the surgical unit, University of Turku Hospital.

homogenizer. The centrifuged extract was drawn to dryness by a rotating film evaporator. The residue was dissolved in a small volume of chloroform, and the phospholipids were precipitated three times by adding a tenfold volume of acetone. The precipitate was again dissolved in chloroform, and a tenfold volume of absolute ethanol was added to remove the bulk of non-lecithin lipids. After 20 h at -15°C the precipitate was discarded, and the supernatant was evaporated to dryness. The crude lecithin obtained was dissolved in a small volume of chloroform-methanol 1:1 v/v and chromatographically purified in a 10 mm. diameter column of basic Al_2O_3 (Merck 1076), recovering the 1:1 v/v chloroform-methanol eluate, thus leaving all the non-choline P-lipids in the adsorbent.³ The eluate was evaporated to dryness, and a sample of the residue was analyzed for phosphorus, nitrogen, and choline. Phosphorus was determined by a modified method of Berenblum and Chain⁴ using methol as reducing agent. The content of phosphorus varied from 3.6 to 3.8%, corresponding to a mean molecular weight of 840 if one atom of P is assumed per molecule of lipid. Nitrogen was analyzed by a micro-Kjeldahl procedure, and choline by the method of Glick. The molar ratio of P:N:choline was 1:1:1 with no more than 6% of variation. These values indicate a highly purified sample of lecithin.

A 100 mg sample of the purified lecithin was dissolved in 60 ml of diethylether. 1 ml of 0.1% venom of *Crotalus durissus terrificus* (phospholipase A, Calbiochem) was added in 0.005 M solution of CaCl_2 .^{4,5} The mixture was kept at 30°C under reflux overnight. The precipitate formed was centrifuged off and washed three times with diethyl ether. No phosphorus was detected in the last washing. The ether supernatant and washings were pooled, drawn to dryness, and tested for their iodide-complexing power, none of which was found. The precipitate which contained lysolecithin was dissolved in chloroform-methanol, and purified with Al_2O_3 absorption procedure as described for lecithin above. The recovery was 55 mg of lysolecithin checked to be pure in thin layer chromatography.¹⁰

The purified lecithin was also subjected to the action of phospholipase C (from *Clostridium Welchii*, Sigma) by the method of Zeller.⁶ Phospholipase C cleaves the linkage between glycerol and phosphate yielding diglyceride and phosphorylcholine as products of hydrolysis.

A further sample of lecithin was treated with phospholipase D⁶ from cabbage (B grade, Calbiochem). The cleavage occurs here between phosphate and choline yielding phosphatidic acid and choline.

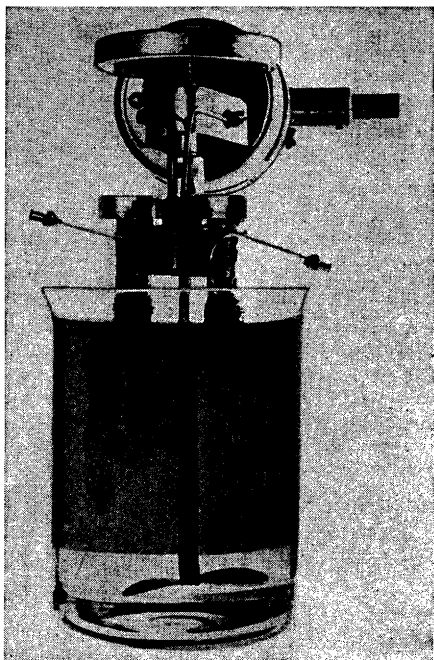


Fig. 1. Dialysis apparatus for determination of the iodide transfer from water phase in the 1 litre glass beaker into chloroform in the two 15 ml cellophane bags. The original concentration of iodide in the water is 10^{-6} M marked with $100 \mu\text{C } ^{131}\text{I}$. 100 mg of sodium hyposulfite is added to water to prevent oxidation of iodide during the reaction. All three spaces are stirred continuously during the run. Samples for determination of ^{131}I activity in chloroform are drawn through polyethene cannulas fitted with hypodermic needles. Without addition of specific lipid to chloroform only negligible amounts of iodide are transferred into the cellophane bags.

All three samples hydrolyzed with various phospholipases were examined for their iodide-concentrating power in an improved two-phase dialysis system (Fig. 1) where untreated lecithin was always run simultaneously as a control. The uptake of ^{131}I into the chloroform phase was plotted against time. The high uptake for purified thyroid lecithin (curve B, Fig. 2) was in all three cases contrasted by negligible uptake into the chloroform phase where lecithin hydrolyzed with one of the phospholipases was added. The percentage of the uptake into the hydrolyzed samples

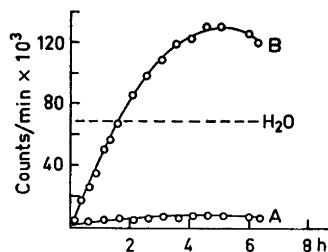


Fig. 2. ^{131}I activity in the chloroform phases against time. H_2O - - - marks the radioactivity in water. *Curve A:* 10 mg of lecithin hydrolyzed with phospholipase A is added to 15 ml of chloroform. *Curve B:* 10 mg of untreated lecithin added to 15 ml of chloroform in the other cellophane bag.

