ACTA CHEMICA SCANDINAVICA 15 (1961) 1595–1603

Neutron Activation Paper Chromatographic Analysis of Phospholipids in Human Liver and Bile

FUMIO NAKAYAMA* and ROLF BLOMSTRAND

The Swedish Medical Research Council, Unit for Biochemical Research for Atherosclerosis, University of Lund, Lund, Sweden

Phospholipids in human liver and bile were analyzed using a neutron activation paper chromatographic technique. Phosphatidylserine, phosphoinositide and choline plasmalogen as well as lecithin, phosphatidylethanolamine and sphingomyelin were found to be present. Further evidence for the presence of lysolecithin in normal human bile is also presented. Similar results were obtained with rat bile using radioactive phosphorus.

Lecithin comprises more than 80 % of the phospholipids in human bile in contrast to liver, where it comprises 45 %, indicating a selective secretion of phospholipid classes from liver into bile. The difference found in the fatty acid composition of the total phospholipids in human liver and bile by gas chromatography, coincides with the findings on the differences in phospholipid composition.

Phospholipids have been known to be present in bile from as early as 1862 [1]. However, the nature of the phospholipids has long remained obscure. The first attempt at characterisation of the phospholipids in bile was made in 1939 by Johnston and coworkers [2]. After careful study, they concluded that phospholipids in bile are composed solely of lecithin. This view was shared by several investigators [3,4] until quite recently. Later, in 1960, we presented evidence indicating the presence of not only lecithin but also of sphingomyelin, cephalin and lysolecithin using a combination of radioactive phosphorus, silicic acid impregnated paper chromatography, and hydrolysis with snake venom [5]. Independently, Philips [6] published paperchromatograms suggesting the presence of lysolecithin, sphingomyelin, lecithin and cephalin.

The study of the phospolipids in bile is important not only for intestinal fat absorption [7,8], but also for a better understanding of gallstone disease, since they have been shown to have a definite influence on the solubilisation of the cholesterol in bile [9–11]. Dog, a species known not to be affected by gallstone

* Visiting Investigator of the Swedish Medical Research Council. Present address: Department of Surgery I, Kyushu University, Faculty of Medicine, Fukuoka, Japan, and Department of Surgery, Wayne State University College of Medicine, Detroit, Michigan, U.S.A.

disease, has been shown to have considerably higher concentration of bile phospholipids compared with human beings [12].

The use of paper chromatography as a microanalytical tool is well established. However, in some cases its resolving power is hampered by the lack of suitable chemical methods of detection. Phospholipid analysis is a good example. In recent work [13] we have shown that after mild hydrolysis of phospholipids a combination of paper chromatography followed by neutron activation permits the separated components not only to be located and characterized but also subsequently determined quantitatively. This technique is most advantageous when phospholipid labeling with ^{32}P is not possible and only a small quantity of sample is available, as is usual in clinical investigations. This technique is therefore applied here to the qualitative and quantitative determination of phospholipids in human liver and bile.

EXPERIMENTAL

Materials. Following accidental trauma normal human hepatic bile was obtained from the bile duct of a patient using a choledochostomy tube. Radioactive rat bile was obtained from common bile duct drainage of animals given ^{32}P-orthophosphate (30 μC per g) intraperitoneally. The bile was run directly into methanol in order to prevent disintegration of biliary phospholipids which is known to occur even under refrigerated conditions [2]. Chloroform was added to the bile-methanol mixture in order to bring the ratio of bile, chloroform and methanol to 1:12:6. The mixture was brought to boiling on the water bath and filtered through a Whatman No. 1 paper. The filtrate was partitioned with one third of a volume of distilled water. The chloroform layer was concentrated under reduced pressure, the residue was taken up in a small volume of chloroform and was subjected to silicic acid column chromatography using a 2:1 mixture of silicic acid and Hyflo Super-Cel (about 100 mg of total bile lipids were run per gram of silicic acid) in order to separate the phospolipids [14].

