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STUDIES ON THE METABOLISM OF LIPIDS:
I. INCORPORATION OF LINOLEIC ACID INTO
LIPIDS OF RAT TISSUES. II. MECHANISM OF
ACTION OF PHOSPHOLIPASE B.

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I. INCORPORATION OF LINOLEIC ACID INTO LIPIDS
OF RAT TISSUES
II. MECHANISM OF ACTION OF PHOSPHOLIPASE B

by
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A thesis submitted to the Faculty
of Graduate Studies in partial fulfillment
of the requirements for the degree
of
Doctor of Philosophy

Carleton University
Ottawa, Ontario
1966

PETROLOGY OF ADAMANT PLUTON,

BRITISH COLUMBIA

By

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A thesis submitted to the Faculty of Graduate
Studies in partial fulfillment of the requirements for the
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Carleton University

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ABSTRACT

Part I.

Changes in the proportion of linoleic acid in the total fatty acids of rats fed varying levels of corn oil in a purified basal diet were followed with time. The greatest increase in carcass linoleic acid in response to a high level of dietary linoleic acid occurred within the first three weeks. After nine weeks, the proportion of linoleic acid in the carcass fatty acids of rats receiving no fat was 2%, whereas in those receiving 20% corn oil the proportion was 46%; this level was not exceeded when 30% corn oil was fed for the same time. In rats fed 2 or 20% corn oil for intervals up to 24 days, the proportion of linoleic acid in the liver fatty acids reached a maximum more quickly than that in the carcass. The concentration of linoleic acid in liver neutral lipid, phosphatidyl choline, and phosphatidyl ethanolamine, determined after separation by paper chromatography, was influenced to different degrees by the dietary level of that acid. The greatest rate of increase in the proportion of linoleic acid occurred in the liver neutral lipids of rats supplied with 20% corn oil. In this lipid fraction the concentration of linoleic acid approached that of the dietary oil. The linoleate content of

phosphatidyl choline appeared to be influenced by the level of exogenous linoleate within three days, and that of phosphatidyl ethanolamine within six days. In all lipid fractions studied, the proportion of linoleic acid deposited was a function of the level of linoleate intake.

Part II.

The degradation of glycerylphosphatides was studied with phospholipase B from Penicillium notatum. This enzyme rapidly deacylated purified egg lecithin in the absence of lipid activators when the substrate was ultrasonically dispersed. Decrease in acyl ester groups was accompanied by release of water-soluble phosphate, identified chromatographically as glycerylphosphorylcholine; no lysolecithin was detected by thin-layer chromatography at any stage of the reaction. The optimum pH in acetate buffer was 4.0, and the apparent K_m was 6.3 mM for the ultrasonically dispersed substrate. The rate of enzymic hydrolysis depended upon the degree of dispersion and unsaturation of the substrate. In order of decreasing ease of hydrolysis, the substrates were lysolecithins, egg lecithin, egg hydrolecithin, dimyristoyl cephalin, and dimyristoyl lecithin. Enzymic activity was strongly inhibited by glutathione, cysteine, iron, or prolonged dialysis. Since inhibition by glutathione was

completely reversed by ferricyanide, the enzyme appeared to require -S-S- linkages for activity.

Phospholipase B preparations from rat liver were found to have relatively low activity and to catalyse the deacylation of lysolecithin more readily than that of lecithin. The activity of phospholipase B in liver may therefore not be of major physiological significance.

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INTRODUCTION

It has been known for more than thirty years that animals require a supply of certain fatty acids in their diet. The most common of these essential substances are linoleic and arachidonic acids. Without an exogenous source of these polyunsaturated acids, epithelial abnormalities and other physiological disturbances appear, and death eventually follows. Except for such gross manifestations, however, the biological role of essential fatty acids remains obscure.

As the composition of complex lipids in cellular membranes has become known, it is apparent that linoleic acid, or arachidonic acid derived from it, are constituents of membrane phosphatides. Although the mechanism for in vitro biosynthesis of phosphatides is now well established, little information is available concerning their metabolism in the intact animal. In particular, it is not known how the dietary essential fatty acids enter the cellular phospholipid and neutral lipid molecules. The first part of the present investigation was therefore undertaken to elucidate the pattern of incorporation of linoleic acid in the body of the rat. This involved a study of the changes in fatty acid composition of tissue from animals supplied with different levels of dietary

corn oil for various time intervals. From initial studies on the composition of carcass fatty acids, it became apparent that the response to linoleic acid, the principal fatty acid in corn oil, occurred within the first three weeks of the feeding period. The rate of incorporation of linoleic acid during the early phase of corn oil administration was therefore investigated in detail.

The composition of membrane phosphatides and the functioning of mitochondria as influenced by a dietary deficiency of essential fatty acids have received attention by other investigators. A study of the opposite situation, namely, the effect of feeding an essential fatty acid on the degree and rate of change in fatty acid composition of specific phosphatides has not been made. In the present work, the incorporation of dietary linoleic acid into lecithin and phosphatidyl ethanolamine was examined in liver cells which are rich in membraneous structures. Changes in total and neutral lipid fatty acids were also studied simultaneously.

Fatty acids, including linoleic acid, occur in living organisms in esterified forms. Two specific enzymes, phospholipase A and phospholipase B, are known to catalyse the hydrolysis of fatty acid ester linkages in phosphatides. The action of phospholipase A, which is

involved in the degradation of lecithin to lysolecithin and the release of free fatty acid from the β -position, is well understood, but the action of phospholipase B is still controversial.

Early investigators demonstrated that phospholipase B deacylated only lysolecithin. Subsequently, it was found that lecithin could also be attacked by this enzyme if activators were added to the reaction system. A theory then arose that the action of phospholipase B could be accounted for by a combination of phospholipase A and lysolecithinase activities. The confusion resulted in several names being applied to the enzyme, for example, lecithinase B, lysophospholipase, lysolecithinase and α -lysolecithinase. In the 1961 report by the Commission on Enzyme Nomenclature, phospholipase B was designated as lysolecithin acyl-hydrolase (EC 3.1.1.5), and described as the enzyme which catalyses the hydrolysis of lysolecithin to glycerylphosphorylcholine and fatty acid. This decision, however, did not resolve the controversy concerning the mode of action of the enzyme.

As the second part of this research project, phospholipase B was investigated to clarify its properties and substrate specificity. To do this, it was first necessary to develop a reliable in vitro assay system for the enzyme. A preparation from Penicillium notatum was employed because it was the most potent source of

phospholipase B known, and was relatively easy to prepare. Since all the assay systems used in previous work were complicated by their requirement for activators, it was desirable to devise a simpler system. This was developed in the present investigation, and was used to study the properties of phospholipase B and to examine its mode of action.

I. INCORPORATION OF LINOLEIC ACID INTO THE LIPIDS
OF RAT TISSUES

A. REVIEW OF LITERATURE

1. Function of essential fatty acids

a. Description of deficiency

The classical experiments of Burr and Burr (1929, 1930) demonstrated the necessity of exogenous fat for growth and survival of animals. When they rigidly excluded all sources of fatty acids from the diet of the rat, the skin became scaly, caudal necrosis developed, kidneys degenerated, growth ceased in about three months, and death eventually ensued. Other symptoms of fat-deficient rats included impaired fertility, increased water consumption and decreased urine production. The increased loss of water was attributed to the abnormal permeability of the skin (Sinclair, 1958). Changes in the skin involved a hyperplasia of the surface epithelium and a plugging of the orifices of hair follicles, which would be associated with the dry appearance of the skin. The detailed histology of the skin of rats deficient in essential fatty acids was reviewed by Sinclair (1964). With regard to reproductive failure, Evans, Lepkovsky and Murphy (1934) found that rats deprived of fat for five to six months showed arrested spermatogenesis at the spermatocyte stage, and a restoration of fertility when a source of essential fatty acids was provided.

Sinclair (1964) has defined an essential fatty acid as "...an unsaturated fatty acid (or closely related compound such as the corresponding alcohol) that cannot be synthesized in the body in sufficient amount for health and must therefore be provided in relatively small amount unless a closely related substance is available to the body..."

b. Chemical nature of essential fatty acids

Linoleic acid (cis,cis-9,12-octadecadienoic acid) was demonstrated to be essential for the rat by Burr and Burr (1930). In the same study, arachidonic acid (cis,cis,cis-5,8,11,14-eicostatetraenoic acid) appeared to be without effect, but Turpeinen (1947) found methyl arachidonate to be three times more effective in promoting growth than methyl linoleate. Three years later, Burr, Brown, Kass and Lundberg (1940), Hume, Nunn, Smedley-Maclean and Smith (1940) and Smedley-Maclean and Nunn (1940) confirmed that arachidonic acid was indeed an essential fatty acid. It is not surprising that conflicting results were obtained in this early work, for highly unsaturated acids are notoriously unstable. The extensive bioassay studies of Thomasson (1953) also established γ -linolenic acid (cis,cis,cis-6,19,12-octadecatrienoic acid) as an essential fatty acid. Hume et al. (1940) attributed essential fatty acid activity to linusic and isolinusic acids (hydroxy

derivatives of linoleic acid), but these acids were probably contaminated with linoleic acid since Thomasson (1953) later found the pure hydroxy acids to be inactive. Cis,cis-11,14-eicosadienoic acid was partially active, although it was formerly reported to be inactive (Karrer and Koenig, 1943). Essential fatty acid activity also appeared in the C₂₂-hexaenoic fraction of brain glycerylphosphatides (DeLongh and Thomasson, 1956).

The possibility of α -linolenic acid acting as an essential fatty acid was suggested by the work of Burr and Burr (1930), but their evidence was based only on weight gain and not the correction of dermal symptoms. The utilization of α -linolenic acid, as an essential fatty acid was reputed to be 'sparked' by linoleic acid (Greenberg, Calbert, Savage and Deuel, 1950). As judged by dermal symptoms (Privett, Pusch and Holman, 1955), α -linolenic acid had no potency in the presence of linoleic acid, yet as judged by growth, both fatty acids were better than either alone.

Many investigations were concerned with the elucidation of the specific chemical structure responsible for biological activity. Conjugated isomers (Aaes-Jorgensen, 1958) and hydrocarbon analogues of linoleic and α -linolenic acid (Aaes-Jorgensen, Privett and

Holman, 1959) were inactive, but linoleyl alcohol was active. Geometric isomers containing at least one trans-double bond lacked activity (Holman, 1951; Privett et al., 1955). Holman and Aaes-Jorgensen (1956) concluded that geometric isomers interfere with the activity of essential fatty acids, but this result was based on an experiment using only two rats per experimental group. A better controlled experiment was reported by Mattson (1960) who found that geometric isomers did not affect the utilization of cis,cis-linoleic acid. However, non-essential fatty acids were known to hasten the development of the deficiency syndrome by increasing the rate of turnover or mobilization of essential fatty acids (Aaes-Jorgensen, 1958).

As pointed out by Thomasson (1953) all compounds having essential fatty acid activity possess common structural features. These include (1) a system of methylene-interrupted bonds of the cis-configuration, and (2) the location of these double bonds at C₆ and C₉, counting from the terminal methyl end of the fatty acid instead of the carboxyl end as is customary. Thomasson's deduction provided a relationship between structure and activity which allowed predictions for untested fatty acids. In a later study, Thomasson (1962) surveyed other possible essential fatty acids.

A summary of the fatty acids used and their relative biological potency is given in Table 1. It is apparent that in general Thomasson's theory is substantiated but that quantitative differences in potency exist among the compounds which meet the known criteria for essential fatty acids. Therefore, biological activity of essential fatty acids must depend on considerations other than these features of chemical structure.

Table 1. Bio-potencies of essential fatty acids
(data from Thomasson, 1962)

Fatty acid		
No. of carbons and double bonds	Location of double bond ¹	Average relative bio-potency ²
18:2	6,9	100
18:3	3,6,9	9
18:3	6,9,12	115
19:2	6,9	9
19:3	6,9,12	6
20:2	6,9	46
20:3	6,9,12	102
20:4	6,9,12,15	139
22:6	? , 6, 9, ? , ? , ?	20-50

1. Numbering from terminal end of chain.
2. Measured by weight gain after development of essential fatty acid deficiency with water restriction.

c. Assay procedures

The early assays for essential fatty acids depended on biological activity. Greenberg et al., (1950) used a time-consuming assay in which male rats were deprived of fat for about three months and then dosed with the material to be tested for essential fatty acid activity. The logarithm of linoleate dose was found to be proportional to the weight gain. By restricting the water intake of the test rats, Thomasson (1953) shortened the experimental period to nine weeks. These assays depended on the measurement of an overall response of the animal, namely weight gain, rather than any specific effect of essential fatty acids.

A definite metabolic effect of essential fatty acid deficiency was early observed by Nunn and Smedley-Maclean (1938) who showed that eicosatrienoic acid accumulated in the livers of deficient rats. The presence of this acid was confirmed by spectrophotometric analyses (Riekehoff, Holman and Burr, 1949; Widmer and Holman, 1950). The greatest changes in tissue fatty acids were thought to occur in the heart until Holman and Greenberg (1953) discovered that the lipids of the testes were more affected by a lack of essential fatty acids. This finding was in accord with the relatively

severe histological changes in testicular tissue. Before impairment in spermatogenic epithelium became obvious, dienoic acids decreased and trienoic acids increased, as observed by Aaes-Jorgensen and Holman (1958). These workers therefore proposed that trienoic acids in the testes or heart should be used as an indicator of essential fatty acid deficiency. Analysis of testes, however, is impractical in young animals. As a further refinement in the assessment of essential fatty acid status, Holman (1960) advocated the use of the ratio of trienoic to tetraenoic acids in heart lipids. In essence, this method of choice involves determination of the proportion of eicosatrienoic acid to arachidonic acid. A ratio of less than 0.4 is taken to indicate that the minimum requirement for linoleic acid had been met.

d. Pathways for biosynthesis of polyunsaturated acids

Early work on the incorporation of deuterium into fatty acids (Bernhard and Schoenheimer, 1940; Bernhard, Steinhauser and Bullet, 1942) and later studies on the development of essential fatty acid deficiency (Barki, Nath, Hart and Elvehjem, 1947) substantiated the observations of Burr and Burr (1930) that there was little or no synthesis of linoleic acid in the animal

body. Mead, Steinberg and Howton (1953) found that only traces of labelled acetate were incorporated into linoleic acid, but that acetate combined with a C₁₈ precursor to form arachidonic acid.

The metabolism of linoleic acid in animal tissues was elucidated by Mead et al. (1953), Mead, Slaton and Decker (1956), Mead (1961) and Steinberg, Slaton and Howton (1956). When either linoleic-1-¹⁴C or γ -linolenic-1-¹⁴C were precursors, the label appeared at C₃ of arachidonic acid; when eicosatrienoic-1-¹⁴C was used, the label appeared at C₁ of arachidonic acid. On the basis of these experiments, the pathway for the conversion of linoleic acid to arachidonic acid was shown to be as outlined in Fig. 1. (Mead 1961). A corresponding series of reactions commencing with linolenic instead of linoleic acid (Fig. 2) was shown to yield docosapentaenoic and docosahexaenoic acid (Mead 1961).

In the absence of dietary linoleic or linolenic acids, polyunsaturated fatty acids were synthesized from oleic acid, and lacked essential fatty acid activity. The sequence of reactions (Fig. 3) established by Fulco and Mead (1959), provided an explanation for the formation of eicosatrienoic acid in essential fatty acid deficiency.

Mead (1961) showed that another series of

polyunsaturated acids might arise from palmitoleic acid (Fig. 4). In this case, the C_{20} trienoic acid was 7,10,13-eicosatrienoic acid. The ratio of this isomer to 5,8,11-eicosatrienoic acid formed from oleic acid was similar to the ratio of palmitoleic to oleic acid. Since both C_{16} and C_{18} monoenoic acids were precursors for C_{20} trienoic acids, the enzymes involved might not be absolutely substrate specific.

Fig. 1 Linoleic acid family; pathway for conversion of linoleic acid to arachidonic acid (Mead, 1961).

Fig. 2 Linolenic acid family; pathway for conversion of linolenic acid to docosahexaenoic acid (Mead, 1961).

Fig. 3 Oleic acid family; pathway for conversion of oleic acid to eicosatrienoic acid (Mead, 1961).

Fig. 4. Palmitoleic family; pathway for conversion of palmitoleic acid to eicosatrienoic acid (Mead, 1961).

