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A continuing study of a chemically induced model of "nephrosis" raises some interesting possibilities in the understanding of membrane mechanochemistry and resultant transport abnormalities.—Ed.

Effects of the Potent Nephrotogenic Aminonucleoside of Puromycin on Phospholipid Metabolism in Rat Kidney Cortex Subcellular Fractions*

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Induction of the nephrotic syndrome in the rat with the aminonucleoside of puromycin, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine, is accompanied by several significant alterations in enzymes and enzyme systems associated with cellular bioenergetics,[1-4] by striking alterations in glomerular and mitochondrial membrane ultrastructure,[5] by changes in mitochondrial mechanochemical parameters such as swelling and U-factor formation,[6] and by significant reduction in the phospholipid fraction of kidney total lipids.[7] During recent years a phospholipid requirement has been demonstrated for a variety of enzymatic activities closely associated with the maintenance and function of cellular and membranous structures and particularly in the function of mitochondrial enzymes and enzyme systems associated with cellular bioenergetics and membrane mechanochemistry.[8-19]

Acting directly as a precursor of the phosphatidyl moiety in phosphatidyl inositol (PI)[20] and indirectly, through dephosphorylation catalyzed by phosphatidate phosphohydrolase (PA-Pase) (E.C.3.1.3.4.), as a precursor of the diglyceride moiety of phosphatidyl choline (PC),[21] phosphatidyl ethanolamine (PE),[21] and triglycerides,[22] phosphatidic acid (PA) occupies a key position in the biosynthesis of phospholipids (See Fig. 1). Thus situated, its metabolism, whether it involves entry into one of the indicated metabolic pathways as a precursor of other phospholipids or participation in a cyclic mechanism for the utilization of the energy of adenosine triphosphate

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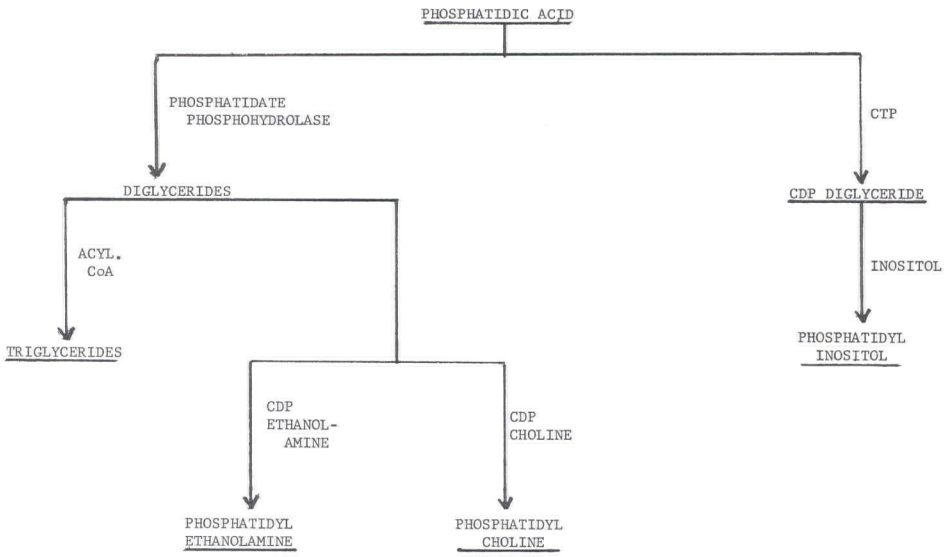

Figure 1
KEY POSITION OF PHOSPHATIDIC ACID
IN PHOSPHOLIPID AND TRIGLYCERIDE FORMATION
CDP = CYTILINE DIPHOSPHATE CTP = CYTIDINE TRIOPHOSPHATE

(ATP) in the transport of hydrophilic substances across lipophilic membranes as proposed by the Hokins[23-25] may significantly affect membrane dynamics. In this context, comparative studies of relative levels of PA-Pase activity in mitochondrial and microsomal fractions and of ^{32}P-orthophosphate incorporation into the various phosphatides of such fractions prepared from kidneys of normal rats and of aminonucleoside-nephrotic rats in which membrane transport is significantly altered were undertaken with the hope that new insight into the pathogenesis of the aminonucleoside-induced disease and into normal membrane transport might be provided.