Paper chromatography. Silicic acid impregnated paper chromatography of phospholipids was performed at room temperature according to the method of Reed *et al.*[15] using *n*-butyl ether, di*iso*butylketone, acetic acid and water (20:20:20:3).

A paper chromatography technique for deacylated phospholipid hydrolysates was used which was essentially based on the method of Dawson [16]. 3.0 mg of phospholipids was transferred into a 14 ml Pyrex tube with a glass stopper and dissolved in 0.2 ml of carbon tetrachloride. 1.9 ml of ethanol was added followed by the addition of 0.16 ml of water and 0.06 ml of 1 N aqueous sodium hydroxide solution. After the solution was thoroughly mixed by swirling, the tube was placed in a water bath maintained at 37°. After 20 min, 0.1 ml of ethyl formate was added and incubated for an additional 5 min in order to neutralize excess alkali. The solvent was evaporated under reduced pressure and the residue was partitioned by the addition of 0.5 ml of water and 1.0 ml of an *iso*-butanol/chloroform (1:2) mixture. After centrifugation, the aqueous layer containing alkali labile phospholipid hydrolysate was separated. The lower chloroform/*iso*butanol layer was washed with 0.5 ml of water twice, and the washings combined with the aqueous layer separated previously. The combined extract was concentrated under reduced pressure and the residue, dissolved in 80 % aqueous ethanol, was made up to 1.0 ml. Half the solution was quantitatively loaded on a Whatman No. 1 paper using a Carlsberg micropipette for subsequent paper chromatographic analysis [16], with the solvent system phenol saturated with water, acetic acid, and ethanol (100:10:12). When two dimensional paper chromatography was necessary, the paper chromatogram was, after drying, run using methanol, 98 % formic acid and water (80:13:7) in the second direction.

The chloroform-*iso*butanol layer was hydrolyzed at 37° by vigourously shaking with 0.2 ml 10 % aqueous trichloroacetic acid solution for 30 min. After hydrolysis 1.0 ml of petroleum ether was added, the resulting aqueous layer containing alkali stable and

acid labile phospholipid hydrolysate was concentrated and loaded on a Whatman No. 1 paper. The paper chromatogram was developed [16] using phenol saturated with water, acetic acid and ethanol (100:10:12).

The upper organic solvent layer containing alkali and acid stable phospholipids was transferred quantitatively into a Pyrex tube (190 × 18 mm). After the solvent was evaporated under reduced pressure, 0.35 ml of 0.7 N HCl in methanol was added. The tube was sealed and placed in an oven at 100°. After 4 h the hydrolysate was concentrated and one half of the hydrolysate loaded on the paper and developed with phenol saturated with water [16].

Neutron activation analysis. For neutron activation analysis the paper chromatogram was rolled and sealed in an aluminium container. After irradiation with a stream of slow neutrons of 5×10^{11} neutron/cm^2/sec for 15 h (irradiation was carried out at the Research Reactor, AB Atomenergi, Stockholm), autoradiograms were prepared using a thick plastic sheet of 10.84 mg per cm^2 as filter and standard Kodak X-ray film. After identification of the individual phospholipid hydrolysates, the corresponding spots were cut from the paper chromatogram and eluted four times with a small volume of warm 80 % ethanol. The extracts were combined and concentrated and an aliquot taken for phosphorus determinations using the method of Chen *et al.*[17]

An experiment was carried out in order to determine whether the induced radioactivity on the paper chromatogram could be used for the quantitative determination of the phospholipids separated. It was found, however, that back ground radioactivity induced in the filter paper, was too high to allow an accurate determination of the smaller components. A control experiment carried out in order to examine the extraction procedure showed a recovery of 92—95 % phosphorus. The phosphorus content of the filterpaper used was of the order of 0.02—0.03 μg per cm^2 so that it does not interfere with the final results.

When pure compounds are irradiated with neutrons, rearrangement of the chemical bonds sometimes take place [18]. However, here we have utilized neutron activation after the separation of each component by paper chromatography and thus this possibility should not interfere with the results.