A chain-shortening process for conversion of 4,7,10,13,16-docosapentaenoic acid to arachidonic acid was demonstrated by Verdino, Bland, Privett and Lundberg (1964). They speculated that these two fatty acids were in equilibrium with each other.

e. Metabolic role of essential fatty acids

Wesson and Burr (1931) found a high metabolic rate without an increase in the respiratory quotient in rats deficient in essential fatty acids. Sinclair (1952) concluded that the high metabolic rate was due to loss of water through the skin of deficient animals. Cytochrome oxidase exhibited increased activity in deficient rats (Kunkel and Williams, 1951). Although

there was an increased consumption of oxygen, oxidative phosphorylation dependent upon nicotinamide adenine dinucleotide (NAD) was reduced (Klein and Johnson, 1954). This represented some uncoupling of oxidative phosphorylation, but provided no indication of a specific role for essential fatty acids.

Examination of the effect of fat deficiency on biological oxidation (Tulpule and Patwardhan, 1952; Tulpule and Williams, 1955) showed reduced butyric dehydrogenase, little change in succinic dehydrogenase, and reduced cytochrome oxidase activities. Therefore essential fatty acids appeared to be involved in the activity of butyric dehydrogenase and cytochrome components.

Although it had been suggested that essential fatty acids might act as coenzymes (Aaes-Jorgensen et al. 1959), no direct evidence was forthcoming. The speculation of Richardson, Tappel and Gruger (1961) seemed more plausible: 'Conceivably, the numerous metabolic defects observed in essential fatty acid-deficient animals might be a secondary effect of deranged membrane structure'.

Evidence suggesting a structural role for essential fatty acids may be found in the early work of Sinclair (1935), who demonstrated that polyenoic acids remained

as constituents of phosphatides, known to be membrane components, during fasting or after feeding saturated fat. More direct evidence for membrane changes in essential fatty acid deficiency was the increased permeability of the capillaries (Kramer and Levin, 1953) and of the skin (Sinclair, 1958). Of particular interest were the studies on mitochondria from livers of deficient rats. Levin, Johnson and Albert (1957) found such mitochondria to be larger and more easily damaged than normal ones; Hayashida and Portman (1960) observed a 79% decrease in essential fatty acids in mitochondria but only a 23% drop in unsaturation due to the compensatory synthesis of eicosatrienoic acid. In photomicrographs of myocardium sections from deficient rats (Stein and Stein, 1964) the mitochondria appeared swollen and the cristae disorganized.

Collins (1962) demonstrated that the uptake of inorganic ^{32}P into phosphatides of rats deficient in essential fatty acids was greater than that of normal controls. Accordingly, he speculated that the increased phosphatide turnover resulted in partial disorganization of enzymes associated with the lipoprotein membrane. The essential nature of membrane lipids was evident from the studies of Green and Fleischer (1963). Phosphatides were removed from mitochondria with loss of electron

transfer properties which were restored upon the addition of phosphatides.

It seems highly likely that a structural alteration of mitochondrial membrane in essential fatty acid deficiency might be responsible for a reduction of enzymic activity associated with electron transport.

2. INCORPORATION INTO TISSUES

a. Source of linoleic acid and effect on total lipids

The most physiological and economical manner of supplying linoleic acid to an animal is by adding triglycerides having a high proportion of that fatty acid to the diet. Cottonseed oil (Spadola and Ellis, 1936), corn oil (Longenecker, 1939) and safflower oil (Wagner, Seelig and Bernhard, 1958; Okey, Lee, Hampton and Miljanick, 1960; Ostwald, Okey, Shannon and Tinoco, 1962) have been used to elevate the levels of tissue dienoic acids. Within one week of feeding coconut oil (a saturated fat) or safflower oil (a relatively unsaturated fat), Ostwald et al. (1962) noted that the fatty acid composition of adipose tissue reflected characteristics of the respective vegetable oils. High levels of linoleic acid appeared in tissues of rats supplied with corn

oil for three or more weeks (Bhalerao, Endres and Kummerow, 1961; Beare, 1961; Perkins, Endres and Kummerow, 1961; Century, Witting, Harvey and Horwitt, 1961). Mohrhauer and Holman (1963 a,b) employed low levels of ethyl linoleate, arachidonate or linolenate, and showed that only the linoleate influenced the concentrations of linoleic acid in rat liver lipids. Ethyl linolenate appeared to suppress the conversion of linoleate to arachidonate. A high level of linoleic acid, however, resulted in decreased proportions of arachidonic acid (Rahm and Holman, 1964), and also inhibited the transformation of oleic acid to 5,8,11-eicosatrienoic acid (Lowry and Tinsely, 1966).

Holman (1964) considered the suppression of synthesis of eicosatrienoic acid to be the result of competition between oleic, linoleic and linolenic acids for the enzymes involved in desaturation or elongation of fatty acids. At the desaturation step of linoleic to γ -linolenic acid other polyenoic acids appeared to be inhibitory (Brenner, De Thomas and Peluffo, 1965). These studies were based on changes in total fatty acid composition without respect to class of lipid.

b. Effect on phosphatides

Dawson (1957) reviewed the early work on the structure and metabolism of animal phosphatides. The

general formulae of these compounds in liver are given in Fig. 5. Little attention was paid by Dawson (1957) to their fatty acid constituents of phosphatides.

In an old and apparently forgotten study Joannovics and Pick (1910) observed that the iodine number of total tissue phosphatides increased with iodine number of the dietary fat. Despite this demonstration of fatty acid renewal, Terroine and Belin (1927) called Phosphatides the 'élément constant' on the basis of their non disappearance under conditions of extreme starvation. These ubiquitous and essential components of tissue however were not inert, but metabolically active. Sinclair (1931) verified that exogenous fat influenced the fatty acid composition of rat liver phosphatides.

The fatty acids of rat liver were studied by several investigators (Klein and Johnson, 1954 b; Dittmer and Hanahan, 1959) using ultraviolet spectroscopy or reversed-phase chromatography which did not distinguish individual fatty acids. The latter authors reported that the relative distribution of major fatty acids between phosphatides and neutral lipids was independent of the oils fed. Using the more precise technique of gas-liquid chromatography, Getz and Bartley (1961) found similar fatty acid compositions in different subcellular liver

Fig. 5. Chemical structures of rat liver phosphatides.

fractions. This finding was confirmed by Pascaud (1964). In an examination of the fatty acids of different lipid classes, Getz, Bartley, Stirpe, Notton and Renshaw (1961) showed that the phosphatides were relatively high in stearic acid and C_{18} and C_{20} polyunsaturated acids, and that the neutral lipid contained high levels of palmitic and C_{18} unsaturated acids. These differences in fatty acid composition for various types of lipid contrasted with similarities in fatty acids of cell organelles. Further studies (Getz, Bartley, Stirpe, Notton and Renshaw, 1962) revealed that mitochondria contained about equal amounts of lecithin and 'kephalin' and appreciable quantities of cardiolipin (16 equiv./g of dry weight) in contrast to microsomes in which the lecithin comprised more than half of the total phosphatides. These results were confirmed by Fleischer and Rouser (1965) who also showed that good preparations of microsomes lack cardiolipin.

Macfarlane, Gray and Wheeldon (1960) and Veerkamp, Mulder and van Deenen (1962) also observed a remarkable similarity of fatty acid composition in different cellular organelles of one tissue. Since the phosphatides are predominantly associated with membranes Veerkamp et al. (1962) concluded that information about fatty acids of membranes could be obtained from phosphatide analyses involving the whole tissue.

Incorporation of labelled palmitic and oleic acids into rat liver lecithin was studied by Hanahan and Blomstrand (1956). The radioactivity of the lecithin was ten times greater with the saturated acid than with the monounsaturated acid. Glenn, Opalka and Tischer (1963) employing labelled palmitic acid detected radioactivity in all normal rat liver phosphatides except phosphatidylserine.

Specific patterns of types of phosphatides in various cellular components are becoming evident. For example, Skipski, Barclay and Archibald (1965) prepared plasma membranes by the procedure of Neville (1960), and found that the membrane fraction of density 1.16-1.22 g/ml contained more sphingomyelin and phosphatidylserine than the total cellular phosphatides.

By the use of phospholipase A which catalyzes the release of fatty acids in the β -position of glyceryl phosphatides, it was possible to distinguish the fatty acids in the α' -and β -positions. De Tomas, Brenner and Peluffo (1963) demonstrated that arachidonic and eicosatrienoic acids were esterified in the β -position. The trans, trans-linoleic acid was found in the α' -position in lecithin whereas the cis,cis-linoleic acid occurred in the β -position (Selinger and Holman, 1965). In an essential fatty acid deficient rat, the distribution of

saturated fatty acids in liver lecithins differed from that of the normal rat at both the α' - and β -positions (Johnson and Ito, 1965). Using a procedure for separating different classes of lecithins, Collins (1966) demonstrated that arachidonic acid or eicosatrienoic acid occurred in the β -position of lecithins in which the α' -position was occupied by stearic or oleic acid. It was recently suggested by Lands and Hart (1966) that the composition of glycerylphosphatides is controlled by the supply of fatty acids and the specificities of acyltransferases. Investigation of these factors should help to elucidate the metabolic control of the fatty acid moieties of glycerylphosphatides.

B. EXPERIMENTAL

1. Materials and Methods

a. General animal procedures

Male, weanling rats of the Wistar strain from the colony of the Food and Drug Directorate were housed in individual, screen-bottomed cages, and supplied with food and water ad libitum. The diet consisted of 19% casein, 1% vitamin mixture in casein, 4% U.S.P. salt mixture, 6% alphacel (non nutritive cellulose), various proportions of corn oil (0 to 30%), and sucrose (70 to 40%). The vitamin mixture provided in each kilogram of diet contained 10 mg thiamine hydrochloride, 10 mg riboflavin, 10 mg pyridoxine hydrochloride, 30 mg calcium pantothenate, 500 mg inositol, 50 mg niacin, 100 mg para-aminobenzoic acid, 0.2 mg biotin, 0.02 mg vitamin B₁₂, 2 g choline tartrate, 1.5 mg vitamin A, 0.025 mg vitamin D, and 91 mg DL- α -tocopherol. The source of these materials was Nutritional Biochemicals Corporation. The fatty acid composition of Mazola corn oil as determined by gas-liquid chromatography was 8.1% palmitic, 2.4% palmitoleic, 1.2% stearic, 31.8% oleic, 53.1% linoleic, 1.5% linolenic, 0.9% arachidonic, and 1.0% eicosenoic.

Diets were refrigerated until fed. Food dishes were emptied and re-filled twice weekly with weighed quantities of diet. To prevent spillage, each aluminum container was fitted with a 3-mesh wire screen resting on

the food and covered with an aluminum lid having a central hole of 4.2 cm diameter.

At various time intervals, rats were anesthetized with ether and bled from a neck incision. The liver and gastrointestinal tract were removed and the latter discarded. Eviscerated carcasses and livers were frozen at -30°C until analyzed.

b. Analytical procedures for carcass lipids

i. Preparation of lipid sample

Frozen, eviscerated rats were minced in an electric food cutter (Hobart, Model 84141) having vertically rotating double blades in a horizontally rotating bowl. The ground tissue was collected with a broad rubber spatula and thoroughly mixed. Lipid was extracted from the ground tissue by a modification of the procedure of Bligh and Dyer (1959). To 15 g of tissue, which contained approximately 10.5 g of water, were added 5.5 ml of water to provide a total of 16 g of water. The mixture was blended in a Lourdes homogenizer with 60 ml of a mixture of methanol and chloroform (2:1,v/v) for 2 min.; 20 ml of water were added and the mixture blended for 30 sec.; 20 ml of chloroform were added and the mixture blended for 30 sec. The mixture was filtered under suction through Whatman No. 1 paper on a Büchner funnel. The filtrate was transferred to a 200 ml centrifuge tube and spun for 10 min. at 2,000 rpm. The supernatant phase of aqueous methanol was removed by aspiration and the chloroform phase was diluted

with benzene and evaporated in a rotary evaporator at 40°C. The resulting lipid material was diluted to 25 ml with chloroform and used for the determination of total fat.

ii. Total lipid determination

A 10 ml-aliquot of lipid extract was placed in a tared 25 ml Erlenmeyer flask which had been previously heated and desiccated. Solvent was removed in a heated vacuum desiccator (45°C), and the flask and contents dried to constant weight.

iii. Preparation of methyl esters of fatty acids

Methyl esters of fatty acids were prepared by transesterification (Stoffel, Chu and Ahrens, 1959). The solvent from 1 or 2 ml of lipid extract was evaporated, and the residue was treated with 10 ml of methanol and 1 ml of 5% hydrogen chloride in methanol. The mixture was gently refluxed for 1 hr. becoming a clear solution as the triglycerides were converted to methyl esters. Methanol and hydrogen chloride were removed in a rotary evaporator at approximately 45°C. The methyl esters were extracted into petroleum ether, and the solvent was subsequently evaporated under a stream of nitrogen. Approximately 1 μ l of the mixture of methyl esters was injected into the gas-liquid chromatograph.

iv. Gas-liquid Chromatography

Preparation of column

Butanediolsuccinate polyester was prepared according to a procedure kindly supplied by B. M. Craig, National Research Council, Saskatoon, Saskatchewan. In

a three-necked round-bottom flask equipped with condenser, stirrer and nitrogen inlet were placed 50 g of 1,4-butanediol, 50 g of succinic acid, and 100 mg of zinc chloride. The mixture was heated for 3 hr at 160°C and 2 hr at 190°C. Water resulting from the esterification was swept out of the reaction vessel by the stream of nitrogen. During the last 2 hr of the reaction a suction pump was connected to the reaction vessel to remove excess 1,4-butanediol. The polyester product was a white, crystalline solid. The zinc chloride was removed by dissolving the polyester in chloroform and treating it with activated charcoal. The melting-point of the butanediolsuccinate was 115°C.

The column packing was prepared from 10 g butanediolsuccinate dissolved in 200 ml chloroform and 60 g chromosorb W. The mixture was thoroughly stirred under a fume hood until the solvent was evaporated.

Copper tubing, 6 feet in length and 1/4 inch in outside diameter, was plugged with glass wool at one end and then filled with butanediolsuccinate on chromosorb W (1:6) through a funnel attached to the other end. The tube was gently tapped as the filling progressed, but care was taken not to pack the column so tightly as to prohibit a suitable gas flow when in operation. The column was coiled and fitted into a Beckman GC-2 chromatograph equipped with a thermal conductivity detector.

The injector block of this instrument was located directly at the head of the column.

Operation of chromatograph

The detector filament current was set at 230 or 250 milliamperes, and the Minneapolis-Honeywell recorder operated at 0-1 millivolt. For most samples, an attenuation of 2 was employed. The silicone rubber septum in the inlet port was replaced after 10 or 12 injections.

The temperatures of the injector chamber and of the column which was operated isothermally were 232°C and 206°C respectively, and the column flow was 80 ml of helium per min, determined by a soap bubble meter at the exit of the column.

Calculation of fatty acid composition

The method of Carroll (1961) was employed for the determination of area of the chromatographic peaks. It was found that the product of peak height and retention time (measured in distance along the baseline) was proportional to peak area. The percentage of a fatty acid (FA)_i in a mixture of n fatty acids was calculated as follows:

$$\% (FA)_i = \frac{h_i d_i}{\sum_{i=1}^n h_i d_i} \times 100$$

where h is the peak height and d is the distance from

point of sample injection to the peak. The peaks were identified by the linear relationship between the logarithm of the retention time and the chain length of the fatty acid (Woodford and van Gent, 1960) and comparison of retention times with those of reference compounds.

c. Analytical procedures for liver lipids

i. Total liver lipid

Total lipids of liver were obtained by a modification of the method of Bligh and Dyer (1959). To approximately 7 g of liver tissue were added 3 ml of water to give 8 g of water including that contributed by the tissue. This was blended with 20 ml of methanol and 10 ml of chloroform in a Lourdes homogenizer for 2 minutes. The mixture was centrifuged for 10 minutes at 2,000 rpm. in a clinical centrifuge. The supernatant fluid was saved and the residue re-extracted with 8 ml water, 20 ml methanol and 10 ml chloroform by homogenization for 2 minutes. After centrifugation, the supernatant extracts were pooled and the volume recorded before transferring the liquid to a separatory funnel. A mixture of methanol, chloroform and water (2:1:0.8) was used for quantitative transfer and to give a final volume of 76 ml. Another 20 ml portion of chloroform was added, the mixture was shaken, 20 ml of water added, and the

resulting two phases allowed to separate. The chloroform phase was removed, concentrated in a rotary evaporator, and the residue was dissolved in 10 ml of chloroform.

ii. Fatty acid composition

Total liver fatty acids were analyzed by gas-liquid chromatography as described for carcass fatty acids except that a Pye Argon Chromatograph with an ionization detector operated at 1,000 volts and argon as a carrier gas was employed. The column containing 10% butanediol succinate on chromosorb W was operated isothermally at 190°C.

iii. Separation of phosphatides

Silicic acid impregnated paper

Strips of Whatman 3MM paper, 4.5 inches X 8 inches were impregnated with silicic acid by the procedure of Marinetti, Erbland and Kochen (1957). A solution of sodium silicate was prepared by dissolving 310 g silicic acid in 1 litre of 7.2 N sodium hydroxide and diluting the resulting solution to 1.6 litres. A 3 cm border at one end of the paper was marked by pencil and used for handling as the rest of the strip was gradually passed twice through the sodium silicate in a petri dish. To convert the sodium silicate on the paper to silicic acid, the coated strips were submerged for 30 minutes in 6N HCl

in a 10 in. glass tray. They were then washed with running water for 90 minutes followed by 4 to 6 washings in distilled water. The papers were suspended by clips and dried overnight under a fume hood. The impregnated papers were subjected to an ascending wash of chloroform-methanol (2:1,v/v) in a chromatographic jar for 16 to 18 hours. To remove traces of acid and materials soluble in organic solvents, the dried papers were washed in a similar way with diisobutyl ketone-acetic acid-water (40:25:5,v/v/v) for approximately 18 hours.