Methods

Sprague-Dawley virgin female rats, four to six months of age, maintained on Rockland Rat Checkers (*ad libitum* feeding) were used in all studies. In experiments in which the effects of induction of aminonucleoside-nephrosis on renal PA-Pase were investigated, the rats were injected subcutaneously with 5 μM aminonucleoside per 100 gm body weight per day for a period of 10 days. Following the final injection the animals were given no food for 24 hours and then sacrificed by stunning and decapitation. In experiments in which ^{32}P-orthophosphate incorporation into the various phospholipids was studied in normal and aminonucleoside-nephrotic rats, a similar course of treatment with aminonucleoside was pursued and four hours prior to sacrifice the animals were injected intraperitoneally with ^{32}P-orthophosphate solution, pH 7.4, in doses of 200 μC (microcuries) per 100 gm body weight. Specific activity of the labeled compound was 200 μC per μM. The four-hour period was employed to permit orthophosphate uptake on the ascending portion of the uptake curve.[26,27] Immediately following death, the kidneys were excised, decapsulated, and chilled in

ice-cold 0.25 M sucrose. Mitochondria were prepared immediately from chilled kidney cortex following the precautions described by Weinbach.[28] The pellet was sedimented at 3500 x g and washed 2 x with ice-cold 0.25 M sucrose. Microsomes were sedimented from the mitochondrial pellet supernatant by centrifugation at 54,000 x g.

Preparation and assay of deoxycholate extracts of mitochondria and microsomes for PA-Pase activity were accomplished as described by Hokin and Hokin.[29] All extracts were dialyzed for 16 hours in the cold room at 3-5°C. to reduce orthophosphate blanks to a minimum. Substrate phosphatidic acid was synthesized essentially as described by Baer and Buchnea,[30] purified on silicic acid columns[31] and used as the sodium salt. Diolein used in the synthesis was a commercial preparation (D47-DO) supplied through the courtesy of Distillation Products Industries and contained 70% 1,3-diolein and 30% 1,2-diolein.[32] PA-Pase activities expressed in terms of mμM orthophosphate released per hour per milligram extract protein were in all experiments blank corrected. Blanks were run not only on reaction mixtures minus substrate but also on reaction mixtures minus extract.

In experiments in which the incorporation of ^{32}P-orthophosphate into the various phosphatides was studied, the phospholipids were extracted from subcellular fractions as described by Biezenski and Spaet.[33] Non-lipid contaminants were removed from the extracts by eluting aliquots of the phospholipid extracts from silicic acid-impregnated paper with methanol-chloroform (20:80) as described by Biezenski.[34] The purified extracts were resolved by thin-layer chromatography on basic plates of silica gel G[35] or by 2-dimensional thin-layer chromatography (TLC) as described by Abramson and Blecher.[36] The resolved phosphatides were visualized by exposure of the TLCs to iodine vapor, the spots outlined, removed from the plates, and extracted with acidified 3 N methanol. Extracts were evaporated to dryness and phosphorus determined on the residues by the Marinetti modification of the Bartlett procedure.[37] ^{32}P activity was determined on similarly resolved fractions of the purified extracts by removing the silica gel G spots from the plate and resuspending the material directly in the solvent system described by Kinard[38] containing 4% by weight Thixotropic Gel Powder (Cab-O-Sil). Samples were counted with an efficiency of 85% in a Tri-Carb 314 x Scintillation Spectrometer. All counts were corrected for decay and quenching by use of an internal standard.

Results

Table I summarizes the results of PA-Pase studies on deoxycholate extracts of mitochondrial and microsomal fractions prepared from the kidneys of nine groups of normal rats and six groups of aminonucleoside-nephrotic rats (two rats per group). Clearly the PA-Pase activity in such fractions is significantly increased in rats in which the nephrotic syndrome has been induced with the aminonucleoside. Attempts to assess the *in vitro* effects of aminonucleoside on PA-Pase activity of deoxycholate extracts of normal rat kidney mitochondrial and microsomal fractions have thus far yielded information of dubious significance. Relatively high concentrations of amin-

TABLE I

PA-Pase ACTIVITY OF DEOXYCHOLATE EXTRACTS OF KIDNEY CORTEX MITOCHONDRIA AND MICROSOMES

	mμM Orthophosphate Released/hr/mg Extract Protein	
	Mitochondria	Microsomes
Normals (9)*	378 ± 158	365 ± 67
	(104 - 617)†	(290 - 477)
Nephrotic (6)	668 ± 96	505 ± 55
	(498 - 799)	(446 - 579)
P‡ (reference 45)	< 0.01	< 0.01

* Pairs of rats studied.