Gas chromatographic analysis of fatty acids in human liver and bile. The liver biopsy sample and hepatic bile were obtained from the same patient operated on for gall-stone disease. Total fat was extracted with a chloroform/methanol (2:1) mixture according to Folch [19]. After saponification and acidification the total fatty acids from the bile were isolated. The liver lipids were separated on silicic acid into cholesterol esters, glycerides and phospholipids [20]. The fatty acids from the different lipid classes were isolated after saponification and acidification. The fatty acids isolated were methylated with diazomethane and analysed by gas-liquid chromatography using an Argon Pye apparatus with an ionisation chamber as detector. As stationary phase a polar polyester (LAC-R-296) was used, the ratio of Celite/stationary phase being 4:1. The experimental procedures used have otherwise been described earlier [20].

RESULTS

Autoradiograms prepared after neutron activation of two-dimensional paper chromatograms of alkali labile phospholipid hydrolysates of human bile, human liver and rat liver, are shown in Fig. 1 and that of a one dimensional chromatogram in Fig. 2a. The following spots were identified: GPC (glycerylphosphorylcholine, derived from lecithin), GPE (glycerylphosphorylethanolamine, from phosphatidylethanolamine), cyc-GP(cyclic glycerophosphate, from lecithin and phosphoinositide), GPS (glycerylphosphorylserine, phosphatidylserine) and GPI (glycerylphosphorylinositol, from phosphoinositide) as previously shown by Dawson [16] using the same paper chromatographic technique. All these components were found to be present in human bile as well as in human and rat liver. However, there are definite differences between these