A sample of total lipid extract containing approximately 100 µg of phosphorus was evaporated under a stream of nitrogen, and taken up in 100 µl chloroform for spotting on a strip of the silicic acid impregnated paper. For preparative purposes, approximately 5 µl of the chloroform solution (approximately 5 µgP per spot) were applied under a stream of nitrogen to each of 20 spots on the paper. For analytical purposes, approximately 3 µgP were applied per individual spot. The phosphatides were separated by ascending chromatography at room temperature in the solvent system, diisobutyl ketone-acetic acid-water (40:25:5,v/v/v). After 18 hours, the chromatogram was dried for 5 to 10 minutes in an atmosphere of nitrogen in another chromatographic jar, and then dipped for 5 minutes into a 0.0015% solution of

rhodamine 6G. The dye was prepared by diluting 5 ml of a stock solution (150 mg of rhodamine 6G/100 ml) to 500 ml. The excess dye was washed away with water and the papers were partially dried in the nitrogen atmosphere. Papers were viewed under ultraviolet light (366 m μ) and the areas of lipid material outlined in pencil. The fluorescent outlines on an underlying filter paper were marked with pencil for a record of the areas.

To identify lipid components, authentic samples of lecithin, lysolecithin and phosphatidyl ethanolamine were used. Chromatograms were also sprayed with ninhydrin reagent (0.25 g in 100 ml of acetone-butidene, 9:1v/v) for the detection of amino lipids, or dipped in Schiff reagent (Hack and Ferrans, 1959) for the detection of plasmalogens. The Schiff reagent was prepared as follows: 1 g pararosanilin, and 6 g sodium metabisulfite were dissolved in 1 ml concentrated hydrochloric acid and 100 ml water. Charcoal was added, the mixture filtered and the filtrate diluted to 500 ml. To 100 ml of 0.005M sodium metabisulfite were added 1 ml of 0.05M mercuric chloride and 1 ml of fuchsin solution. The chromatogram was dipped in this dye for 10 minutes, washed twice with 0.005M metabisulfite and dried. Plasmalogen positive spots appeared as purple areas on a white background. The identification of phosphatides

was based on relative R_f 's as reported by Marinetti (1958), by characteristic fluorescent colors of chromatograms stained with rhodamine 6G and viewed under UV, and by specific reactions with ninhydrin and Schiff reagents. •

iv. Quantitative analyses of phosphatides

The areas of the partially dried chromatograms containing the separated phosphatides and neutral lipid as well as a blank strip were cut into pieces, approximately 0.5 cm^2 and dropped into a 50 ml Erlenmeyer flask with a 5 ml side arm (Kates, 1964); 4.5 ml of 2.5% hydrogen chloride in anhydrous methanol were added to the flask and the mixture was refluxed for 1 hour. To the flask was added 0.5 ml of water, and additional 90% methanol if necessary, to fill the side arm. Methyl esters of fatty acids were extracted three times with 5 ml of petroleum ether, and the combined extracts evaporated almost to dryness under a stream of nitrogen. The residual methyl esters of fatty acids were analyzed by gas-liquid chromatography. The aqueous-methanolic phase containing the water-soluble products from the phosphatides was centrifuged or filtered through glass wool, and analyzed for phosphorus. Values were corrected for the amount of phosphorus in the blank.

Determination of stability of linoleic acid

To determine the stability of linoleic acid during the chromatographic procedure, 3 to 4 mg of corn oil and a similar amount of purified egg lecithin were chromatographed on paper and the areas containing triglycerides and lecithin respectively were cut out for direct transesterification of the methyl esters, as previously described. The fatty acid composition for each of the two types of lipid before and after chromatography are shown in Table 2. The decrease in the proportion of linoleic acid from corn oil was 7.4%, and from lecithin, 8.0%. Under the conditions employed, auto-oxidation did not appear to be an important factor.

Phosphorus determination

The procedure of Allen (1940) was employed as follows: A solution containing 20 to 80 µg of lipid phosphorus in a straight-walled 'sugar tube' calibrated at 12.5 and 25 ml, was evaporated under a stream of forced air in a hot water bath. The residue was digested with 2.0 ml of 70% perchloric acid. After the tubes were cooled, the walls were washed down with 10 ml of water, and 2 ml of 1% amidol solution (1 g of amidol, Verona Chemical Co., and 20 g of sodium metabisulfite diluted to 100 ml with distilled water) and 1 ml of 8.3% ammonium molybdate. To avoid the production of molybdenum blue

colour, care was taken not to touch the sides of the tube during pipetting. The solution was diluted to the 25 ml mark, mixed well, and read after 5 to 30 minutes against a reagent blank at 680 m μ . A solution of potassium dihydrogen phosphate containing 10 μ g of phosphorus per ml was employed as a standard.

Purified egg lecithin prepared by the method of Lea, Rhodes and Stoll (1955) was carried through the chromatographic and extraction procedures. The recovery of lipid phosphorus was 86%.

d. General experimental plan

i. Effect of level of corn oil

Five groups of 10 weanling rats were fed diets containing 0, 0.5, 1, 2, 4, or 20% corn oil. Half of the rats in each group were killed at 3 weeks and the remainder at 9 weeks. For zero time values, five rats were killed on weaning. Fatty acids of the carcass lipids were analyzed.

ii. Effect of Duration of Feeding

Two groups of 28 rats were fed diets containing 2 or 20% corn oil and 7 rats from each group were killed after 3, 6, 12, and 24 days. For zero time values, the same number were killed on weaning. Fatty acids of carcass, liver, and phosphatidyl choline, phosphatidyl ethanolamine, and neutral lipid of liver were determined.

iii. Maximum level of Deposition

Three groups of 10 rats were each supplied with 7.5, 15, or 30% corn oil, and were killed at 9 weeks. Carcass and liver lipids were analyzed as in the previous experiment.

Table 2. Effect of silicic acid-paper chromatography on the fatty acid composition of corn oil and egg lecithin.

Lipid	Fatty acid ¹	Percent of fatty acids	
		Before chromatography	After chromatography
Corn oil	myristic 14:0	1.4	3.0
	palmitic 16:0	10.9	10.9
	stearic 18:0	1.9	1.9
	oleic 18:1	21.4	24.3
	linoleic 18:2	63.4	58.7
	linolenic 18:3	1.1	1.2
Lecithin	14:0	4.7	7.2
	16:0	28.7	32.6
	18:0	13.5	12.4
	18:1	36.6	32.5
	18:2	14.2	13.1
	20:4	2.3	2.1

¹ Abbreviation of fatty acids is as follows: a:b where a is the number of carbon atoms and b is the number of double bonds.

2. RESULTS

a. Effect of amount and duration of corn oil intake on carcass fatty acids

i. Level of corn oil

Rats receiving 0 to 20% corn oil for 3 weeks had similar body weights. At 9 weeks, body weights of rats fed no fat were significantly lower ($P = 0.05$) than those of rats fed 0.5 to 20% corn oil. The data are shown in Table 3. Food consumption was inversely related to the level of corn oil, being less with 20% corn oil than with the lower levels of oil tested. While the caloric value of the diets increased from 360 to 460 calories/100 g as the level of fat increased from 0 to 20%, the caloric intake remained fairly constant. The proportion of total fat in the rat carcass did not change progressively with increasing amount of corn oil in the diet. The concentration of linoleic acid in the body fat, however, increased greatly (Table 4). At 3 weeks the major changes in fatty acids, in response to increases in exogenous fatty acids from corn oil, were a higher proportion of linoleic acid and decreased proportions of palmitic, palmitoleic and oleic acids. Eicosatrienoic acid appeared to be absent when the amount of corn oil exceeded 0.5% of the diet. The general pattern of changes in fatty acids with increasing amounts of corn

Table 3. Food consumption, body weight and carcass fat of rats fed different levels of corn oil (CO) for 3 or 9 weeks.¹

Dietary CO	Time	Body wt.	Food consumption	Carcass fat
%	wk.	g	g	%
-	0	39 ± 1	-	8.3 ± 0.5
0	3	112 ± 3	190 ± 5	5.4 ± 0.5
0.5	3	113 ± 4	191 ± 6	4.7 ± 0.6
1	3	116 ± 5	189 ± 6	5.9 ± 0.4
2	3	112 ± 3	180 ± 5	4.6 ± 0.5
4	3	116 ± 3	182 ± 5	6.5 ± 0.6
20	3	114 ± 2	157 ± 3	6.5 ± 0.4
0	9	195 ± 8	775 ± 32	6.0 ± 0.4
0.5	9	238 ± 7	820 ± 14	5.9 ± 0.5
1	9	232 ± 3	789 ± 13	5.9 ± 1.0
2	9	215 ± 7	735 ± 23	4.4 ± 0.4
4	9	221 ± 10	725 ± 22	5.3 ± 0.4
20	9	247 ± 5	638 ± 14	7.5 ± 0.3

¹ Values are means ± standard errors.

Table 4. Average percent of individual fatty acids in total carcass fatty acids of rats fed different amounts of corn oil for 3 weeks.

Fatty acid	% dietary corn oil					
	0	0.5	1	2	4	20
12:0 ¹	0.8	0.6	0.6	0.5	0.6	0.6
14:0	3.0	2.5	2.7	2.4	2.1	1.4
14:1	0.9	0.7	0.6	0.6	0.5	0.5
15:0	0.4	0.3	0.3	0.4	0.3	0.2
16:0	26.2	25.2	25.7	23.4	20.4	13.4
16:1	14.5	12.5	12.3	9.8	6.8	3.4
18:0	5.5	6.3	6.1	6.4	6.2	4.9
18:1	41.3	38.4	36.4	34.6	31.4	29.4
18:2	4.9	9.0	12.1	18.8	27.9	42.1
18:3	0.3	1.5	0.3	0.4	0.6	0.8
20:2	0.6	0.7	0.8	0.8	0.5	0.8
20:3	0.5	0.2	-	-	-	-
20:4	1.1	2.2	2.0	2.4	2.6	2.5

¹ Number of carbon atoms: number of double bonds

Dash means not detected

oil in the diet was similar at 3 and 9 weeks, but the differences between the 0% and 20% corn oil were more pronounced at 9 weeks (Fig. 6). Thus, at 9 weeks the animals fed the fat-free ration contained 2.0% linoleic acid, 48.1% oleic acid and 0.8% eicosatrienoic acid in the carcass fatty acids while the corresponding proportions in those fed 20% corn oil were 46.2% linoleic acid, 31.2% oleic acid, and no detectable eicosatrienoic acid. With the high intake of corn oil, linoleic acid increased from 42 to 46% during the 3 to 9 week period. Mean linoleate values are plotted against time in Fig. 7. The level of dietary linoleate is seen to be of prime importance in determining the final concentration of tissue linoleate, but the greatest rate of change occurred within the first 3 weeks of the experiment.

ii. Effect of duration of feeding

As seen in Table 5, rats fed 2% or 20% corn oil for intervals up to 24 days had similar body and liver weights throughout the experiment.

At 6 days and thereafter, rats receiving 2% oil consumed greater amounts of diet and possessed a somewhat lower proportion of body fat than rats receiving 20% oil (Table 6).

Changes in the proportion of linoleic acid in the total fatty acids of carcass from 0 to 24 days are shown

Fig. 6. Changes in the proportions of major fatty acids in the carcass of rats fed different levels of corn oil for 3 or 9 weeks. Height of vertical lines represents standard error of the mean values.

Fig. 7. Proportions of linoleic acid in carcasses of rats fed different levels of corn oil (CO).

Table 5. Body and liver weights of rats fed 2% or 20% corn oil (CO) for different lengths of time.

<u>Days</u>	<u>Body weight (g)</u>		<u>Liver weight (g)</u>	
	2% CO	20% CO	2% CO	20% CO
0	43 ± 1		1.65±0.12	
3	52±2	52±2	2.33±0.08	2.21±0.09
6	64±2	64±3	3.20±0.11	2.96±0.08
12	85±1	85±2	3.77±0.09	3.82±0.11
24	125±4	135±2	5.20±0.14	6.08±0.23

Table 6. Food consumption and carcass fat of rats fed 2% or 20% corn oil (CO) for different lengths of time.

<u>Days</u>	<u>Food consumption (g)</u>		<u>Carcass fat (%)</u>	
	2% CO	20% CO	2% CO	20% CO
0				
3	17±1 ^x	15±1	7.3±0.7	8.5±0.3
6	42±1	34±1	6.9±0.2	8.4±0.4
12	93±2	80±3	5.8±0.4	6.9±0.2
24	227±6	198±5	5.3±0.1	7.2±0.5

^x Mean ± S.E.

in Fig. 8. The dietary supply of linoleic acid had a marked influence within the first 3 days, as evidenced by the significantly larger proportions of linoleic acid in rats which had received the higher level of oil. The concentration of linoleic acid in the carcass of rats fed 20% corn oil increased progressively throughout the experiment while that in rats fed 2% corn oil remained at a relatively constant level. The levels of linoleic acid attained at 24 days were similar to those found at 3 weeks in the previous experiment.

b. Linoleic acid in the total lipids, neutral lipids and phosphatides of liver

Levels of linoleic acid in the total fatty acids of liver are shown in Fig. 9. Unlike the situation in the carcass, the concentration of linoleic acid in the liver of rats fed 20% corn oil reached a maximum at 12 days, and thereafter showed no change. In animals receiving 2% corn oil, the level of linoleic acid decreased within the first three days, and returned to almost the initial value after 12 days.

Total liver lipids from rats fed 20% corn oil were separated into various phosphatides and neutral lipid by chromatography on silicic-acid impregnated paper as illustrated in Fig. 10. The diet of the rats had no apparent influence on the chromatographic pattern.

Fig. 8. Effect of time (0 to 24 days) on the proportion of linoleic acid in total fatty acids of carcass of rats fed 2% (open circles) or 20% (closed circles) corn oil.

Fig. 9. Effect of time on the proportion of linoleic acid in the total fatty acids of liver of rats fed 2% (open circles) or 20% (closed circles) corn oil.

Fig. 10. Tracing of chromatogram of liver lipids stained with Rhodamine 6G and viewed under ultraviolet light (360 mμ); fluorescent colours: B, blue; Y, yellow.

The distribution of lipid phosphorus among the phosphatide components recovered from the chromatogram was particularly variable for the combined sphingomyelin and lysophosphatidyl ethanolamine, for phosphatidyl serine and for phosphatidic acid (Table 7). Phosphatidyl choline (lecithin) constituted approximately half of the total liver phosphatides. A tracing of a chromatogram stained with specific reagents is shown in Fig. 11. There was a positive ninhydrin reaction for components designated as phosphatidyl ethanolamine, and a positive Schiff plasmalogen reaction for phosphatidyl and lysophosphatidyl choline, and for phosphatidyl and lysophosphatidyl ethanolamine, indicating that these phosphoglycerides were present in both plasmalogen and diacyl ester forms.

The linoleic acid in the fatty acids of phosphatidyl choline and phosphatidyl ethanolamine from liver varied with the linoleate intake as shown in Fig. 12. Each point represents analyses of liver phosphatides from a pair of randomly chosen rats within each experimental group. The major phosphatide, phosphatidyl choline, showed an increase in linoleic acid with the high corn oil diet within the first 3 days. Phosphatidyl ethanolamine contained a lower proportion of linoleic acid than lecithin and showed less well defined differences between the two experimental groups.

Table 7. Distribution of lipid phosphorus among liver phosphatides of 3 pairs of rats.