† Range of values.

± Values are standard deviations.

‡ P = Significance of difference of mean PA-Pase activities for nephrotic and normal rats.

onucleoside (10-20 mM) though appearing to inhibit PA-Pase activity also stimulated to a striking degree the release of orthophosphate from deoxycholate extract controls assayed in the absence of phosphatidate. Though interesting, this finding still remains unexplained and until more relevant information becomes available, decision concerning direct versus indirect effects of aminonucleoside on PA-Pase activity must necessarily be held in abeyance.

Employing experimental conditions identical to those utilized in the assay of PA-Pase activity but with α- or β-glycerophosphate substrate substituted for phosphatidate the possibility of the existence of an alternative mechanism of dephosphorylation of the latter by deoxycholate extracts has also been investigated. Sequential deacylation of phosphatidate to glycerophosphate followed by phosphomonoesterase catalyzed dephosphorylation of the latter, for example, would constitute such a mechanism. Although the results of such studies, shown in Table II, clearly indicate the presence of considerable phosphomonoesterase activity in deoxycholate extracts of normal rat kidney mitochondrial and microsomal fractions, such activity remains relatively unaffected as a result of induction of aminonucleoside-nephrosis in the rat, whereas PA-Pase activity is virtually doubled. Moreover failure to obtain any evidence for the release of fatty acid ester groups from PA by hydroxaminolysis and spectrophotometric determination of ferric hydroxamate complexes during incubation periods comparable to those employed in the PA-Pase assays further substantiates the conclusion that we are, indeed, assaying PA-Pase activity and not the suggested alternative dephosphorylation process.

TABLE II

NONSPECIFIC PHOSPHOMONOESTERASE ACTIVITY OF DEOXYCHOLATE EXTRACTS OF KIDNEY MITOCHONDRIA AND MICROSOMES

	Orthophosphate Released mμM/hr/mg Extract Protein			
Substrate*	Normal Rat Kidney		Aminomucleoside-Nephrotic Rat Kidney	
	Mitochondria	Microsomes	Mitochondria	Microsomes
α-Glycerophosphate	240	98	190	92
β-Glycerophosphate	707	375	871	386

* Substrate concentration 0.002 M in all experiments.

Table III shows the distribution of ^{32}P-orthophosphate incorporated into the phospholipids of mitochondrial and microsomal fractions prepared from the kidney cortex of both normal and aminonucleoside-nephrotic rats. Certainly the most striking alteration seen in this data is in the percentage of the total ^{32}P incorporated into the PI fraction. In aminonucleoside-nephrotic rats this percentage is almost double that seen in normal rats.

TABLE III

DISTRIBUTION OF ^{32}P-ORTHOPHOSPHATE INCORPORATED INTO KIDNEY CORTEX PHOSPHOLIPIDS*

	Percentage of Total ^{32}P Incorporated into Phospholipid			
Phospholipid Fraction	Normal Rat Kidney		Aminonucleoside-Nephrotic Rat Kidney	
	Mitochondria	Microsomes	Mitochondria	Microsomes
PC	73.53	79.75	70.39	72.68
PE	14.34	9.09	14.01	12.67
PI	6.05	5.72	11.94	10.91
PA	1.69	2.34	1.46	1.70
Sph	2.94	1.48	2.10	1.86

* Phosphatidyl serine (PS) was not detected on the TLCs.

Tables IV and V summarize specific activity and relative specific activity data for the several phospholipids isolated from kidney cortex mitochondria and microsomes of normal and aminonucleoside-nephrotic rats. Specific activity of kidney mitochondrial PC is reduced 11% as a result of induction of aminonucleoside-nephrosis in the rat. Specific activities of the PI and PE fractions, on the other hand, are increased 9 and 35% respectively in aminonucleoside-nephrotic rat kidney mitochondria. Specific