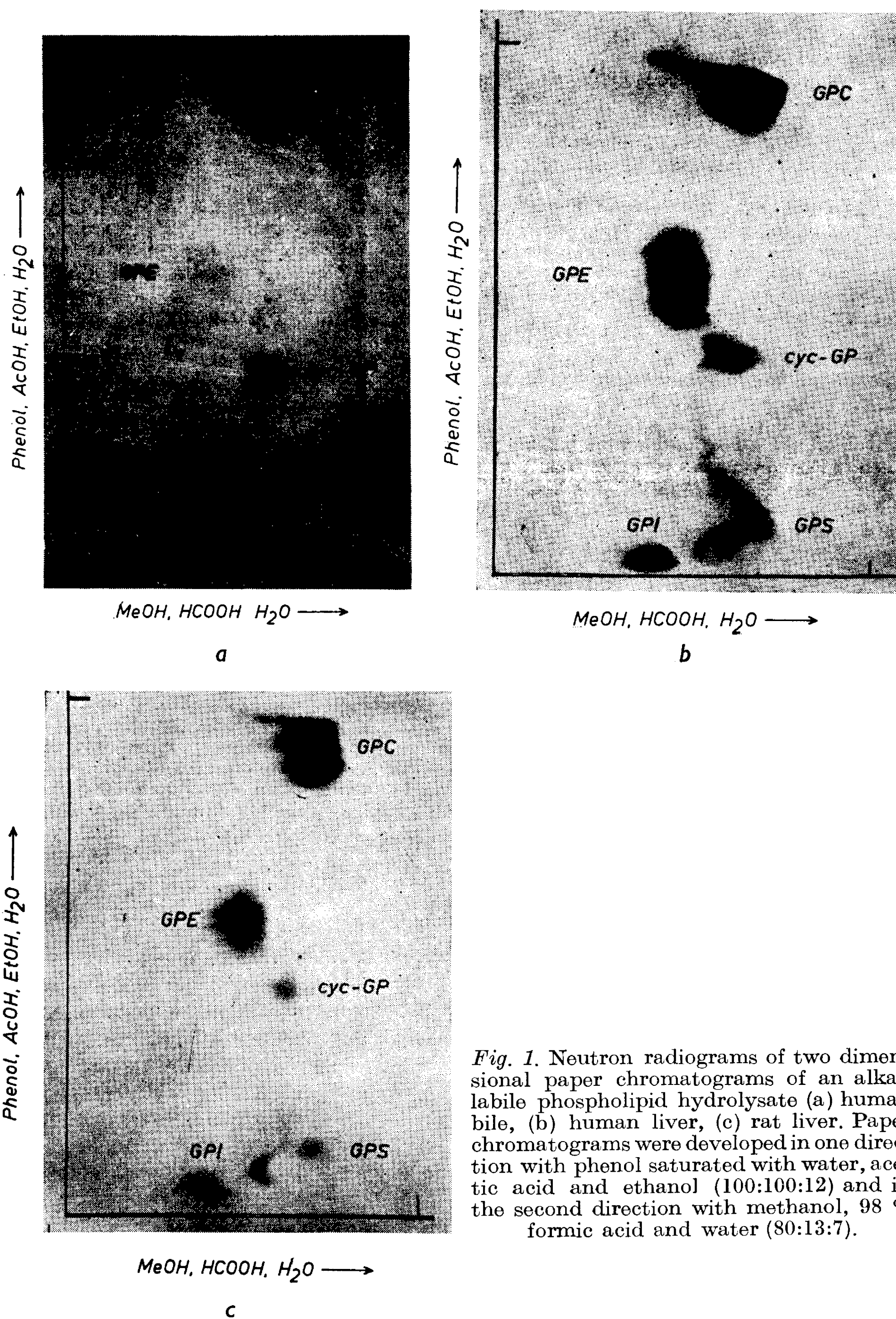

Fig. 1. Neutron radiograms of two dimensional paper chromatograms of an alkali labile phospholipid hydrolysate (a) human bile, (b) human liver, (c) rat liver. Paper chromatograms were developed in one direction with phenol saturated with water, acetic acid and ethanol (100:100:12) and in the second direction with methanol, 98 % formic acid and water (80:13:7).

autoradiograms, *e.g.* in human bile only faint glycerylphosphorylethanolamine, glycerylphosphorylserine and glycerylphosphorylinositol spots are visible as distinct from definite glycerylphosphorylcholine spot (Figs. 1 and 2a). The autoradiogram of alkali stable and acid labile phospholipid hydrolysate showed a glycerylphosphorylcholine spot indicating the presence of choline plasmalogen in human bile as well as in human and rat liver (Fig. 2b). Fig. 2c shows the autoradiogram prepared from a neutron activated paper chromatogram of alkali and acid stable phospholipid hydrolysates from human bile, human and rat liver. Phosphorylcholine, a hydrolysis product of sphingomyelin, was identified in all three. The results presented indicated the presence of lecithin, phosphatidylethanolamine, phosphatidylserine, phosphoinositide, choline plasmalogen and sphingomyelin in human bile as well as in human liver and rat liver.

Similar experiments, carried out on rats using radioactive phosphorus instead of neutron activation, indicated the presence of lecithin, phospatidylethanolamine, phosphatidylserine and phosphatidylionisitide (Figs. 3 and 4a) choline plasmalogen (Fig. 4b) and sphingomyelin (Fig. 4c).

Some preliminary quantitative data are given in Table 1. A comparison between phospholipid composition in normal human bile and liver reveals one striking difference; phospholipids in bile are predominantly composed of lecithin (over 80 %) in contrast to those in liver (48 %). Phosphatidylethanolamine, phosphatidylserine, phosphoinositide, choline plasmalogen and sphingomyelin are also present in human bile but in minute amounts.