Phosphatide Fraction	% of lipid P		
Phosphatidyl inositol and lysophosphatidyl choline	10.0	10.1	10.9
Sphingomyelin and lysophosphatidyl ethanolamine	17.6	4.4	5.3
Phosphatidyl choline	42.3	49.5	55.9
Phosphatidyl serine	5.5	14.3	4.3
Phosphatidyl ethanolamine	15.6	18.9	18.3
Cardiolipin (polyglycerol phosphatide)	1.5	2.4	4.4
Phosphatidic acid	7.5	0.4	0.9

Fig. 12. Effect of time on the proportion of linoleic acid in the fatty acids of liver phosphatidyl choline or phosphatidyl ethanolamine of rats fed 2% or 20% corn oil.

In Fig. 13 are shown the proportions of linoleic acid in the fatty acids of the liver neutral lipids. There was a striking, twofold increase in linoleate concentration within 3 days in rats fed 20% corn oil. Thereafter, no further increase in the proportion of linoleate occurred. Rats receiving the low level of corn oil showed a loss of linoleate in the neutral lipids, particularly during the first days of the experiment; after six days the linoleate tended to increase.

iii. Maximum level of deposition

When diets containing 7.5, 15, or 30% corn oil were fed for 9 weeks, the concentration of linoleic acid increased in the fatty acids of carcass and liver (Table 8).—In the liver, the relative proportion of arachidonic acid was lowest at the highest intake of linoleate. The concentration of linoleic acid in each of the phosphatides examined was unaffected by changes in the amount of corn oil in the diet (Table 9). Simultaneously the proportion of arachidonic acid in the phosphatides decreased. The neutral lipid fraction exhibited the greatest response to increased linoleate in the diet, but did not appear to change its content of arachidonic acid. The relative levels of linoleic acid in each of the lipid fractions of liver and in the total carcass lipids are shown in Fig. 14. Corresponding values for

Fig. 13. Effect of time on the proportion of linoleic acid in the total fatty acids of liver neutral lipid of rats fed 2% or 20% corn oil.

Table 8. Major fatty acids of carcass and liver lipids from rats fed 7.5, 15 or 30% corn oil for nine weeks.¹

Fatty acid ²	Dietary corn oil	Carcass total lipids	Liver total lipids
	%		
16:0	7.5	16.0 ± 0.2	16.0 ± 0.3
	15	14.1 ± 0.4	13.7 ± 0.8
	30	11.0 ± 0.6	12.2 ± 0.2
18:0	7.5	5.2 ± 0.1	18.2 ± 0.4
	15	5.9 ± 0.4	17.7 ± 0.5
	30	4.6 ± 0.3	15.4 ± 0.5
18:1	7.5	32.7 ± 0.4	14.9 ± 0.4
	15	32.0 ± 1.0	17.1 ± 0.6
	30	33.9 ± 0.3	17.5 ± 0.8
18:2	7.5	35.4 ± 0.4	18.2 ± 0.7
	15	39.0 ± 0.5	23.0 ± 0.5
	30	43.3 ± 0.2	28.4 ± 1.5
20:4	7.5	2.3 ± 0.1	20.1 ± 0.3
	15	2.5 ± 0.5	18.7 ± 1.0
	30	2.1 ± 0.2	14.9 ± 0.8

¹ Values are means ± standard errors

² Number of carbon atoms: number of double bonds

Table 9. Major fatty acids of liver phosphatidyl choline, phosphatidyl ethanolamine and neutral lipids.¹

Fatty acid	Dietary corn oil	Phosphatidyl choline	Phosphatidyl ethanolamine	Neutral lipids
	%			
16:0	7.5	20.0 ± 0.5	15.4 ± 0.6	22.6 ± 0.6
	15	18.3 ± 0.1	17.5 ± 0.3	21.3 ± 1.1
	30	18.9 ± 1.1	14.5 ± 1.0	15.6 ± 1.4
18:0	7.5	23.5 ± 0.1	28.9 ± 0.9	3.6 ± 0.3
	15	26.8 ± 0.5	32.1 ± 1.4	3.5 ± 0.4
	30	27.1 ± 2.6	32.7 ± 1.3	3.2 ± 0.1
18:1	7.5	7.3 ± 0.1	7.3 ± 0.5	26.5 ± 0.2
	15	6.0 ± 0.8	9.1 ± 0.8	22.0 ± 0.9
	30	8.0 ± 1.2	7.9 ± 0.2	21.5 ± 0.2
18:2	7.5	15.3 ± 0.4	9.0 ± 0.3	36.6 ± 2.1
	15	16.3 ± 1.9	9.7 ± 0.5	44.0 ± 1.0
	30	17.3 ± 1.5	11.8 ± 1.3	50.3 ± 0.5
20:4	7.5	29.4 ± 0.8	37.3 ± 0.5	7.8 ± 0.6
	15	29.1 ± 0.3	26.4 ± 0.6	7.0 ± 0.6
	30	24.5 ± 1.7	26.6 ± 0.6	6.0 ± 0.5

¹ Values are means ± standard errors

Fig. 14. Effect of linoleate intake on the proportion of linoleic acid in the fatty acids of neutral lipid (NL), carcass (C), liver (L), phosphatidyl choline (PC), and phosphatidyl ethanolamine (PE).

arachidonic acid are given in Fig. 15. The phosphatides had the lowest linoleate values and the highest arachidonate values of the lipid fractions studied.

Fig. 15. Effect of linoleate intake on the proportion of arachidonic acid in the fatty acids of phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), liver (L), neutral lipid (NL), and carcass (C).

C. DISCUSSION

The present experiments have demonstrated that the linoleate content of rat tissue lipids was dependent on the level of corn oil in the diet and on the duration of its administration. This relationship, however, was found to vary with different tissue lipids. Of particular interest was the response of the liver phosphatides, phosphatidyl choline and phosphatidyl ethanolamine to the level of dietary fat. The linoleic acid in these membrane components clearly exchanged to only a limited extent with the corn oil lineolate, whereas that in the neutral cellular lipids equilibrated rapidly. This relatively small change in the linoleate content of membrane phosphatides may be related to the maintenance of the permeability characteristics of liver cells.

Young rats were used in this investigation because it was known that changes in fatty acid patterns were easier to impose on young animals than upon adults (Aaes-Jorgensen, Leppik, Hayes and Holman, 1958). Furthermore, previous work had shown that when the external supply of linoleate was restricted, older animals were able to preferentially retain their content of this fatty acid obtained from previous diets (Beare, 1961; Ostwald et al., 1962). For these reasons then, the present experimental treatments were begun immediately after the animals were weaned. In the absence of dietary fat, the rats lost

some weight during the nine-week feeding period, but did not have sufficient time to develop the classical symptoms of essential fatty acid deficiency. When 0.5% corn oil was supplied in the diet, rats maintained good growth throughout the experimental period. As the level of corn oil in the diet and so the proportion of linoleic acid in the carcass increased, the proportion of other tissue fatty acids decreased, but not the absolute amounts of these acids since the animals continued to gain weight and deposit fat.

It is apparent from the results in Fig. 7 that the greatest increase in carcass linoleate in response to a high level of dietary corn oil occurred within the first three weeks. During this period, the animals appeared to make most of the metabolic adjustment to the dietary regimen. On the other hand, the maximum proportion of linoleic acid in the carcass was not detected until the end of the nine-week feeding period and then only in rats supplied with corn oil at the 20% level. This level of linoleate intake appeared to be the maximum that could affect the fatty acid composition of the rat since no more linoleic acid was deposited in animals fed corn oil at the 30% level over the same period of time.

It is known that rats supplied with fat high in linoleic acid and low in linolenic acid, such as corn oil, deposit primarily palmitic, stearic, oleic, linoleic and

arachidonic acids in the tissue lipids (Klenk and Mohrhauer, 1960; Beare, 1961; Collins, 1963). Linolenic acid and the C_{20} and C_{22} acids derived from it are in this circumstance reduced to trace components. Since the five major fatty acids mentioned above constitute well over 90% of the tissue fatty acids in the rat, it is evident that when corn oil is provided, the need for biosynthesis of fatty acids is greatly reduced. The work of Hill, Linazasoro, Chevallier and Chaikoff (1958) provides an excellent example of this situation. These workers found a measurable depression in hepatic synthesis of fatty acids from acetate when rats were fed 2.5% fat in the diet, and about 90% decrease with 15% fat. In the present study the increase in tissue linoleate associated with increasing amounts of dietary corn oil similarly appeared to be related to the suppression of lipogenesis

The initial fall in linoleate of liver lipids when rats were fed 2% corn oil probably reflects an adjustment from rat milk with its high content of fat and relatively high concentration of linoleic acid (Cox and Mueller, 1937; Luckey, Mende and Pleasants, 1954; Beare, Gregory, Smith and Campbell, 1961).

Since linoleic acid found in the rat does not arise from biosynthesis, this acid could be considered as a label. After nine weeks, rats fed 20 or 30% corn oil incorporated linoleic acid to the extent of 43-46% of the

total fatty acids of the carcass. Previous studies showed 39% linoleic acid (Bhalerao et al., 1961) and 43% linoleic acid (Beare, 1961) in the carcass fatty acids of rats fed 20% corn oil for four weeks, and 36% linoleic acid in rats fed 10% corn oil for eight and a half weeks (Perkins et al., 1961). There is some evidence that a dietary oil containing more linoleic acid than corn oil can further elevate the tissue levels of linoleic acid. Ostwald et al. (1962) and Okey et al. (1962) fed 10% safflower oil to rats for three weeks and demonstrated approximately 53% linoleic acid in the fatty acids of adipose tissue and 18% linoleic acid in total liver phosphatides. It appears then, that when oils high in linoleic acid are fed, the concentration of this acid in the tissue varies not only with the dietary level but also with its relative proportion to other fatty acids in the oil. When the fat intake is high enough to render lipogenesis of little consequence, the proportion of linoleic acid in the tissue tends to approach that in the dietary fat.

These relationships, however, do not apply to all classes of lipids in a particular tissue. Okey, Shannon, Tinoco, Ostwald and Miljanick (1961) observed a lesser response to linoleate intake in the total phosphatide fraction than in the neutral lipids of rat liver. Similar results were obtained for rat chylomicrons (Whyte,

Karmen and Goodman, 1963). More recently, Yamamoto, Isozaki, Hirayama and Sakai (1965) found 13% linoleic acid in the fatty acids of both mitochondria and microsomes from livers of rats fed 7% ethyl linoleate for three weeks. In the present study, phosphatidyl choline showed a maximum linoleate content (15%) after three days and phosphatidyl ethanolamine (9%) after six days. These phosphatides reflected differences in dietary linoleate to a much smaller extent than the neutral lipids in which the proportion of linoleic acid (40%) increased within three days to values approaching that of the diet. The maximum level of linoleic acid in phosphatidyl choline was higher than that in phosphatidyl ethanolamine, in agreement with the finding of Getz et al. (1961) for phosphatidyl choline and the combined phosphatidyl serine and phosphatidyl ethanolamine. The linoleic acid in the total lipids reached a peak level after 12 days, which is considerably later than the time at which the maximum linoleate concentration had been attained in phosphatidyl choline, phosphatidyl ethanolamine and neutral lipid. It is not known whether the fatty acids of other phosphatides accounted for the relatively slower changes in linoleate concentration of the total lipids.

According to Kennedy (1961), diglycerides are intermediates both in the synthesis of triglycerides and of phosphatidyl choline or ethanolamine. Since the linoleic

Fig. 16. Biosynthetic pathways for some glyceryl phosphatides (Kennedy, 1961).

acid content of the liver triglycerides (neutral lipid) was at all times higher than that of the phosphatides, it follows that either the phosphatides and triglycerides do not arise from a common diglyceride pool, or the enzymes converting diglycerides to phosphatides have substrate specificities different from that of triglyceride synthetase (Weiss, Kennedy and Kiyasu, 1960). Another process which may affect the fatty acid composition of phosphatides is the acylation of lysophosphatidyl choline or ethanolamine by liver acyl transferases (Lands, 1960; Lands and Hart, 1964, 1965, 1966). As proposed by Brockerhoff, Ackman and Hoyle (1963), this process might be responsible for incorporation of the essential fatty acids, linoleic and arachidonic acids, into phosphatides. Although differences existed in metabolic turnover rates,

Lands, Blank, Nutter and Privett (1966) suggested that the diglyceride portions of triglycerides and lecithins tended to equilibrate. An entirely opposite situation was apparent in the studies reported here, for much larger quantities of linoleic acid were incorporated into neutral lipids than into phosphatides.

In summary, the present results indicate that the fatty acid pattern of liver membrane phosphatides varies with the level of dietary linoleate and with the duration of the feeding period, but that the degree of variation in fatty acid composition is restricted, thereby ensuring the maintenance of membrane properties.

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II. MECHANISM OF ACTION OF PHOSPHOLIPASE B

A. REVIEW OF LITERATURE

1. Action and occurrence

The conventional designations of positions in an L- α -glycerylphosphatide are:

where R' and R'' are fatty acids and X is usually choline or ethanolamine.

The existence of enzymes which catalyze the hydrolysis of lecithin and other phosphatides to fatty acids, glycerol, choline, and phosphate has been known for many years. From studies with rice bran, rice embryos and Aspergillus oryzae, Contardi and Ercoli (1933) proposed a scheme for enzymic degradation of lecithin (Fig. 17). Lecithinase A catalyzed the release of one fatty acid from lecithin giving rise to lysolecithin, which in turn was deacylated by lecithinase B to glycerylphosphorylcholine. Lecithinase B also catalyzed the complete deacylation of lecithin to glycerylphosphorylcholine.

Hanahan (1957 a) criticized the claim of Contardi and Ercoli (1933) that lecithinase B attacked both

Fig. 17. Enzymic degradation of lecithin.

lecithin and lysolecithin, and attributed their results to contamination of the lecithinase B preparation with lecithinase A. Hanahan's thesis was that the action of lecithinase B was the result of the combined activities of lecithinase A and a lysolecithinase. This theory was based in part on the investigations of Fairbairn (1948) who demonstrated the presence of an enzyme in Penicillium notatum specific for lysolecithin. Fairbairn introduced the term lysophospholipase which he considered to be synonymous with the term, lecithinase B (Ercoli, 1940). Uziel and Hanahan (1956) confirmed the findings of Fairbairn with the P. notatum enzyme and established the product of the action of phospholipase B on lysolecithin as being L- α -glycerylphosphorylcholine.

The activity of both lysolecithinase and lecithinase was demonstrated in aqueous extracts of the fungus, Lycoperdon giganteum by Francioli (1935).

When Serratia plymuthicum was grown in a medium containing lecithin, enzymes were produced for the degradation of this phosphatide (Hayaishi and Kornberg, 1954). Since lecithin is not normally present in this bacterium this experiment appears to have been an early example of enzyme induction. Extracts of S. plymuthicum were capable of hydrolyzing both lysolecithin and lecithin.

Shapiro (1952) reported two different enzyme systems in acetone powders of ox pancreas which catalyzed the deacylation of lysolecithin and the formation of glycerylphosphorylcholine. One of these hydrolyzed lysolecithin and had no effect on lecithin; the second enzyme hydrolyzed lecithin without a concomitant decrease in ester content, and was presumed to transfer the acyl group to an unknown carrier in the enzymic preparation. This finding has not been substantiated by other workers.

Uziel and Hanahan (1957) detected no lysolecithinase in commercial pancreatin, but only a lysolecithin migratase which they also found in P. notatum. This enzyme presumably catalyzed the reversible migration of the fatty acyl group from the α' - to the β -position of the phosphatide.

Autolysis of beef pancreas of rat liver mucosa

resulted in large increases in glycerylphosphorylcholine (Schmidt, Greenbaum, Fallot, Walker and Thannhauser, 1955). The appearance of this compound was indicative of phospholipase B activity. This enzyme was presumed to be absent in lamb liver homogenates since glycerylphosphorylcholine was not formed on autolysis.

Dawson (1956 b) studied the deacylating enzyme in extracts of acetone-dried rat liver and sheep brain using lysolecithin and lysophosphatidylethanolamine as substrates. This liver enzyme was thought to be required for the rapid removal of lysophosphatides which otherwise might disrupt the cell membranes. Dawson's results were in accord with the following pathway for the degradation of lecithin:

Marinetti, Erbland and Witter (1958 a) detected lysolecithinase activity in a soluble rat-liver system. Using fresh homogenates, Marples and Thompson (1960) found appreciable lysolecithinase activity in rat ileum, lung, spleen, liver, and pancreas, and low activity in skeletal muscle, kidney, testes, brain, and blood. These results were contrary to the earlier negative findings of Francioli (1934) and Noguchi (1944) for mammalian tissue.