TABLE IV

SPECIFIC ACTIVITY AND RELATIVE SPECIFIC ACTIVITY OF RAT KIDNEY CORTEX MITOCHONDRIAL PHOSPHOLIPIDS

Phospholipid Fraction	Specific Activity (CPM/ugm.P)		Relative Specific Activity * (x 10)	
	Normal	Aminonucleoside-Nephrotic	Normal	Aminonucleoside-Nephrotic
PC	7,031	6,256	9.3	6.3
PI	4,822	5,255	6.3	5.3
PE	1,714	2,313	2.3	2.3
PA	758	746	1.0	0.7
Sph	681	713	0.9	0.8

* Relative Specific Activity = $\frac{\text{Specific Activity of Phospholipid-P}}{\text{Specific Activity of Acid-Soluble-P}}$

activities of PA and Sph (sphingomyelin) fractions remain unchanged. Relative specific activities of the PC, PI, and PE fractions were reduced respectively 32, 15, and 0% while that of the PA fraction was decreased 30% in mitochondria.

TABLE V

SPECIFIC ACTIVITY AND RELATIVE SPECIFIC ACTIVITY OF RAT KIDNEY CORTEX MICROSOMAL PHOSPHOLIPIDS

Phospholipid Fraction	Specific Activity (CPM/ugm.P)		Relative Specific Activity * (x 10)	
	Normal	Aminonucleoside-Nephrotic	Normal	Aminonucleoside-Nephrotic
PC	4,829	4,861	6.6	4.9
PI	2,508	3,306	3.4	3.3
PE	1,720	2,331	2.3	2.4
PA	381	694	0.5	0.7
Sph	392	337	0.5	0.3

* See footnote in Table IV.

In the case of kidney microsomal fractions, induction of aminonucleoside-disease had no effect on the specific activity of the PC fraction but produced marked and striking elevations in the specific activities of the PI, PE, and PA fractions. These were respectively 32, 35, and 82% higher in the fractions prepared from aminonucleoside-nephrotic rat kidney microsomes. Relative specific activities remained unchanged in PI and PE fractions, increased 40% in the PA fraction, and decreased 26% in the PC fraction as a result of induction of the aminonucleoside disease.

Discussion

The striking increase in PA-Pase activity in deoxycholate extracts of kidney mitochondria and microsomes of rats treated with the aminonucleoside of puromycin and the apparent inhibition of such activity in extracts prepared from normal rat kidney subcellular fractions assayed in the presence of added aminonucleoside bears a striking resemblance to previously reported effects of aminonucleoside on ATPase activity.[27] Thus, both PA-Pase and ATPase activity are significantly elevated in aminonucleoside-nephrotic rat kidney mitochondrial and microsomal fractions and paradoxically both appear to be inhibited in assays conducted in the presence of added aminonucleoside. To some degree, as earlier reported, the inhibitory effects of aminonucleoside on the ATPase activity of rat kidney cortex mitochondria appear to be dependent on the presence of exogenously added activating cations and/or on the structural state of the mitochondria.[39] Indeed, ATPase activity assayed in the absence of exogenously added activating cations, but in the presence of added aminonucleoside was actually stimulated.[39] In the absence of experimental information pertaining to the effects of ionic environment on PA-Pase activity of deoxycholate extracts of kidney mitochondrial and microsomal fractions, we can only speculate concerning a similar dependency. Relatively recent studies of partially purified soluble rat liver phosphatidate phosphohydrolase activity, however, indicate stimulation of such activity by monovalent cations such as Na^+ and K^+.[40] So, it is tempting to speculate that in this respect also the PA-Pase and ATPase activities of kidney mitochondrial and microsomal fractions are also strikingly similar, and that similar phosphohydrolase-aminonucleoside interaction sites are probably involved in the observed alterations.

Since increased PA-Pase activity might be expected to enhance the pool of diglyceride precursors available for the formation of PC, PE, and triglycerides, stimulation of the formation of these lipids might also be expected. As judged by relative specific activity data, biosynthesis of PC is actually reduced, contributing to the reduction of kidney total phospholipids in the aminonucleoside-nephrotic rat as reported by Levy.[7] Biosynthesis of PE was not affected. Marked hyperlipemia in aminonucleoside-nephrotic rats to which a several-fold increase in serum triglycerides is a major contributing factor[7] might, however, be at least partially ascribable to the stimulating effect of increased diglycerides on triglyceride formation.