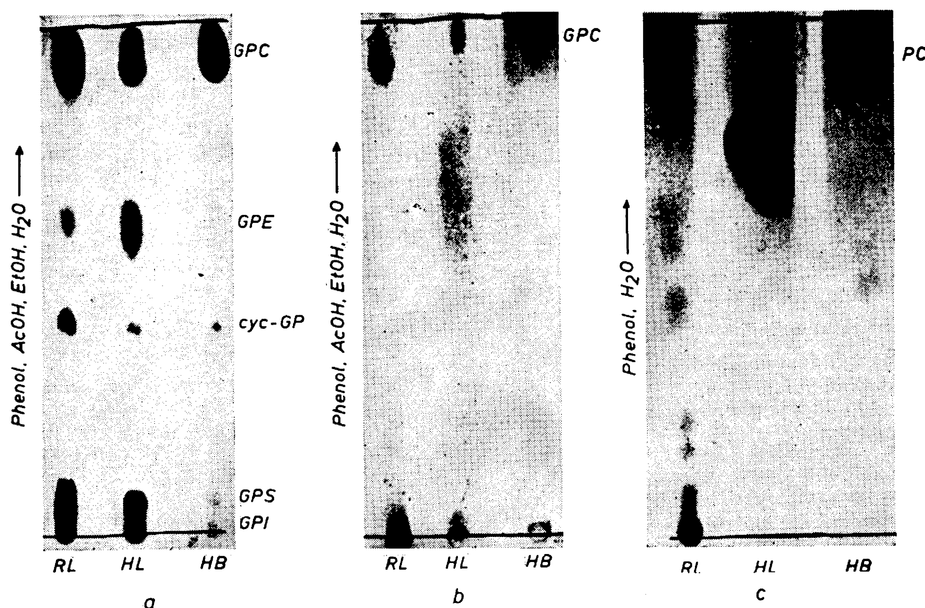

Fig. 2. Neutron radiograms of a phospholipid hydrolysate of rat liver (RL), human liver (HL) and human bile (HB), (a) alkali labile phospholipids, (b) alkali stable acid labile, (c) alkali and acid stable.

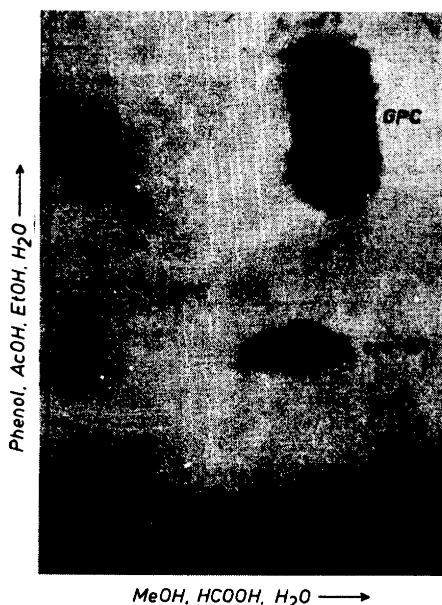

Fig. 3. Autoradiogram of two dimensional paper chromatogram of a ^{32}P-labelled alkali labile phospholipid hydrolysate of rat bile.

Similar experiments were carried out on human bile using silicic acid impregnated paper chromatography and hydrolysis with snake venom, as outlined previously [5], followed by neutron activation. Lysolecithin was also found to be present in human bile as in rat bile.

Figs. 5a and b show a comparison between the fatty acid composition in liver phospholipids and the total fatty acids in bile. Since more than 90 % of the fatty acids in bile are associated with phospholipids, the total fatty acid

Table 1. Phospholipid composition of human bile and liver as determined by neutron activation paper chromatographic analysis *.

	Human bile	Human liver	Rat liver
Alkali labile phospholipids			
Phosphoinositide	2.65	14.93	14.43
Phosphatidylserine	traces	6.54	6.09
Phosphatidylethanolamine	3.42	18.12	8.10
Phosphatidylcholine	81.36	48.36	61.57
Alkali stable acid labile phospholipids			
Choline plasmalogen	3.79	1.49	1.16
	0.36	2.35	1.48
Alkali acid stable phospholipids			
Sphingomyelin	2.52	6.93	4.97
Unidentified	5.90	1.45	3.20

* expressed as % phosphorus per total phospholipid phosphorus.