Lysolecithinase activity appeared to be present in increasing amounts in the acetone powders of rat placenta, fetal liver, and maternal liver (Winkler, 1964). The low enzymic activity in the placenta paralleled the relatively high content of lysophosphatides.

Cod muscle was thoroughly investigated by Yurkowski and Brockerhoff (1965), and found to possess more lysolecithinase than lecithinase activity.

Other studies on phospholipase B from animal sources involved homogenates of rabbit polymorphonuclear leukocytes which catalyzed the release of fatty acids from both lecithin and lysolecithin (Elsbach and Rizack, 1963).

Rabbit erythrocytes hemolyzed by cobra venom were found to hydrolyze lysolecithin (Klibansky and de Vries, 1964). While studying the action of phospholipase A of snake venom and bee venom on lysolecithin or dimyristoyllecithin, Doery and Pearson (1964) observed that in addition to the expected lyso-compounds, glycerylphosphorylcholine was formed, indicating phospholipase B activity. This enzyme appeared to be ten times more active in Pseudechis porphyriacus than in Crotalus adamenteus.

The role of lipases in catalyzing the release of the α - and α' -acyl groups of triglycerides has been well established, but it has recently been found that lipase

preparations from different sources appear also to possess phospholipase activity. De Haas, Sarda and Roger (1965) found that a pancreatic lipase preparation degraded egg lecithin to lysolecithin and mainly saturated fatty acids. Since it is known that snake venom releases the fatty acid from the β -position of lecithin (Tattrie, 1959; Hanahan, Brokerhoff and Barron, 1960) and that this fatty acid is probably unsaturated, the pancreatic lipase appeared to hydrolyze the ester linkage in the α' -position. This was confirmed by using synthetic α' -oleoyl- β -stearoyl-glycerolphosphorylcholine as substrate. The enzyme catalyzed the release of oleic acid and the formation of lysolecithin containing stearic acid. Therefore, the site of attack by the lipase preparation on lecithin was conclusively shown to be the α' -position and was independent of the nature of the fatty acid moieties. Laboureur and Labrousse (1964) found that lipase from a mold of the genus Rhizopus catalyzed the hydrolysis of serum phosphatides. Since most of these investigations were made with impure lipase preparations, it seems likely that the phospholipase B activity associated with them was due to contamination and not to the lipase itself.

2. Purification and cellular distribution

Crude enzymic preparations have been employed in most investigations of phospholipase B. Contardi and Ercoli (1933) concentrated the enzyme from A. oryzae or

fresh rice bran by precipitation with lead acetate followed by removal of the lead with hydrogen sulfide. Shapiro (1952) precipitated the enzyme from ox pancreas by diluting the glycerol extract with water and adjusting the pH to 5.0 with acetic acid. Subsequently, Shapiro (1953) used ammonium sulfate fractionation to obtain a partially purified lysolecithinase preparation showing no lecithinase activity.

Phospholipase B of germinating barley appeared to be particulate bound, and could be separated from the accompanying soluble phospholipase D by ultracentrifugation or passage through a column of permutite (Acker and Bucking, 1957). A potent particulate lecithinase preparation from rat intestinal mucosa was obtained by centrifugation of a homogenate of this tissue at 800 X g. (Epstein and Shapiro, 1957). Ottolenghi (1964) determined the subcellular distribution of lecithinase activity in rat intestinal mucosa, using a sonicated lecithin-oleate suspensions stabilized with ethylenediaminetetra-acetic acid (EDTA) as substrate. He found that most of the enzymic activity was associated with particulate material which exhibited sedimentation characteristics of ribosomes. This fraction, however, was not examined by electron microscopy.

To study the deacylating enzymes of rat liver, Dawson (1956 b) employed glycerol extracts of acetone-dried tissue. Marinetti et al. (1958) on the other hand

used a soluble fraction obtained by homogenization of liver with ground glass and differential centrifugation to remove mitochondria and microsomes. The supernatant fluid was capable of converting ^{32}P -lysolecithin into ^{32}P -lecithin as well as to ^{32}P -glycerylphosphorylcholine which is water-soluble. The reactions involved were considered to be as follows:

It was not established whether the lecithin was formed directly from lysolecithin or from glycerylphosphorylcholine. This enzyme preparation showed 'extremely weak activity' toward labelled lecithin. In the subcellular fractions of rat liver examined by Shibko and Tappel (1964), low lysolecithinase activity, determined by the release of choline, was associated with microsomal and soluble constituents. Lysosomes were found to possess 1.2% of the total lysolecithinase activity. A different cytological distribution for lecithinase and lysolecithinase in leukocytes was suggested by Elsback and Rizack (1963) since lecithin was hydrolyzed by both the supernatant (8,200 X g) and the granules of the leukocytes, whereas the lysolecithin was hydrolyzed mainly by the supernatant fluid.

The most successful attempt to purify phospholipase B appears to have been that of Oi and Satomura (1963) who worked with the fungus Sclerotinia. By column chromatography on anion exchange resin and DEAE-cellulose the phospholipases were separated into a phospholipase A and two 'phospholipases B', one of which was crystallized. Oi (1963) demonstrated that the phospholipase A specifically hydrolyzed the ester bond of the unsaturated fatty acid in soybean lecithin, presumably the β -ester linkage, whereas the crystalline 'phospholipase B' hydrolyzed the ester bond of the saturated fatty acid in soybean lecithin, presumably at the α' -linkage. It seems likely that this 'phospholipase B' is similar to the phospholipase which hydrolyzes only the α' -fatty acid of lecithin, as described by Van den Bosch and van Deenen (1965). The assignment of two 'phospholipases B's' may not have been justified, particularly since the only criterion for phospholipase B activity was the liberation of free fatty acids.

The most rigorous proof for the detection of phospholipase B activity is the formation of glycerylphosphorylcholine. Free fatty acids may arise from the action of phospholipase A or phospholipase B. Choline may be indicative of the formation of diglyceride and phosphorylcholine (phospholipase C) or of phosphatidic acid and choline (phospholipase D). Therefore reports based on only the release of free fatty acid or choline do not specifically designate phospholipase B activity.

3. Enzymic properties

In most of the studies on phospholipase B, the degree of purification of the enzyme was not commensurate with the assignment of definite properties. Variable results for the cellular distribution of the enzyme may have been the result of inadequate separation of sub-cellular fractions or disruption of membranes during fractionation. Electron microscopy studies of subcellular preparations used for phospholipase studies have not been reported.

The characteristics of phospholipase B varied with the source of the enzyme. Contardi and Ercoli (1933) found a pH optimum of 3.5 for the enzyme from A. oryzae. The lysolecithinase from P. notatum described by Fairbairn (1948) possessed optimum activity at pH 4.0. Lecithinase from the same source showed peak activity at pH 3.1 to 3.4 when cardiolipin or monophosphoinositide was employed as an activating lipid, and pH 4.2 when the activating lipid was tripalmitin (Dawson, 1958 c). For the enzyme of S. plymuthicum the optimum pH was 5.6 - 5.7 (Hayaishi and Kornberg, 1954), and for that of barley, pH 6.0 to 6.3.

The optimum pH's for the enzyme derived from animal tissues are also relatively high. The partially purified lysolecithinase obtained by Shapiro (1953) from ox pancreas had a pH optimum of about 6.0. Maximal hydrolysis of lysolecithin by rat liver occurred at pH 6.2 (Dawson, 1956 b),

and by cod muscle at pH 7.6 (Yurkowski and Brockerhoff, 1965). The optimum pH's for the lecithinase, and lysolecithinase of polymorphonuclear leukocytes were 6.0 and 7.0 respectively (Elsback and Rizack, 1963). The latter authors speculated that this pH difference could be important during phagocytosis when the intracellular pH falls. During this process, lysolecithin might accumulate and cause lysis.

The phospholipase B of snake and bee venoms showed maximum activity in the alkaline region, pH 8.5 to 10 (Doery and Pearson, 1964), and appears therefore to be appreciably different from the enzymes derived from other sources.

Heat-sensitivity is another variable characteristic of phospholipase B. A preparation from pig lung lost its activity after 30 minutes at 60°C. After 10 minutes at 50°C, the enzymic activity of rat intestinal mucosa was destroyed (Epstein and Shapiro, 1959). The enzyme from rat liver (Dawson, 1956 b) was stable after 10 minutes at 50°C, but inactive at 55°C. At temperatures of 41°C or over and a pH above 7, the enzymic activity of P. notatum was quickly destroyed (Fairbairn, 1948). In contrast, the enzymic activity of S. plymuthicum was 30 to 50% retained after heating to 100°C for 10 minutes (Hayaishi and Kornberg, 1954). Similar heat stability was found for the enzymic activity of venoms (Doery and Pearson, 1964).

Fairbairn (1948) observed a strong inhibition of the

P. notatum enzyme by cyanide, a finding confirmed by Uziel and Hanahan (1956) but not by Dawson (1958 c). Dawson, in turn, found that the enzyme was sensitive to fluoride. Cyanide and fluoride were not inhibitors of cod lysolecithinase (Yurkowski and Brockerhoff, 1965). Intestinal lecithinase was inhibited by mercury, cadmium and calcium (Epstein and Shapiro, 1959). The phospholipase B of Sclerotinia was markedly inhibited by zinc and tin (Oi and Satomura, 1963).

Francioli (1937) found physostigmine to be an inhibitor of lysolecithinase in Lycoperdon giganteum, but this inhibitor was ineffective for the lysolecithinase of P. notatum (Fairbairn, 1948).

4. Activation

In some enzymic preparations, calcium appeared to be an activating factor. When Hayaishi and Kornberg (1954) centrifuged the extracts of Serratia plymuthicum at 110,000 X g, two complementary components essential for enzymic activity were separated. The soluble fraction could be partially replaced by ferric ions, whereas the residue required calcium for activity.

Divalent cations enhanced the phospholipase activity of the venoms studied by Doery and Pearson (1964). On the other hand, calcium or magnesium had no effect on phospholipase B of P. notatum (Fairbairn, 1948), of rat liver (Dawson, 1956 b) or of ox pancreas (Shapiro, 1953). The activity of a phospholipase B isolated by Oi and Satomura

(1963) was markedly increased by calcium, magnesium and manganese, whereas the activity of the other phospholipase B was inhibited by these metals.

Dawson (1957 b) showed that 'activating lipids' obtained from liver permitted phospholipase B from P. notatum to deacylate lecithin. These lipids were isolated and identified (Dawson, 1958 a) as phosphatidyl inositol and a polyglycerol phospholipid (cardiolipin). The addition of either of these lipids in low concentration to a system containing the P. notatum extract and lecithin resulted in rapid hydrolysis of the substrate with the liberation of free fatty acids and glycerylphosphorylcholine. Monophosphoinositide was also attacked by the enzyme, the products being free fatty acids and glycerylphosphorylinositol (Dawson, 1958 b). To account for the activating effect, Dawson (1958 c) suggested that these lipids imparted negative charges to the surface of the lecithin particles, thereby aiding the enzymic attack. Liver cardiolipin lipid and monophosphoinositide were found to be effective activators, tripalmitin and tristearin, weak activators, and all other lipids tested were ineffective.

Using microelectrophoresis, Bangham and Dawson (1959) found a correlation between the charge on the substrate and enzymic activity. Pure lecithin particles exhibited a slight net positive charge at pH 3.3 but the activating lipids imparted to them a net negative charge.

Phospholipase B activity was also promoted by a number of other anionic molecules of an amphipathic nature, that is, having both hydrophobic and hydrophilic groups. Surface-film studies employing ^{32}P -labelled lecithin, obtained by growing yeast in the presence of ^{32}P -phosphorus, were used to follow the action of phospholipase B (Dawson and Bangham, 1959). The loss of radioactivity as the water-soluble glycerylphosphorylcholine- ^{32}P diffused into the aqueous phase was a measure of enzymic activity. The collapsed film of lecithin by itself was not attacked but addition of dicetylphosphoric acid resulted in hydrolysis. With a unimolecular layer of lecithin on a Langmuir-type trough (Bangham and Dawson, 1960), the enzyme deacylated pure lecithin at low pressures. Enzymic activity increased with pressure up to 30 dynes/cm, and then rapidly decreased to zero at higher pressures. Dicetylphosphoric acid was presumed to promote the enzymic attack on films of substrate under high pressure, by imparting negative charges to the layer. Dawson (1964) argued that the negative charge on the substrate surface, rather than the dispersion of the molecules, was responsible for activation because a cationic, amphipathic substance was ineffective. Great significance was therefore attributed to the electrostatic situation at the lipid-water interface when the enzyme encountered the lipid substrate.

Fatty acids in roughly equimolar concentrations to the lecithin substrate were required for lecithinase activity of intestinal mucosa (Epstein and Shapiro, 1959). This intestinal mucosal enzyme was sensitive to X-irradiation of the whole rat (Ottolenghi and Bernheim, 1960, 1961). Lecithinase was more readily inactivated than was lysolecithinase, but both types of activity were restored by the addition of normal mucosa or chymotrypsin. This treatment presumably uncovered the enzymic active site. In further investigations with the mucosal preparation, Ottolenghi (1962, 1963) separated an activating fraction from the enzymic material by centrifugation in a glycerol density gradient. The nature of the activator was undetermined.

5. Substrate specificity

The rate of hydrolysis catalyzed by lysolecithinase was greater with unsaturated than with saturated substrate (Uziel and Hanahan, 1956). As previously discussed, lecithinase activity could sometimes be demonstrated in enzyme preparations once thought to have only lysolecithinase activity. Schmidt, Bessman and Thannhauser (1957) found that beef brain cephalins were more readily hydrolyzed than egg lecithin, and brain plasmalogens were unaffected.

A recent study by Bjornstad (1966) demonstrated that endogenous phosphatidyl ethanolamine was deacylated to glycerylphosphorylethanolamine by liver microsomes in

vitro. The principal degradation product of lecithin was free choline. In such a system, phospholipase B activity with a lecithin substrate could not be discerned. Enzymic preparations of P. notatum (Dawson, 1953 b) hydrolyzed phosphatidyl inositol. Phospholipase B from rat spleen hydrolyzed phosphatidyl glycerol and lysophosphatidyl glycerol to produce glycerylphosphoryl glycerol (Haverkate, Houtsmuller and van Deenen, 1962).

Working on the assumption that complete deacylation of lecithin is effected by two different enzymes, one for the β - and one for the α' -position, Robertson and Lands (1962) suggested that whichever lysolecithin was formed must be quickly removed. A lysolecithin intermediate could not be detected in homogenates of rat intestine, spleen, lung, heart, liver or brain. Subsequently, the existence of an α' -lecithinase was demonstrated in rat liver (Van den Bosch and van Deenen, 1965) and in rat intestine (Robertson, 1966). Synthetic lecithins containing a labelled fatty acid in the β -position were hydrolyzed by rat liver homogenates to lysolecithins with or without this label. Thin-layer chromatography confirmed the structure of two isomeric lysolecithins (Van den Bosch and van Deenen, 1965). Using rat intestine Robertson (1966) demonstrated the deacylation of α' -lysolecithin, but was unable to separate the lysolecithinase specific for the α' -fatty acid from the enzyme which removes the β -fatty

acid of lecithin. It is not known whether a similar enzymic system exists in liver.

Whether the phospholipase B activity of P. notatum or rat liver involves one or more enzymes for the deacylation of glycerylphosphatides is a problem yet to be solved.

B. EXPERIMENTAL

1. Materials and methods

a. Preparation of phospholipase B

i. From *Penicillium notatum*

According to the procedure of Fairbairn (1948) as modified by Dawson (1958 a), mycelia of *P. notatum* were ground in a mortar to a slurry in water and autolyzed for 24 hrs. The autolysate was filtered and the filtrate dialyzed against water at 4°C for 24 hrs. The dialysis sac was then immersed in glycerol until the volume of its contents was reduced to about one quarter. This glycerinated preparation was stored at -10°C, and was stable indefinitely under these conditions. Prior to use, the glycerol was removed by dialyzing 10 ml of the preparation against 4 litres of distilled water at room temperature for two hours. The volume of the dialyzed enzymic preparation was 33 ml. The protein concentration was approximately 1 mg/ml; glycerol and inorganic phosphate were present in only trace quantities. This dialyzed preparation was stable for several weeks at 5°C.

ii. From rat liver

Acetone powder

The liver was quickly removed from stunned rats which were bled from a neck incision, and was immediately placed on dry-ice. As described by Dawson (1956 a), livers and dry-ice were broken into small pieces in a mortar cooled to -20°C, and two volumes of ice-cold water

were added. After homogenization, the suspension was frozen and thawed. Five volumes of acetone at -20°C were added to the suspension which was immediately filtered under reduced pressure. The filter cake was washed with acetone; the residue was dried in a vacuum desiccator.