(Table 1) is not significantly higher than that for pure chloroform, or for any other P-lipid (except lecithin) tested.

Table 1. The maximum of the iodide uptake curve for hydrolyzed lecithin expressed as percentage of the same for untreated lecithin.

Sample No.	Maximal iodine uptake into lecithin hydrolyzed with phospholipase, %		
	A	C	D
1	5.4	0.5	6.2
2	5.7	7.4	1.6
3	8.0	7.6	4.7
4	2.9	8.9	6.4
mean	5.5	6.1	4.7

In the three cases where the molecular structure of lecithin is destroyed by any of the specific enzymes, the iodide-complexing power of the preparation is lost. Phospholipase A attacks on α -lecithin and cephalin, and not on sphingomyelin.^{6,7} Thus it seems that a phosphodiglyceride group is essential for iodide complex. Phospholipase C attacks lecithin and sphingomyelin, but not cephalin,^{6,7} cleaving off phosphorylcholine which so is needed in the iodide-complexing molecule. Phospholipase D splits off choline from lecithin. As all these enzymatic actions destroy the iodide-complexing power of the thyroidal lecithin preparation, no doubt is left that

the active principle is lecithin (or one of the lecithins), and not lysolecithin or one of the minor contaminants of the preparation.

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1. Vilkki, P. *Arch. Biochem. Biophys.* **97** (1962) 425.
2. Vilkki, P. *Suomen Kemistilehti B* **33** (1960) 209.
3. Rhodes, D. N. and Lea, C. H. *Biochem. J.* **64** (1957) 526.
4. Tattrie, N. H. *J. Lipid Res.* **1** (1959) 60.
5. Lands, W. E. M. and Merkl, I. *J. Biol. Chem.* **238** (1963) 898.
6. Zeller, E. A. In Sumner, J. B. and Myrbäck, K. *The Enzymes*, Academic Press, New York 1951, Vol. 1 part 2, p. 986.
7. van Deenen, L. L. M. and de Haas, G. H. *Biochem. Biophys. Acta* **70** (1964) 538.
8. Berenblum, I. and Chain, E. *Biochem. J.* **32** (1938) 295.
9. Glick, D. J. *J. Biol. Chem.* **156** (1944) 643.
10. Skipski, V. P., Peterson, R. F. and Barclay, M. *Biochem. J.* **90** (1964) 374.

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Separation of the Aminoethylated A and B Chains of Insulin

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An investigation of the structure of a protein frequently involves the cleavage of one or more disulfide bridges. When tryptophan is absent, as in insulin¹ and ribonuclease,² oxidation with performic acid has been used as a convenient tool for cleaving S—S-bonds. Proteins containing tryptophan may, however, after oxidation give undesirable side products. Since tryptophan is normally stable to reduction

several investigators have studied the reduction of —S—S— to —SH followed by protection of the sulfhydryl groups. Reduction with mercaptoethanol or thio-glycolic acid seems to be the method of choice in this connection.³ The SH-groups can then be stabilized by alkylation.

The present report describes a convenient method for the separation of the two chains of insulin obtained after reduction and aminoethylation of the SH-groups.

Procedure. The general method of Cavallini *et al.*⁴ and Hofman⁵ was used.

a) *Reduction.* The insulin (250 mg = 40 μ moles) was dissolved in 25 ml of 0.05 M tris-HCl-buffer pH 8 which was 8 M with respect to urea in a tube fitted with a three-way stopcock. Air was removed with an oil pump and substituted by argon. Mercaptoethanol (2.5 ml = 40 mmoles) was added and the reaction mixture was again carefully deaerated and left for 24 h at +2°C.

b) *Coupling.* β -Aminoethylbromide-HBr (40 g = 200 mmoles) was completely dissolved in the reduction mixture. After 1 h at room temperature the solution was transferred to a water bath and incubated at 37°C for 4.5 h. It was placed in a coldroom over night. During the first 5.5 h the pH was kept at 8 (indicator paper) with 10 N sodium hydroxide. The final volume was 49 ml. The part of the solution which was not chromatographed immediately was stored at -20°C.

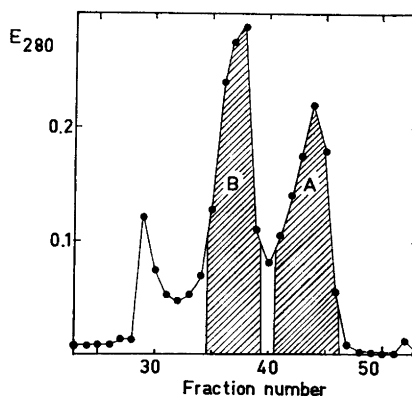


Fig. 1. A chromatogram of the aminoethylated chains from insulin. The shadowed areas were pooled and correspond to the A- and B-chains, respectively. Column: Sephadex G-50, 4 \times 100 cm, eluted with 50% acetic acid; flow rate 25 ml/h, fraction volume 12 ml.