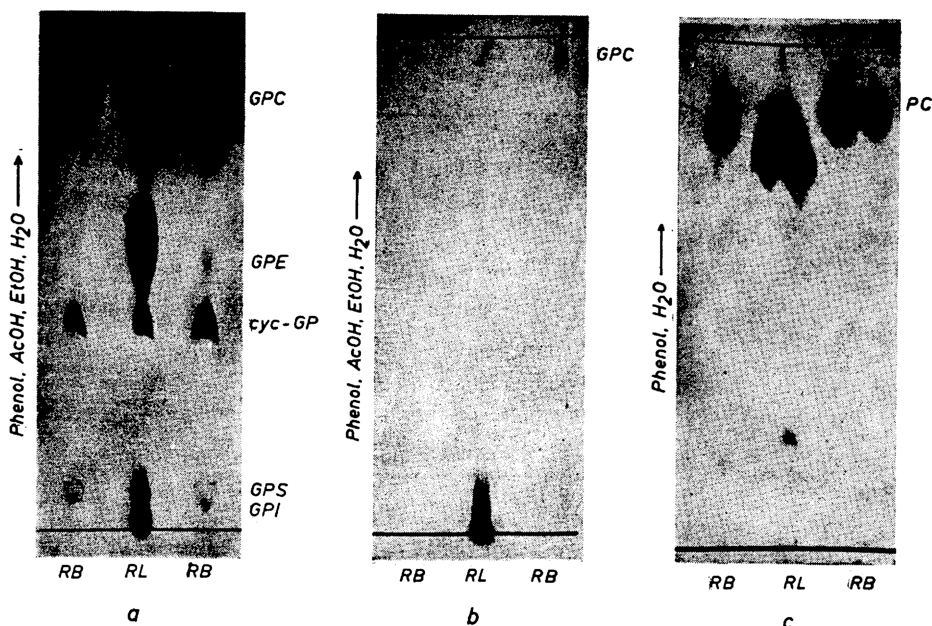

Fig. 4. Autodioragram of a ^{32}P-labelled phospholipid hydrolysate from rat liver (RL) and rat bile (RB), (b) alkali labile phospholipids, (c) alkali stable acid labile, (a) alkali and acid stable.

composition of bile could be expected to be similar to the fatty acid composition of phospholipids in bile. In fact, the fatty acid composition of purified biliary phospholipids is very similar to the total fatty acid composition of bile.

Certain differences could be seen in fatty acid composition of human liver and bile; the relative percentage of stearic acid in the liver phospholipids is greater (20.9 %) than in bile (8.5 %), while linoleic acid is smaller in liver (15.8 %) than in bile (27.8 %).

DISCUSSION

Phospholipids are secreted from the liver and enter the intestinal tract *via* bile. There are indications that phospholipids facilitate the solubilisation of the cholesterol in bile [9–11], thus preventing the formation of gall-stones. During passage in the intestinal tract, phospholipids undergo extensive hydrolysis [21,22], and may interact with fat absorption. Since bile phospholipids comprise an important part of the phospholipids present in the intestine, the mechanism of phospholipid absorption and the way phospholipids interact with fat absorption will not be fully understood, so long as the nature of the phospholipids in bile remains obscure.

The present results on the phospholipid analysis of human and rat bile confirm and extend our earlier findings [5]. Paper chromatography of phospholipids after mild hydrolysis in combination with neutron activation gives a

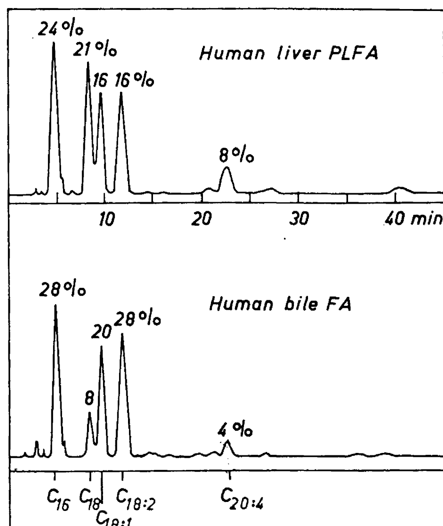

Fig. 5. Total fatty acid composition of, (a) human liver phospholipids, (b) human bile phospholipids.

better separation and identification of each of the phospholipids. Using this technique, choline plasmalogen, phosphatidylserine and phosphatidylinositol are found to be present in human bile as well as in rat bile in addition to lecithin. phosphatidylethanolamine, sphingomyelin, lysolecithin, identified earlier[6]. Gas chromatograms of fatty acids isolated from purified bile lecithin indicated the presence of several kinds of lecithins [20,23]. Among these phospholipids present in bile, the presence of a considerable amount of lysolecithin is of special interest, since this compound has been shown to have a marked solubilising effect on cholesterol [11], which is a major component of gall-stones.