Subcellular fractions of liver

Liver tissue was fractionated into mitochondrial, microsomal and soluble constituents by ultracentrifugation according to the procedure of Dounce, Witter, Monty, Pate and Cottone (1955).

To 10 g of rat liver were added 40 ml of 0.44M sucrose in 0.0018M citric acid. The mixture was homogenized by a few strokes of the plunger in a Dounce homogenizer, diluted with an equal volume of 0.44M sucrose, and spun at $675 \times g$ for 20 minutes at 0°C in a Lourdes centrifuge to remove whole cells, nuclei and debris. The mitochondrial fraction was sedimented by centrifugation at $13,000 \times g$ for 10 minutes. The supernatant fluid was then spun at $78,000 \times g$ for one hour to separate microsomes from the 'soluble' fraction. These crude cellular fractions were tested for their ability to deacylate lecithin.

The cellular fractionation technique of Sewant, Shibko, Kumta and Tappel (1964) was used to isolate a lysosomal fraction. Rat liver tissue (74 g) from 15 fasted rats was homogenized in 8 vol. of 0.25M sucrose in 0.001M EDTA

(diaminoethanetetra-acetic acid, disodium salt, Analar reagent grade) in a Waring blender for 20 seconds and then adjusted to pH 7.0. After the removal of unbroken cells, nuclei and debris by centrifugation at 750 X g for 10 minutes, the homogenate was spun at 3,300 X g for 10 minutes to remove 'heavy mitochondria'. The light mitochondrial fraction containing lysosomes was sedimented by centrifugation at 16,300 X g for 20 minutes, resuspended in 0.3M sucrose in 0.001M EDTA, pH 7.0, and recentrifuged at 9,500 X g for 10 minutes. The supernatant fluid was discarded, and the pellet of light mitochondria and lysosomes resuspended in 25 ml of 0.4M sucrose - 0.001M EDTA (pH 7). This constituted the top layer of a discontinuous sucrose gradient in which the middle layer was 30 ml of 0.6M sucrose - 0.001M EDTA and the bottom layer was 35 ml of 0.7M sucrose - 0.001M EDTA. The pellet obtained by centrifugation of this gradient at 9,500 X g for 30 minutes was resuspended in 0.7M sucrose, and centrifuged at 5,900 X g for 30 minutes. This step sedimented the light mitochondria leaving the lysosomes in the supernatant; the latter were subsequently sedimented at 17,000 X g for 20 minutes. The lysosomal fraction was resuspended in 0.7M sucrose - 0.001M EDTA and spun at 17,000 X g for 20 minutes. Total homogenate, homogenate after removal of heavy and light mitochondria, the two mitochondrial fractions and lysosomes were assayed for phospholipase B activity at a

concentration approximating that in the whole liver homogenate.

b. Preparation of substrates

Egg lecithin

The substrate most studied was egg lecithin which was prepared by the procedure of Lea et al. (1955), and stored in methanol. It was chromatographically homogenous and had the following analyses: 3.8% P; 1.72% N; N/P atomic ratio, 0.99; 15.2% choline; choline N/P atomic ratio, 1.00; 69.5% fatty acids; fatty acids/P mole ratio, 1.98; fatty acids consisting of 3.2% myristic, 38.5% palmitic, 11.0% stearic, 31.7% oleic, 12.2% linoleic, 0.9% linolenic, 2.5% arachidonic.

To obtain an aqueous dispersion of substrate, an aliquot of methanolic solution of the phosphatide containing 20 to 70 μ moles of lipid phosphorus was placed in a thick-walled glass tube having an inside diameter of 15 mm and a length of 62 mm. The solvent was evaporated under a stream of nitrogen, and 2 to 5 ml of distilled water were added to give a final substrate concentration of 2.0 to 2.8 μ moles/ml. The mixture was placed in an ice-salt bath at -15°C , and dispersed by a Branson sonifier (20 K cycles/sec) at an output of 110W for 3 to 5 minutes. The translucent dispersion was stable for 2 to 3 days at 4°C .

Rat liver lecithin

Lecithin from rat livers was separated from other

lipids by a modification of the thin-layer chromatographic procedure of Skipski, Peterson and Barclay (1964). Silica gel without calcium sulfate binder (Silica Gel H, E. Merck AG., Darmstadt, Germany) was slurried with a 1 mM sodium carbonate solution (1 g of silica gel to 2.25 ml), and a coating 500 μ thick was applied to 20 cm X 20 cm glass plates with a Unoplan Colab Applicator (Consolidated Laboratories Ltd.). The plates were allowed to dry, and before use, were activated at 110°C for 1 hour. Approximately 500 μ l of lipid extract were applied by spotting across the line of origin. The chromatogram was developed in the solvent system of chloroform-methanol-acetic acid-water (99:50:15:7, v/v/v/v) and one part of 0.5% butylated hydroxy toluene (4-methyl-2,6-ditertiary butylphenol) in chloroform, as recommended by Wren and Szczepanowski (1964) to prevent oxidation during chromatography. The lipids were detected by placing the chromatographic plate in a jar containing iodine vapour (Sims and Larose, 1962) for 20 to 30 seconds. The lecithin fraction as determined by R_f , was delineated with a dissecting needle, and recovered by removing the silica gel with a razor blade, suspending the powder in 3 ml of developing solvent, and centrifuging. The solvent was removed with a capillary pipette, and the silica gel eluted successively with 2 ml of developing solvent, 2 ml of methanol and then 2 ml of methanol-acetic acid-water (94:1:5, v/v/v). The phosphatide in the total solvent fractions was partitioned

according to Bligh and Dyer (1959) in a solvent mixture adjusted to give methanol-chloroform-water, 2:1:0.8, v/v/v. Upon the addition of one part of chloroform and one part of water to give a final composition of methanol-chloroform-water, 2:2:1.8, v/v/v, the chloroform phase containing the lecithin separated from the aqueous methanol phase. The chloroform phase was evaporated under a stream of nitrogen and the lecithin dissolved in chloroform-methanol (1:1, v/v) for storage. Before use, the solvent was evaporated, and the lecithin dispersed ultrasonically in water as described for egg lecithin.

Other substrates

Hydrogenated egg lecithin (hydrolecithin) was prepared by Dr. Morris Kates. Egg lysolecithin was obtained from Pierce Chemical Company, and beef cephalin from Sylvana Chemical Company.

Synthetic phosphatides used included dimyristoyl cephalin and dipalmitoyl lecithin from Dr. E. Baer, University of Toronto, and monopalmitoyl lecithin and monopalmitoleyl lecithin from Dr. D. J. Hanahan, University of Washington. Dimyristoyl lecithin was obtained from La Motte Chemical Products Company.

c. Enzyme assay

For most determinations with the P. notatum enzyme, each reaction tube contained 0.20 ml of aqueous lecithin solution (equivalent to 2.5-2.8 μ moles), 0.20 ml of 1M acetate buffer (pH 4.0), and 0.60 ml of enzyme preparation

(0.6 mg of protein). Variation in amount of any one component was compensated by the addition of distilled water to maintain a constant total volume of 1.00 ml. The mixture was shaken during incubation at 37°C. At the required time, the reaction was stopped by the addition of 2.50 ml of methanol and the sample was shaken in a water bath at 80°C for 30 seconds. The lipids in the enzyme digest were extracted by a modification of the method of Bligh and Dyer (1959): the cooled reaction mixture was diluted with 1.25 ml of chloroform, the mixture vibrated for 10 seconds, another 1.25 ml of water added, and the mixture shaken and centrifuged. The final chloroform phase was therefore 3.0 ml, and the aqueous methanol phase was 4.75 ml. A 3 ml aliquot of the methanolic phase was taken for phosphorus determination and a 1 or 2 ml aliquot of the chloroform phase taken for ester determination.

Enzyme activity was determined by the release of organic phosphorus into the methanolic phase, which is an index of glycerylphosphorylcholine formation, and by the reduction of acyl ester in the chloroform phase after appropriate corrections for enzyme and substrate blanks.

The assay conditions with rat liver preparations were the same as those used with the P. notatum enzyme except that the pH was maintained at 6.4 with 1M phosphate, or tris maleate buffer. In the presence of phosphate

buffer, reduction of acyl ester was the only criterion employed for enzymic activity.

d. Analytical methods

i. Total phosphorus

The solution to be analyzed was evaporated to dryness in a hot water-bath under a stream of air. The method of Allen (1940) was used as described in Part I.

ii. Acyl esters

Acyl esters in lipids were determined by conversion to hydroxamic acids which were complexed with acid ferric perchlorate to give a purple iron chelate. The method used was a modification of that of Snyder and Stephens (1959). To remove all traces of chloroform from the lipid sample, the solvent was first evaporated under a stream of nitrogen in a water bath below 40°C; 0.5 ml of acetone was then added and blown off with nitrogen, and the residue was dried under vacuum in a desiccator for 20 to 30 minutes.

Alkaline hydroxylamine was prepared fresh daily from equal volumes of 4% ethanolic hydroxylamine hydrochloride (2.0 g dissolved in 2.5 ml of distilled water, diluted to 50 ml with absolute ethanol) and of 8% ethanolic sodium hydroxide (4 g dissolved with heating in 2.5 ml of distilled water, diluted to 50 ml with absolute alcohol). The solutions were mixed in a stoppered 50 ml centrifuge tube and spun at 3,000 X g for 10 minutes. One ml of the supernatant was added to each dry lipid sample, which was

placed in a water-bath at 65°C for 2.0 minutes, then allowed to cool for 5 minutes.

The colored complex was formed upon the addition to the lipid samples of 3.0 ml of ferric perchlorate reagent. This reagent was prepared by mixing 4 ml of stock (5 g ferric perchlorate dissolved in 10 ml of distilled water, then diluted to 100 ml with absolute ethanol and refrigerated) with 3 ml of 70% perchloric acid diluted to 100 ml with absolute ethanol. The resulting purple color was read 30 minutes later against a reagent blank at 530 mμ.

A standard curve obtained with egg lecithin is illustrated in Fig. 18.

iii. Protein

The method of Lowry, Rosebrough, Farr and Randall (1951) was employed. Material to be analyzed was diluted to 1.0 ml with distilled water and treated with 5 ml alkaline copper solution (50 parts of 0.5% copper sulfate in 1% sodium tartrate and 1 part of 2% sodium carbonate in 0.1N sodium hydroxide). After 15 minutes, 0.5 ml of 0.5N Folin and Ciocaltau phenol reagent was added, and the solution vigorously vibrated. The color was allowed to develop for 30 minutes and read at 700 mμ.

iv. Paper chromatography of phosphate esters

Approximately 0.5 ml of aqueous solution containing 0.5-1.0 μg of phosphate ester was spotted on acid-washed Whatman No. 1 filter paper, 17 cm X 45 cm. The solvent

Fig. 18. Standard curve for determination of ester groups in lecithin.

system of butanol: acetic acid: water (5:3:1,v/v/v), as described by Kates (1964), was used for descending chromatography. The chromatogram was removed after 18 hours, allowed to dry, and passed through the molybdate reagent described by Burrows, Grylls and Harrison (1952). The reagent consisted of 1 g ammonium molybdate in 8 ml water, 3 ml concentrated hydrochloric acid and 3 ml 70% perchloric acid, diluted to 100 ml with acetone.

v. Thin-layer chromatography

Lecithin and its hydrolytic products were separated on glass plates (20 cm X 20 cm) coated with silica gel G, 250 μ thick, using chloroform-methanol-water, 65:25:4,v/v/v as solvent. The efficiency of separation was optimal 2 to 4 days after mixing the solvent. Spots were detected by spraying with water, which distinguished differences in refractive index between silicic acid with and without added material; with a 0.2% solution of Rhodamine 6G for viewing under ultraviolet light; with 40% sulfuric acid for subsequent charring of organic material in an oven at 125°C - 130°C for 10 minutes; or with the molybdenum stain described by Dittmer and Lester (1964). Equal volumes of molybdenum trioxide (20.055 g) in 500 ml of 25N sulfuric acid (solution 1) and of powdered metallic molybdenum (0.890 g) dissolved in 250 ml of solution 1 were mixed and diluted with water until the combined solution was dark green. The preparation stained phosphatides blue.

vi. Gas-liquid chromatography

Free fatty acids were converted to methyl esters with diazomethane for analysis by gas-liquid chromatography with the Pye chromatograph as described in Part I. To prepare diazomethane, 25 ml of diethyl ether were added to 3 g of potassium hydroxide and 4 ml of water in a 100 ml round bottom flask cooled in an ice-bath. Nitrous methyl urea (2 g) was slowly added, and diazomethane in ether was distilled into a receiving flask in ice upon gentle warming of the reaction flask.

Lipid residue (usually obtained from 2 ml of chloroform phase) was treated with 1 ml of diethyl ether and sufficient ethereal diazomethane to give a permanent yellow color. After 15 to 20 minutes, methyl behenate (10 μ moles / ml in chloroform) was added as an internal standard for the chromatographic analysis.

2. RESULTS WITH P. NOTATUM PHOSPHOLIPASE B

a. Stability of enzyme to dialysis

The glycerinated, undialyzed enzymic preparation was found to be highly active towards the ultrasonically dispersed egg lecithin. When the enzyme was dialyzed against water, however, considerable variation in rate of hydrolysis was observed. This was subsequently found to be dependent on the duration of dialysis. Within the first 6 hours of dialysis no appreciable change in activity occurred, but subsequently the activity decreased until only 8% remained after 44 hours (Fig. 19). Since glycerol in the assay system interfered with subsequent phosphorus analysis, but could be mostly removed within the first 2 hours, the enzyme preparation used in subsequent experiments was dialyzed for that period.

b. pH optimum

As shown in Fig. 20, maximal activity of the enzyme occurred between pH 3.8 and 4.0 in acetate buffer.

c. Identification of products

The products of the reaction catalyzed by phospholipase B were separated into water-soluble and chloroform-soluble material. Thin-layer chromatography of the chloroform-soluble products (see Fig. 23) revealed the presence of only free fatty acids and unreacted lecithin. Methylation of the chloroform-soluble material with diazomethane and analysis by gas-liquid chromatography confirmed the formation of free fatty acids.

Fig. 19. Effect of dialysis on the phospholipase B activity of *P. notatum* enzymic preparation. Glycerinated enzyme (10 ml) was dialyzed against 4 liters of distilled water. Activity was measured on samples containing 0.2 mg of protein at the indicated times.

Fig. 20. Effect of pH on phospholipase B activity. Reaction mixture contained 2.8 μ moles of lecithin in 0.2 ml of water, 0.2 ml of 1M acetate buffer (pH 4.0) and 0.6 ml of glycerinated enzyme preparation (equivalent to 2.0 mg protein). Incubation period, 20 minutes.

Paper chromatography of the water-soluble phosphates (Fig. 21) demonstrated that glycerylphosphorylcholine ($R_f = 0.28$) was the only water-soluble phosphate formed and that it increased in concentration as the enzymic reaction progressed. No lysolecithin was detected in the reaction mixture even at the early times (0 to 6 minutes). A substance having an R_f of approximately 0.5 and another with an R_f of about 0.08 were initially present in the enzyme preparation but gradually disappeared during the reaction. No inorganic phosphate appeared on the chromatogram, and only traces were found by quantitative analysis by the method of Allen (1940).

d. Dispersion and charge on substrate

To determine the effect of degree of dispersion of the substrate, the enzyme was incubated with an aqueous suspension of egg lecithin, prepared by shaking on a Vortex mixer for 1 to 2 minutes, and with an ultrasonicated dispersion of the same substrate. The particle diameter of the former dispersion was in the range of 5 to 10 μ , whereas that of the ultrasonicated dispersion was in the range of 1 to 3 μ . As shown in Fig. 22, the ultrasonically dispersed egg lecithin was more readily hydrolyzed by the enzyme than was the non-ultrasonified lecithin. Not only was the rate of hydrolysis of the poorly dispersed substrate lower, but much of it appeared to be unavailable for hydrolysis even after an extensive period of time. The progress of the reaction was followed by thin-layer chromatography (Fig. 23).

Fig. 21. Tracing of paper chromatogram of water-soluble phosphate esters released during phospholipase B hydrolysis of egg lecithin. GPC = glycerylphosphorylcholine; GP = glycerylphosphate; E = enzyme; S = substrate; LL = lysolecithin. Stippling indicates relative density of stain. (R_f of GPC $>$ R_f of GP)

Fig. 22. Effect of ultrasonically dispersed egg lecithin on the activity of phospholipase B. The reaction mixture contained 2.8 μ moles of lecithin in 0.2 ml of water, 0.2 ml of 1M acetate buffer (pH 4.0) and 0.6 ml of enzyme preparation (0.6 mg protein).