Acting directly as a precursor of the phosphatidyl moiety in the biosynthesis of PI,[20] PA might be expected to be more highly labeled with ^{32}P than PI. Data

presented in Tables IV and V, however, clearly indicate that the opposite situation prevails. In both rat kidney cortex mitochondrial and microsomal fractions of normal and of aminonucleoside-nephrotic rats, the specific activity and the relative specific activity of the PI fraction are five- to seven-fold greater than the corresponding values for the PA fraction. Our findings in rat kidney cortex subcellular fractions are in this respect strikingly similar to those reported by Tinker *et al*[27] in studies on phospholipid metabolism in rabbit kidney cortex slices. The relatively low level of labeling of PA in comparison with that of PI is difficult to explain in terms of a metabolite pathway involving random metabolism of precursor molecules.[41] Existence of an alternative metabolic pathway for the formation of PI, utilization of compartmentalized PA,[23] or selective uptake of newly synthesized radioactive PA for PI formation (equivalent to nonrandom utilization of PA as suggested by Possmayer and Strickland[42]) might be invoked, however, to explain these observations. Possmayer and Strickland[42] in studying the nature of the cytosine nucleotide requirement for the biosynthesis of PI in rat brain have reported PA biosynthesis to be inhibited by cytidine triphosphate (CTP) or some product derived therefrom. Results of their studies indicated the labeling of PA from α-glycerophosphate to be substantially reduced in the presence of CTP but labeling of PI to be enhanced and above that in the PA fraction. Should it prove possible to demonstrate similar effects of CTP on the labeling of the same phosphatides in kidney tissue the observed differences in the labeling of PA and PI might be similarly explained.

As to the significance of the striking alterations induced in phospholipid metabolism in kidney cortex mitochondria and microsomes as a result of induction of the nephrotic syndrome in the rat with the aminonucleoside of puromycin we can only speculate at this time. Clearly, however, under experimental conditions (i.e., induction of the nephrotic syndrome) known to be accompanied by profound alterations in glomerular membrane permeability to albumin and in tubular water and electrolyte transport, shifts in the metabolic activity of PC, PI, PE, and PA focus attention again on the possible involvement of phospholipids in membrane mechanochemistry. In this context the findings of Vignais et al,[19] that PI is a required factor for ATP-induced contraction of pre-aged or KCl-extracted rat liver mitochondria and the recently reported observations of Martonosi *et al* [43] on the role of phospholipids and especially of lecithin (PC) and lysolecithin in the ATPase activity and Ca^{++} transport of sarcoplasmic reticulum and on kidney microsomal ATPase isolated as described by Skou,[44] may in some as yet unknown manner be relevant. Thus far, the preponderance of experimental information available does not appear to support direct participation of phospholipids as phosphorylated intermediates in the reactions of cellular bioenergetics. Alternatively their involvement may well prove to be relatable to a physical role in specific tertiary or quaternary conformation of membrane proteins.[25,43] Increasing tempo of investigations focusing on this aspect of phospholipid involvement in membrane mechanochemistry will hopefully provide new insight into what might be classified as membrane transport abnormalities.

REFERENCES

1. Bartlett, P.; Keegan, J.; and Schaefer, H.: Mechanism of aminonucleoside-induced nephrosis in the rat. III. Kidney mitochondrial phosphorylation and dephosphorylation activity, *Proc Soc Exp Biol Med* 112:96-101, Jan 1963.

2. Bartlett, P.; Tsujimoto, T.; and Schaefer, H.: Observations of rat kidney mitochondrial adenosine triphosphatase activity during induction of aminonucleoside nephrosis, *Life Sci* 4:1590-14, Aug 1965.

3. Johnston, C.C., and Bartlett, P.: Respiratory control in kidney and liver mitochondria isolated from rats treated with the potent nephrotogenic aminonucleoside of puromycin, *Biochem Pharmacol* 14:1231-6, Aug 1965.

4. Johnston, C.C., and Podsiadly, C.J.: Oxidative enzyme content of kidneys from normal and aminonucleoside-nephrotic rats, *Proc Soc Exp Biol Med* 124:988-91, Mar 1967.

5. Metcoff, J.; Kaiser, E.; and Spargo, B.: "Correlation of ultrastructure and enzyme activities in kidneys of rats with aminonucleoside nephrosis," in *Proceedings of the Second International Congress of Nephrology, Prague, August 26-30, 1963,* publication 78, International Congress Series, Amsterdam: Excerpta Medica Foundation, 1964, pp 511-3.