A comparison between the phospholipid composition in human liver and bile reveals the presence of a much greater proportion of lecithin (phosphatidylcholine) in the bile (Table 1), which would indicate that phospholipids are selectively secreted from liver into bile. The significance of the presence of a small amount of other phospholipids has to be investigated.

Since lecithin comprises more than 80 % of bile phospholipids in contrast to that of liver (48 %) and as liver contains an appreciable amount of phospholipids other than lecithin, the differences found between liver and bile (Fig. 7) in the fatty acid composition of the total phospholipids could very well be explained by the difference in phospholipid composition in liver and bile.

This work is part of investigations supported by *The Swedish Medical Research Council.*

REFERENCES

1. Thudichum, J. L. W. *A treatise on gallstones*, John Churchil and Sons, London 1863.
2. Johnston, C. G., Irvin, J. L. and Walton, C. *J. Biol. Chem.* **131** (1939) 425.
3. Isaksson, B. *Acta Soc. Med. Upsaliensis* **56** (1952) 177.
4. Borgström, B. *Acta Chem. Scand.* **11** (1957) 749.
5. Nakayama, F. and Blomstrand, R. *Acta Chem. Scand.* **14** (1960) 1211.
6. Philips, G. B. *Biochim. et Biophys. Acta* **41** (1960) 361.
7. Nakayama, F. *Am. J. Physiol.* **196** (1959) 319.
8. Nakayama, F. and Johnston, C. G. *Am. J. Physiol.* **197** (1959) 327.
9. Isaksson, B. *On the lipids and bile acids in normal and pathological bladder bile: A study of the main cholesterol dissolving components of human bile.* Thesis, Lund, Sweden, 1954.
10. Johnston, C. G. and Nakayama, F. *Arch. Surgery* **75** (1957) 436.
11. Nakayama, F. *Solubility of cholesterol in bile in relation to gallstone formation.* Thesis, Wayne State University College of Medicine, Detroit, Michigan, U.S.A. 1957.
12. Nakayama, F. and Johnston, C. G. *Proc. Soc. Exptl. Biol. Med.* **104** (1960) 73.
13. Blomstrand, R. and Nakayama, F. *Scand. J. Clin. & Lab. Invest. In print.*
14. Borgström, B. *Acta Physiol. Scand.* **25** (1952) 101.
15. Reed, C. F., Swisher, S. N., Marinetti, G. V. and Eden, E. G. *J. Lab. Clin. Med.* **56** (1960) 281.
16. Dawson, R. M. C. *Biochem. J.* **75** (1960) 45.
17. Chen, P. A., Jr., Toribara, T. Y. K. and Warner, H. *Anal. Chem.* **28** (1956) 175b.
18. Sellers, P. S., Sato, T. R. and Strain, H. H. *J. Inorg. & Nuclear Chem.* **5** (1957) 31.
19. Floch, J., Ascoli, I., Lees, M., Meath, J. A. and Lebaron, F. N. *J. Biol. Chem.* **191** (1951) 833.
20. Blomstrand, R. *Acta Chem. Scand.* **14** (1960) 1006.
21. Blomstrand, R. *Acta Chem. Scand.* **8** (1954) 1945.
22. Blomstrand, R. and Nakayama, F. *To be published.*
23. Blomstrand, R. and Ekdahl, P. *Proc. Soc. Exptl. Biol. Med.* **104** (1960) 205.

Received April 19, 1961.