Fig. 23. Tracing of thin-layer chromatogram chloroform-soluble products of hydrolysis of egg lecithin from reaction mixtures described in Fig. 22. 100 μ l of each chloroform extract were spotted. Solvent system: chloroform-methanol-water (65:25:4, v/v/v), molybdenum stain.

The effect of cardiolipin added to ultrasonified or non-ultrasonified lecithin is shown in Table 10. Cardiolipin in the reaction mixture with the non-ultrasonified substrate increased the rate of hydrolysis to a value close to that of the ultrasonified substrate. When cardiolipin was added to ultrasonified lecithin, the initial rate of hydrolysis increased to a relatively small extent.

To determine whether the effects of cardiolipin were attributable to the surface charge on the substrate, the electrophoretic mobilities of ultrasonified and non-ultrasonified lecithin particles in 0.2M acetate buffer at pH 4.0 were measured. The results are shown in Table 11. There was no correlation between negative charge on the substrate particles and enzymic activity. The non-ultrasonified particles possessed a greater negative charge than the ultrasonified particles, but the latter were hydrolyzed more readily. Addition of cardiolipin to the non-ultrasonified substrate slightly increased the net negative charge but resulted in a six-fold increase in rate of hydrolysis. On the other hand, addition of cardiolipin to the ultrasonified substrate increased the net negative charge but only increased the initial rate of hydrolysis to a small extent.

In contrast, the enzymic activity was found to vary directly with the % transmittance of the substrate

Table 10. Influence of cardiolipin on rate of hydrolysis of egg lecithin by phospholipase B

Substrate	Time Min.	<u>M of P released</u>	<u>M of</u> <u>ester hydrolyzed</u>
		M of substrate	M of substrate
Sonified lecithin ¹	15	0.40	0.71
	30	0.60	1.15
	60	0.83	1.51
	90	0.92	1.76
Sonified lecithin ¹ and cardio- lipin ²	15	0.45	0.87
	30	-	1.06
	60	0.81	1.55
	90	0.88	1.82
Non- sonified lecithin ¹	15	0.06	0.10
	30	0.09	0.24
	60	0.22	0.38
	90	0.29	0.44
Non- sonified lecithin ¹ and cardio- lipin ²	15	0.38	0.83
	30	0.61	1.39
	60	-	1.63
	90	0.86	1.79

¹ 2.8 μ mole of purified egg lecithin

² 0.1 μ mole of cardiolipin (Sylvana Comp.)

suspension. Ultrasonication which reduced particle size and increased light transmittance was associated with an increase in the initial rate of enzymic hydrolysis.

Table 11. Physical properties of lecithin suspensions and their effect on phospholipase B activity

Lecithin substrate	Cardiolipin added	Particle size, μ	Mobility, $\mu/\text{sec}/\text{V}/\text{cm}^1$	% Transmittance ²	Enzyme activity % hydrolysis/15 min
Non-ultrasonified	-	5-10	-1.35 ± 0.03	24.0	6
	+	-	-1.86 ± 0.04	46.3	38
Ultra-sonified	-	1-3	-0.14 ± 0.01	82.4	40
	+	-	-5.09 ± 0.18	92.3	45

¹ Values obtained by Dr. J. R. Madeley are averages (with standard deviations) of measurements on at least thirty-two different particles (field reversed alternately). Mobilities for the substrate suspensions without cardiolipin in 0.15M NaCl adjusted to pH 4.0 with 0.15M HCl were: non-ultrasonified, -0.7; ultrasonified, -0.1.

² At 680 m μ in a 1.0 cm cuvette; relative to distilled water (100% transmittance).

e. Stoichiometry and kinetics of reaction

In Fig. 24 is shown the time-course of enzymic hydrolysis of ester linkages and the accompanying liberation of water-soluble phosphate. The ratio of acyl groups hydrolyzed to phosphate released was approximately 2.

Fig. 24. Time course of liberation of water-soluble phosphate and hydrolysis of ester groups in egg lecithin by phospholipase B. Reaction mixtures contained 2.8 μ moles of substrate in 0.2 ml of water, 0.2 ml of 1M acetate buffer (pH 4.0) and 0.6 ml of enzyme preparation.

Thin-layer chromatography of chloroform-soluble material in the reaction mixture is illustrated in Fig. 25. The concentration of lecithin appeared to decrease as the concentration of free fatty acids increased.

At various time intervals during the enzymic reaction, the relative proportions of fatty acids released were as shown in Table 12. It is apparent that relatively more linoleic acid was liberated from the substrate in the early stages of the reaction. The relevance of this finding will be discussed later.

f. Enzyme concentration

The liberation of organic phosphate from egg lecithin and the hydrolysis of its acyl ester groups at pH 4.0 were dependent on the concentration of the enzyme (Fig. 26). The reaction rate appeared to vary linearly with enzyme concentrations up to 0.2 mg protein/ml and to approach a maximum with 0.5 to 0.6 mg protein/ml of reaction mixture. The progress of the reaction was followed by thin-layer chromatography (Fig. 27).

g. Substrate concentration and Michaelis-Menten constant

The effect of substrate concentration (Fig. 28) at pH 4.0 and with an enzyme concentration of 0.6 mg protein/ml was also shown in the plot of Lineweaver and Burk (1934). From 2.1 to 5.4 μ moles of substrate, $1/v$ was linearly related to $1/s$. The line representing

Fig. 25. Tracing of thin-layer chromatogram of chloroform-soluble products formed during hydrolysis at indicated times; 100 μ l of chloroform extract from each enzymic reaction mixture. Solvent system; chloroform-methanol-water (65:25:4, v/v/v), molybdenum stain.

release of phosphate shared the same point on the $1/s$ axis with that representing hydrolysis of ester groups.

The apparent Michaelis-Menten constant, calculated according to Dixon (1953), was 6.3 mM.

Table 12. Composition of fatty acids released from egg lecithin at various time intervals by phospholipase B.¹
(% of total fatty acids)

Fatty acid	Time of enzymic reaction-minutes					
	1	2	4	6	40	90
16:0	27.0	30.2	24.6	31.0	34.9	35.1
16:1	2.5	3.7	2.9	4.4	2.5	3.2
18:0	10.2	16.0	10.6	16.0	16.6	13.8
18:1	15.4	16.6	27.5	25.0	29.8	31.5
18:2	44.8	33.5	34.4	23.6	16.2	16.3
Total moles/mole substrate	0.10	0.18	0.20	0.29	1.04	1.53

¹ Substrate was 2.7 μ moles.

Fig. 26. Dependence of phospholipase B activity on enzyme concentration $[E]$. Reaction mixtures contained 2.8μ moles of substrate in 0.2 ml of water, 0.2 ml of 1M acetate buffer (pH 4.0), 0.1 to 0.6 ml of enzyme (equivalent to 0.1 to 0.6 mg of protein) and water to 1.0 ml total volume: Incubation period, 30 minutes.

Fig. 27. Tracing of thin-layer chromatogram of chloroform-soluble products from each reaction mixture employed in assay for Fig. 26; 100 μ l of each extract applied. Solvent system: chloroform-methanol-water (65:25:4, v/v/v), molybdenum stain.

Fig. 28. Dependence of phospholipase B activity on substrate concentration. Reaction mixture contained 0.7 to 5.6 μ moles of substrate, 0.2 ml of 1M acetate buffer (pH 4.0), 0.4 ml of enzyme preparation and water to 1.0 ml. Incubation period, 30 minutes.

h. Substrate specificity

It was desirable to study the action of the P. notatum enzyme on phosphatides other than egg lecithin. Various glycerylphosphatide preparations were checked for purity by thin-layer chromatography as shown in Fig. 29. 'Beef cephalin' contained three components, two of which were phosphatidyl ethanolamine and phosphatidyl serine. This mixed phosphatide was therefore not used as a substrate. Synthetic dimyristoyl cephalin was predominantly phosphatidyl ethanolamine but showed a minor component, possibly lysophosphatidyl ethanolamine. Dipalmitoyl lecithin contained three minor components in addition to lecithin, and was obviously partly decomposed. Egg lecithin, the test substrate used in these experiments was chromatographically pure. Egg hydrolecithin was partly decomposed (Fig. 32), but monopalmitoyl lecithin and monopalmitoleyl lecithin appeared to be pure compounds (Fig. 32).

When the P. notatum preparation of phospholipase B was tested with dimyristoyl cephalin, 20 to 25% of the total material was hydrolyzed within 90 minutes (Fig. 30). Dimyristoyl lecithin did not appear to be hydrolyzed to any significant extent (Fig. 31). These saturated diacyl phosphatides were not effectively dispersed by ultrasonification, and were unsatisfactory for the enzyme system employed.

Fig. 29. Tracing of thin-layer chromatogram of various phosphatides: BC, beef cephalin; DMC, dimyristoyl cephalin; DML, dimyristoyl lecithin; DPL, dipalmitoyl lecithin; EL, egg lecithin. Solvent system: chloroform-methanol-acetic acid-water (100,60,15,7,v/v/v), molybdenum stain.

Fig. 30. Hydrolysis of dimyristoyl cephalin by phospholipase B. System contained 2 mg of substrate ultrasonically dispersed in 0.2 ml of water, 0.2 ml of 1M acetate buffer (pH 4.0) and 0.6 ml of enzyme preparation (0.6 mg of protein).

Fig. 31. Phospholipase B hydrolysis of dimyristoyl lecithin by phospholipase B. Enzyme system contained 2 mg of substrate ultrasonically dispersed in 0.2 ml of water, 0.2 ml of 1M acetate buffer (pH 4.0) and 0.6 ml of enzyme preparation (0.6 mg of protein). $\blacktriangle$ indicates not ultrasonically dispersed.

Before studying the action of the enzyme on lysolecithin, it was desirable to determine whether the substrate partitioned into the aqueous methanol phase as well as into the chloroform phase in the assay procedure. The distribution of lysolecithin substrates between the two solvent systems is shown in Fig. 32. Only a minor proportion of each of the lysolecithins partitioned into the aqueous methanol. For comparison, egg hydrolecithin and dimyristoyl cephalin were also partitioned between the two solvents, and each phase was tested chromatographically. The two diacyl phosphatides were contaminated with small amounts of the corresponding lysophosphatides as seen from the chromatogram of the chloroform phase, but no lyso compounds were detected in the methanolic phase. In studies with the lysophosphatides, the correction for the zero-time value was high due to the partitioning of some substrate into the methanolic phase.

The monoacyl ester linkage of monopalmitoyl lecithin showed a high initial rate of hydrolysis without prior ultrasonication. When this substrate was ultrasonified only a slight increase in hydrolysis was observed (Fig. 33). With the unsaturated lysolecithin (Fig. 34) hydrolysis was also rapid with or without ultrasonication.

The hydrogenated egg lecithin (Fig. 35) appeared to be about half hydrolyzed by the enzymic preparation, as judged by the loss of ester groups. This result might

Fig. 32. Partitioning of phosphatide substrates (S) between chloroform and methanol phases as shown by thin-layer chromatography. Substrates tested: monopalmitoyl lecithin (MPL), monopalmitoleyl lecithin (U-MPL), hydrolecithin and cephalin. Solvent system: chloroform-methanol-water (65:25:4, v/v/v), molybdenum stain.

Fig. 33. Hydrolysis of ultrasonicated and non-sonicated monopalmitoyl lecithin by phospholipase B. Enzyme system contained 2 mg of substrate in 0.2 ml of water, 0.2 ml of 1M acetate buffer (pH 4.0) and 0.6 ml of enzyme preparation (0.6 mg of protein).

Fig. 34. Hydrolysis of monopalmitoleyl lecithin by phospholipase B. Enzyme system contained 0.2 mg of substrate in 0.2 ml of water, 0.2 ml of 1M acetate buffer (pH 4.0) and 0.6 ml of enzyme preparation (0.6 mg of protein). Curve shown is for both sonified and unsonified substrate.

Fig. 35. Hydrolysis of egg hydrolecithin by phospholipase B. Enzyme system contained 2 mg of substrate in 0.2 ml of water, 0.2 ml of 1M acetate buffer (pH 4.0) and 0.6 ml of enzyme preparation (0.6 mg of protein).

have indicated a phospholipase A action to yield lysolecithin and fatty acid. However, since an almost corresponding amount of water-soluble phosphate was formed, the enzyme showed phospholipase B activity towards this substrate. The rate of hydrolysis observed was probably high due to the presence of some lysolecithin in the hydrolecithin.

A comparison of the rates of hydrolysis of egg lysolecithin (Pierce Chem. Co.) and the purified egg lecithin is shown in Fig. 36. In the first 5 minutes of enzymic reaction, the rate of hydrolysis of lysolecithin was 36 times faster than that of lecithin.

Rat lecithins

Lecithin from livers of rats fed 20% corn oil was more readily attacked by phospholipase B than that from rats fed little or no fat (Table 13). The rate of hydrolysis appeared to be strongly influenced by the content of polyunsaturated acids, and particularly linoleic acid, in the substrate.

i. Inhibitors and activators

The activity of P. notatum phospholipase B was markedly inhibited by glutathione and cysteine, both of which contain a free sulfhydryl group and are disulfide reducing agents (Table 14). Glutathione was a strong inhibitor at concentrations of 0.5 to 10 mM. Thioglycolate which also contains -SH was an inhibitor at 2.5 mM

Table 13. Relationship between fatty acid constituents of liver lecithins and the rate of hydrolysis by phospholipase B

Fatty acid ¹	Corn oil in rat diet		
	0%	2%	20%
16:0	36.5	35.3	26.7
16:1	4.4	1.6	0.6
18:0	27.7	30.6	33.2
18:1	18.9	13.3	8.6
18:2	1.4	6.1	12.8
20:4	10.1	12.6	16.5
Class of fatty acids			
Saturates	64.2	65.9	59.9
Monounsaturates	23.3	14.9	9.2
Polyunsaturates	11.5	18.7	29.3
% hydrolysis in 90 min. ²	57	45	94

¹ Number of carbon atoms: number of double bonds. Minor quantities of acids tentatively identified as 17:0 and 17:1 were also found.

² Reaction mixture contained 1.0 to 1.4 μ moles of substrate, each obtained from 4 rat livers, 0.2 ml of 1M acetate buffer (pH 4.0), 0.6 ml of 2-hour dialyzed enzyme preparation and water to a total volume of 1.0 ml; mixtures were incubated for 90 minutes at 37°C.

Fig. 36. Comparison of rates of hydrolysis of lysolecithin and lecithin by phospholipase B. Lysolecithin (0.2 mg) was dissolved in 0.2 ml of water. Lecithin (0.2 mg) was ultrasonically dispersed in 0.2 ml of water. To each substrate was added 0.2 ml of 1M acetate buffer, 0.2 ml of water and 0.4 ml of enzyme preparation (0.4 mg of protein).

Table 14. Effect of inhibitors or activators on activity of phospholipase B¹

Substance	Conc mM	% of control ²		
		Expt. 1	Expt. 2	Expt. 3
Glutathione	0.5			14.8
	2.5	19.3		12.1
	5.0		10.0	13.0
	10.0	24.4		17.6
Cystine	2.5	82.7		
	10.0	92.9		
Cysteine	2.5	28.2		
	10.0	27.2		
Thioglycollate	2.5		37.3	71.4
	10.0	—	101.5	101.5
Iodoacetate	2.5	100.0		
	10.0	111.4		
KCN	2.5	100.7		
	10.0	109.7		
NaF	2.5	76.4		
	10.0	58.4		
K ₃ Fe(CN) ₆	2.5			133.2
	5.0		131.4	132.0
	10.0			137.8

¹ System contained 0.2 ml of ultrasonicated suspension of egg lecithin (13.5 μ mole/ml), 0.2 ml of 1N acetate buffer, 0.4 ml of enzymic preparation dialyzed 2 hours, additive and water to give total volume of 1 ml.

² P release per/hr in control of Expt. 1 was 60.6 μ g, of Expt. 2, 60.9 μ g, and of Expt. 3, 62.1 μ g.

but not at 10 mM. Little effect on enzymic activity was obtained with cystine which has a disulfide group or with iodoacetate which combines with sulfhydryl groups. Cyanide appeared to have a negligible effect, and fluoride exerted moderate inhibition. With the mild oxidizing agent, potassium ferricyanide, the enzymic activity was augmented. As shown in Fig. 37, this oxidizing agent completely reversed the inhibition caused by glutathione, and also increased the enzymic activity beyond the control values.