6. Maeda, J., and Bartlett, P.: Effects of the potent nephrotogenic aminonucleoside of puromycin on mitochondrial mechanochemistry, *Biochem Pharmacol* 16:761-8, May 1967.

7. Levy, R.: "Lipids of the kidney, blood, and urine in the nephrotic syndrome," in Metcoff, J. (ed.): *Renal Metabolism and Epidemiology of Some Renal Diseases, Proceedings, Fifteenth Annual Congress on the Kidney,* New York: National Kidney Foundation, 1964, pp 222-30.

8. Brierley, G.P.; Merola, A.J.; and Fleischer, S.: Studies of the electron-transfer system. XLIX. Sites of phospholipid involvement in the electron-transfer chain, *Biochim Biophys Acta* 64:218-28, 1962.

9. Criddle, R.S., et al: Specific interaction of mitochondrial structural protein (S.P.) with cytochromes and lipid, *Biochem Biophys Res Comm* 5:75-80, May 1961.

10. Fleischer, S., and Klouwen, H.: The role of soluble lipid in mitochondrial enzyme systems, *Biochem Biophys Res Comm* 5:378-83, Aug 1961.

11. Green, D.E., and Fleischer, S.: The role of lipids in mitochondrial electron transfer and oxidative phosphorylation, *Biochim Biophys Acta* 70:554-82, 1963.

12. Lester, R.L., and Fleischer, S.: Studies on the electron-transport system. XXVII. The respiratory activity of acetone-extracted beef-heart mitochondria role of coenzyme Q and other lipids, *Biochim Biophys Acta* 47:358-77, 1961.

13. Martonosi, A.: Role of phospolipids in ATPase activity and Ca transport of fragmented sarcoplasmic reticulum, *Fed Proc* 23:913-21, Sept-Oct 1964.

14. McConnell, D.G., et al: Studies on the electron transfer system. LXV. Formation of membranes by purified cytochrome oxidase, *J Biol Chem* 241:2373-82, May 1966.

15. Petrushka, E.; Quastel, J.H.; and Scholefield, P.G.: Role of phospholipids in mitochondria respiration, *Canad J Biochem Physiol* 37:975-87, Aug 1959.

16. Petrushka, E.; Quastel, J.H.; and Scholefield, P.G.: Role of phospholipids in oxidative phosphorylation and mitochondrial structure, *Canad J Biochem Physiol* 37:989-98, Aug 1959.

17. Richardson, S.H.; Hultin, H.O.; and Fleischer, S.: Interactions of mitochondrial structural protein with phospholipids, *Arch Biochem* 105:254-60, May 1964.

18. Tzagoloff, A., and MacLennan, D.H.: Studies of the electron-transfer system. LXIV. Role of phospholipid in cytochrome oxidase, *Biochim Biophys Acta* 99:476-85, Jun 22 1965.

19. Vignais, P.M.; Vignais, P.V.; and Lehninger, A.L.: Identification of phosphatidylinositol as a factor required in mitochondrial contraction, *J Biol Chem* 239:2011-21, Jun 1964.

20. Agranoff, B.W.; Bradley, R.M.; and Brady, R.O.: The enzymatic synthesis of inositol phosphatide, *J Biol Chem* 233:1077-83, Nov 1958.

21. Kennedy, E.P., and Weiss, S.B.: The function of cytidine coenzymes in the biosynthesis of phospholipides, *J Biol Chem* 222:193-214, Sept 1956.

22. Weiss, S.B., and Kennedy, E.P.: The enzymatic synthesis of triglycerides, letter to the editor, *J Amer Chem Soc* 78:3550, Jul 20 1956.

23. Hokin, L.E., and Hokin, M.R.: Phosphatidic acid metabolism and active transport of sodium, *Fed Proc* 22:8-18, Jan-Feb 1963.

24. Hokin, L.E., and Hokin, M.R.: The mechanism of phosphate exchange in phosphatidic acid in response to acetylcholine, *J Biol Chem* 234-1387-90, Jun 1959.

25. Hokin, M.R., and Hokin, L.E.: The synthesis of phosphatidic acid and protein-bound phosphorylserine in salt gland homogenates, *J Biol Chem* 239:2116-22, Jul 1964.