To eliminate the possibility that the ferric ion rather than the oxidizing capability of ferricyanide was responsible for the enhanced enzymic activity, the metal ions listed in Table 15 were tested in the enzyme system. At least partial inhibition was obtained with most cations tested. The ferric and ferrous ions with or without ethylenediaminetetraacetate completely inhibited the action of the enzyme, leaving no doubt that they were not responsible for the activation associated with ferricyanide.

Enzymic preparations which had been partially inactivated by dialysis were unaffected by addition of glycerol but were reactivated to the extent of 52 - 58% of the control by potassium ferricyanide (Table 16). Addition of the concentrated diffusate also partially restored lost enzymic activity.

Fig. 37. Effect of glutathione (GSH) and potassium ferricyanide (2.5μ moles of each) on the deacylation of egg lecithin by phospholipase B of *P. notatum*.

Table 15. Effect of metal ions on activity of phospholipase B

(Concentration of metallic salt or of EDTA was 10 mM)	
<u>Metal ion</u>	<u>% of control</u> ¹
Ca ²⁺	74.1
Mg ²⁺	72.4
Zn ²⁺	70.8
Fe ²⁺	0
Fe ²⁺ + EDTA ²	0
Fe ³⁺	0
Fe ³⁺ + EDTA	0
Mn ²⁺	97.1
Mn ²⁺ + EDTA	85.0
Cu ²⁺	79.9
Cu ²⁺ + EDTA	69.3
Fe(CN) ₆	122.6
Fe(CN) ₆ + EDTA	105.5

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¹ In the control, 43.4 μ g P were released by 0.4 ml of enzyme preparation (0.4 mg of protein) in 60 minutes.

² Ethylenediaminetetraacetate.

Table 16. Effect of various substances on enzyme partially inactivated by dialysis

Substance added	Concentration mM	% of control ¹	
		Expt. 1	Expt. 2
None		62.2	43.0
Glycerol	10.0	62.2	
K ₃ Fe(CN) ₆	10.0	81.1	65.1
Diffusate ²		81.8	76.0

¹ Control enzyme which had been dialyzed for 2 hours hydrolyzed 60.9 μ g of phosphorus/hr in Expt. 1. Partially inactivated enzyme preparations were dialyzed for 44 hours.

² 0.2 ml of concentrated diffusate obtained during 24 to 44 hours of dialyzing 1 ml of glycerinated enzyme preparation against 500 ml of water.

j. Partial fractionation

After centrifugation of the P. notatum enzymic preparation at 15,000 X g for 5 minutes (Table 17), the activity in the supernatant and precipitate together accounted for about half of the activity in the unfractionated preparation. In the assay system with purified substrates, it was therefore advantageous to use the

enzymic preparation with further treatment after a 2-hour dialysis against water.

Table 17. Effect of partial fractionation of phospholipase B preparation on rate of hydrolysis

Enzyme	Release of water-soluble phosphate/60 min.	
	μ moles	% of control
Supernatant	0.43	21.1
Precipitate	0.68	33.5
Unfractionated preparation	2.04	100.0

3. Results with rat liver enzyme

a. Acetone powder

An acetone powder of rat liver catalyzed the release of water-soluble phosphate from lecithin at different pH's as shown in Table 18. The optimum pH appeared to be about 6.4. The activity of the rat liver enzyme was very low relative to that of the P. notatum enzyme.

Table 18. Lecithinase activity of rat liver acetone powder¹

<u>pH</u>	<u>% P released/60 min.</u>
3.6	3.7
4.0	1.9
4.4	1.2
5.0	1.9
5.6	10.8
6.0	12.7
6.4	19.4
6.8	16.7
7.2	17.3

¹ System contained 0.2 ml of sonicated lecithin suspension (10.6 μ mole/ml), 0.2 ml of 1M buffer (acetate for pH's up to 5.0; maleate for pH's 5.6 to 6.6; borate for pH 7.2) 0.4 ml of enzyme preparation (0.4 mg protein and 0.2 ml of water).

b. Cellular fractionation

The total homogenate showed considerable activity towards lysolecithin but relatively little towards lecithin (Table 19). The most effective cellular sub-fraction in hydrolyzing both substrates appeared to be the supernatant fluid, but some activity was also found in the particulate fractions.

Table 19. Lecithinase and lysolecithinase activity of fresh liver homogenate and subcellular fractions

Fraction	% P released/2 hour	
	lyso. ¹	Lec. ²
Total homogenate	40.0	5.8
Mitochondria	5.7	0.5
Microsomes	9.8	0
Supernatant fluid	18.4	5.0

¹ lysolecithin, Pierce Chem. Co.

² egg lecithin

System contained 0.2 ml of 1M Tris-maleate buffer (pH 6.4); 0.2 ml of substrate, 0.4 ml of liver fraction in 0.25M sucrose and 0.2 ml of water.

In another experiment, subcellular fractions including lysosomes were studied (Table 20).

Table 20. Lecithinase and lysolecithinase activity of rat liver subcellular fractions including lysosomes

Fraction	% ester hydrolyzed/2 hr ^x	
	Lyso.	Lec.
Total homogenate	-4.8	1.3
Total homogenate minus Mt ¹	-2.6	9.5
Total homogenate minus Mt ²	-4.2	3.2
Mt ¹	1.9	3.1
Mt ²	8.0	5.5
Lysosomes	10.3	8.0

Mt¹ = heavy mitochondria; Mt² = light mitochondria

^x Phosphate buffer was used. Therefore esters hydrolyzed instead of phosphate released was criterion of activity.

Ester groups did not appear to be hydrolyzed by the total homogenate, but instead, lysolecithin seemed to acquire ester groups. The light mitochondria and the lysosomes most readily catalyzed the hydrolysis of lysolecithin and lecithin, but the activities observed were extremely weak compared to those of P. notatum.

C. DISCUSSION

It is evident from the above experiments that the phospholipase B preparation from P. notatum readily deacylated lecithin to form free fatty acids and glycerylphosphorylcholine, provided the substrate was ultrasonically dispersed. This finding is in contrast to the results of Dawson (1957 a, 1958 a, 1958 c, 1964) and Bangham and Dawson (1959) who were unable to demonstrate enzymic activity towards lecithin in the absence of an activator. Their observation that cardiolipin promotes the enzymic hydrolysis of unsonicated lecithin was confirmed, but this activating substance was found to have little or no effect on the breakdown of sonicated lecithin. Bangham and Dawson (1959) showed that the lipid activator imparted a negative charge to the substrate particles, and this they believed was responsible for the activation. In the present work, however, no correlation was found between the electrostatic charge of the sonicated substrate particles and enzymic activity.

The effect of cardiolipin could equally well be interpreted as being due to penetration and dispersion of lecithin particles by an anionic amphipath. The lack of activation by cationic amphipaths (Bangham and Dawson, 1960) might result from inability to penetrate the lecithin film, or, as found by Kates (1957) for phospholipase D, to inactivation of the enzyme by cationic amphipaths.

Enzymic activity was positively correlated with the % transmittance of the substrate suspension and appeared to be unrelated to the charge on the substrate. It is concluded therefore that the degree of substrate dispersion is the important factor controlling the rate of hydrolysis of phosphatides.

The problem of obtaining a suitable assay system for enzymes attacking lipids in an aqueous medium has been solved in various ways for other lipolytic enzymes. In the measurement of lipase activity, an emulsifying agent was usually added to the assay medium (Ammon and Joarna, 1950). Hanahan (1952 b) discovered that phospholipase A degraded egg lecithin in ethyl ether solution. This solvent was also found to be an activator for phospholipase D, hydrolyzing glycerylphosphatides to phosphatidic acid and nitrogenous base (Kates, 1953). In an assay system for phospholipase B, however, ethyl ether had no activating effect (Dawson, 1958 c).

After Saunders, Perrin and Gammack (1962) demonstrated that stable lecithin sols could be prepared ultrasonically, Dawson (1963) found that such suspensions were rapidly hydrolyzed by phospholipase A. In the present studies, ultrasonication of the substrate was found to be effective in dispersing lecithin for phospholipase B attack in the absence of negatively charged activators. The affinity of the enzyme for the ultra-

sonically dispersed egg lecithin is given by the expression, $\frac{1}{K_m} = \frac{1}{6.3 \times 10^{-3}M} = 160M^{-1}$, and was of the same order as that of other phospholipases in ether-activated systems (Kates, 1960).

Substrates other than egg lecithin also appeared to be accessible to enzymic attack according to their ease of dispersion in an aqueous system. Saturated lecithins were found to be poorly dispersed, even with ultrasonication, and their deacylation was slow. Dimyristoyl lecithin, in particular, was visibly poorly dispersed, and its rate of deacylation was negligible.

The release of proportionately more linoleic acid than other fatty acids during the early stages of the reaction with egg lecithin (Table 12), and the relatively high rate of hydrolysis of rat liver lecithin containing an appreciable quantity of linoleic acid (Table 13) might indicate that the enzyme has a greater affinity for the more unsaturated substrate, or that the extent of dispersion of the substrate is related to its degree of unsaturation. In view of the apparent dependence of phospholipase B activity on the degree of substrate dispersion, the latter factor is probably more important.

The hydrolysis of both the saturated and unsaturated lysolecithins in the present experiments was rapid. It seems likely that the previous findings of a deacylation of lysolecithin but not lecithin (Fairbairn, 1948;

Uziel and Hanahan, 1956) by the P. notatum enzyme may be attributed to the fact that lysolecithin is soluble in water whereas lecithin is only poorly dispersed. By using a system without activators, it was possible for the first time to compare the rate of hydrolysis of lecithin with that of lysolecithin. It was found that lysolecithin was much more rapidly hydrolyzed than lecithin, although even in identical systems the relative solubility of these two substrates may have influenced this result.

Maximal lecithinase activity was observed at approximately pH 4.0, in agreement with the value reported by Fairbairn (1948) for 'lysophospholipase' activity and by Dawson (1958 c) for the phospholipase B activity in P. notatum.

The loss of enzymic activity by prolonged dialysis of the glycerinated preparation may have resulted from a change in the protein tertiary structure or from the loss of an unidentified cofactor such as a divalent metal ion. None of the metal ions tested here showed an activating influence on the enzyme, but were instead somewhat inhibitory. These ions, therefore, could not have been cofactors lost during dialysis, but the possibility that a metal ion not tested might be involved must not be overlooked. The loss of a sulfhydryl group from the active site during dialysis may be excluded by the fact that a

-SH binding reagent, such as iodoacetate, did not inhibit the enzyme.

Ottolenghi (1965) found that phospholipase C, a zinc metallo-enzyme, was inactivated by extensive dialysis, but was reversibly activated by thiol-containing compounds. Although phospholipase B has been shown here to be similarly affected by dialysis, sulfhydryl compounds caused inhibition which was reversed by ferricyanide. Since both ferric and ferrous ions caused complete inhibition, the activating effect of ferricyanide is therefore presumed to be associated with its oxidative capacity.

An inhibition by sulfhydryl agents which is reversed by mild oxidation strongly suggests that disulfide groups are required for enzymic activity. It is possible that the loss of activity during dialysis may be partly due to the reduction of -S-S- linkages in the protein. Enzymes which require disulfide bonds for activity include ribonuclease (Resnick, Carter and Kalnitsky, 1959) and cytochrome oxidase (Cooperstein, 1963) where it is known that the disulfide bonds supply a specific tertiary conformation to the protein which is essential for the maintenance of enzymic activity. It is conceivable that the disulfide bonds in phospholipase B may perform the same function and that sulfhydryl agents would inhibit by disrupting the tertiary structure of the protein. Other possible causes for apparent inhibition might be an interaction of the

inhibitor with an unknown cofactor in the crude enzymic preparation, or a specific reaction with the protein molecule either at the allosteric or active site. If a disulfide bond occurs at the active site, it is conceivable that both acyl groups in the substrate may be added simultaneously across the -S-S- bond to form two acylthiol ester groups which are then hydrolyzed to free fatty acids and the reduced enzyme. This reaction would involve an accompanying oxidation-reduction system to supply electrons for cleaving the disulfide bond and to reoxidize the reduced enzyme. Such a mechanism would explain the increased activity with ferricyanide and the absence of lysolecithin as an intermediate. Whether the thiol-acylated enzyme is an intermediate remains to be proven, but information on this point might be obtained in future work by the use of substrates containing ^{14}C -labelled fatty acids.

The problem of whether one or two enzymes are involved in the deacylation of lecithin has not been resolved. Hanahan (1957) favoured a mechanism involving a different enzyme for each ester linkage, that is, a phospholipase A for the β -position and a lysolecithinase for the α' -position. Phospholipase A has a pH optimum of about 7 whereas the deacylation of lecithin by P. notatum is maximal at pH 4. Furthermore, ethyl ether (Hanahan, 1952) and calcium ion (Long and Penny, 1957) activate

phospholipase A but are without effect on phospholipase B. It seems unlikely, therefore, that the action of P. notatum phospholipase B can be attributed to the combined effects of phospholipase A and lysolecithinase. The fact that lysolecithin was not detected in the very early stages of reaction (1 to 4 minutes) cannot, however, be used as an argument for or against Hanahan's proposal, since the rapid hydrolysis of lysolecithin observed here would preclude its detection even if it had been formed. Resolution of this problem will depend on whether fractionation of the crude preparation for P. notatum yields one or more protein fractions with phospholipase activity.

The relatively weak enzymic activity found in liver tissue may represent the net difference between acylating enzymes (Lands, 1960; Lands and Hart, 1963, 1964, 1965) and the deacylating enzymes. Since a negative value was obtained for the deacylation of lysolecithin with liver homogenate (Table 18), the acylating activity must have been greater than the deacylating activity.

The cellular distribution of liver phospholipase was not clearly defined, and appeared to be predominantly in the supernatant fluid in one experiment (Table 19) and in the light mitochondria and lysosomes in another (Table 20). It seemed possible that phospholipase B might be found in the lysosomes where the enzymes are of a hydro-

lytic nature (De Duve, Pressman, Gianetto, Wattiaux and Appelmans, 1955), but convincing evidence was not forthcoming. The overall low phospholipase activity and the lack of a clear-cut cellular distribution may have been due to incomplete separation of fractions and to particle disruption with resultant release of enzyme protein. Further fractionation studies must be done to elucidate the distribution of phospholipase B in liver tissue.

It may be concluded from the present study that phospholipase B activity in the rat liver does not appear to be of major physiological significance. It seems that this enzyme is more active in pancreatic and intestinal tissues which have a digestive function to perform.

III. CONCLUSIONS

From the observations recorded in Part I of this thesis, the following conclusions were drawn concerning the deposition of linoleic acid in the rat:

1. The linoleate content of the major liver phosphatides, phosphatidyl choline and phosphatidyl ethanolamine, was influenced by the level of corn oil in the diet, but only to a limited extent. These changes are presumed to represent structural alterations in membrane components.
2. With a high level of dietary corn oil (20%), the linoleate content of phosphatidyl choline increased by about 25% within three days and phosphatidyl ethanolamine by about 50% within six days. No further increases in linoleate concentrations during the remaining feeding period were observed.
3. The proportion of linoleic acid in the neutral lipids from liver tissue doubled within the first three days, and thereafter showed no further increase. These lipids apparently exchanged more readily with dietary linoleic acid than did the phosphatides.
4. The linoleate concentration in total liver fatty acids reached a maximum within twelve days whereas that in the carcass showed a continuous increase throughout the experimental period.

Investigations on the mode of action of phospholipase B from Penicillium notatum reported in Part II of this thesis led to the following findings:

1. The hydrolysis of lecithin by phospholipase B was achieved by ultrasonically dispersing the substrate, and without adding any activating substance.
2. The rate of hydrolysis of lecithin was not related to the electrostatic charge on the substrate, as proposed by other workers.
3. The apparent K_m for the ultrasonically dispersed lecithin was 6.3 mM.
4. The rate of hydrolysis of rat liver lecithin was related to its content of polyunsaturated acids.
5. Lysolecithin was much more rapidly hydrolyzed than lecithin in an identical system.
6. Reagents combining with sulfhydryl groups did not affect phospholipase B activity, showing that -SH groups are not involved at the active centre of the enzyme.
7. Phospholipase B activity was inhibited by glutathione, cysteine, iron, or prolonged dialysis. Since ferricyanide reversed the inhibition caused by glutathione, it appears that the enzyme requires -S-S- linkages for activity.
8. Phospholipase B of liver tissue seems to be of minor physiological importance in the rat.

IV. THESIS PUBLICATIONS

The publications arising from the research work reported in this thesis include the following:

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