26. Nelson, D.R., and Cornatzer, W.E.: Effect of digitoxin, aldosterone, and dietary sodium chloride on incorporation of inorganic P^{32} into liver and kidney nuclear and mitochondrial phospholipids, *Proc Soc Exp Biol Med* 116:237-42, May 1964.

27. Tinker, D.O.; Koch, A.; and Hanahan, D.J.: Phospholipid metabolism in kidney. II. Potassium uptake, lipid composition, and P^{32} labelling of the phospholipids in rabbit kidney cortex slices in vitro, and the effects of diphenylhydantoin, *Arch Biochem* 103:66-73, Oct 1963.

28. Weinbach, E.C.: A procedure for isolating stable mitochondria from rat liver and kidney, *Anal Biochem* 2:335-43, Aug 1961.

29. Hokin, M.R., and Hokin, L.E.: The synthesis of phosphatidic acid from diglyceride and adenosine triphosphate in extracts of brain microsomes, *J Biol Chem* 234:1381-6, Jun 1959.

30. Baer, E., and Buchnea, D.: Synthesis of unsaturated α-phosphatidic acids and α-bisphosphatidic acids; cardiolipin substitutes. IV, *Arch Biochem* 78:294-305, Dec 1958.

31. Hanahan, D.J.; Dittmer, J.C.; and Warashina, E.: A column chromatographic separation of classes of phospholipides, *J Biol Chem* 228:685-700, Oct 1957.

32. Hokin, L.E.; Hokin, M.R.; and Mathison, D.: Phosphatidic acid phosphatase in the erythrocyte membrane, *Biochim Biophys Acta* 67:485-97, Mar 12 1963.

33. Biezenski, J.J. and Spaet, T.H.: Phospholipid content of subcellullar fractions in adult rat organs, *Biochim Biophys Acta* 51:221-6, Aug 5 1961.

34. Biezinski, J.J.: A simple chromatographic technique for removal of non-lipid contaminants from lipid extracts, *J Lipid Res* 3:120-5, Jan 1962.

35. Skipski, V.P.; Peterson, R.F.; and Barclay, M.: Separation of phosphatidyl ethanolamine, phosphatidyl serine, and other phospholipids by thin-layer chromatography, *J Lipid Res* 3:467-70, Oct 1962.

36. Abramson, D., and Blecher, M.: Quantitative two-dimensional thin-layer chromatography of naturally occurring phospholipids, *J Lipid Res* 5:628-31, Oct 1964.

37. Marinetti, G.V.: Chromatographic separation, identification, and analysis of phosphatides, *J Lipid Res* 3:1-20, Jan 1962.

38. Kinard, F.E.: Liquid scintillator for the analysis of tritium in water, *Rev Sci Instrum* 28:293-4, Apr 1957.

39. Bartlett, P.; Tsujimoto, T.; and Schaefer, H.: Dependence of aminonucleoside-inhibition of kidney mitochondrial adenosine triphosphatase on activation and structural state of mitochondria, *Proc Soc Exp Bl Med* 114:29-31, Oct 1963.

40. Sedgwick, B., and Hübscher, G.: Metabolism of phospholipids. X. Partial purification and properties of a soluble phosphatidate phosphohydrolase from rat liver, *Biochim Biophys Acta* 144:397-408, Oct 2 1967.

41. Zilversmit, D.B.; Entenman, C.; and Fishler, M.C.: On the calculation of "turnover time" and "turnover rate" from experiments involving the use of labeling agents, *J Gen Physiol* 26:325-31, Jan 1943.

42. Possmayer, F., and Strickland, K.P.: The incorporation of α-glycerophosphate-^{32}P into the lipids of rat brain preparations. II. On the biosynthesis of monophosphoinositide, *Canad J Biochem* 45:63-70, Jan 1967.

43. Martonosi, A.; Donley, J.; and Halpin, R.A.: Sarcoplasmic reticulum. III. The role of phospholipids in the adenosine triphosphatase activity and Ca++ transport, *J Biol Chem* 243:61-70, Jan 10 1968.

44. Skou, J.C.: Preparation from mammallian brain and kidney of the enzyme system involved in active transport of Na ions and K ions, *Biochim Biophys Acta* 58:314-25, Apr 9 1962.

45. Fisher, R.A.: *Statistical Methods for Research Workers,* ed 10, Edinburgh: Oliver and Boyd, Ltd, 1946.