insulins would be separable with great difficulty, if at all, in the system used. This is an interesting observation in trying to assess from a structural standpoint the upper limit of separability. All the differences are small and are in neutral side chains which afford no changes in charge. However, covering or releasing one carboxyl or amino group in a weight of 6,000 causes sufficient differences in physical properties for resolution to be achieved. This has been shown in the separation of the A and B components. These differ only in that B has one more free carboxyl group than A. This conclusion is also supported by the experience with the reaction with fluorodinitrobenzene where covering one free amino group with the dinitrophenyl residue increases the K fivefold. Further unpublished data indicate that the methylation of one carboxyl group in 6,000 reduces the K by a factor of two. The separation of molecules of the size of insulin where there are only very small variations in the neutral amino acid residues, with resulting small changes in the physical properties, would be expected to be very difficult. In the future systems may be found with greater resolving power than that of the 1% aqueous dichloroacetic acid/2-butanol system used for all the work with insulin. However, it should be pointed out that in spite of the possible failure of this system in separating such closely related compounds, considerable purification has been achieved by countercurrent distribution. Several minor impurities have been removed and the A and B components which had not been detected by electrophoresis, the ultracentrifuge, the solubility method, or partition chromatography have been clearly demonstrated and separated.

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[CONTRIBUTION FROM THE BANTING AND BEST DEPARTMENT OF MEDICAL RESEARCH, UNIVERSITY OF TORONTO]

Phosphatide Analogs. The Synthesis of Glycollecithins and Bis-(glycol)-phosphatidic Acids[1]

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A new class of phosphatides and a generally applicable method for the synthesis of its members is described. The new phosphatides, which can be regarded as analogs of both the lecithins and lysolecithins, and which differ from these substances only in that they contain glycol instead of glycerol, have been assigned the generic name "glycollecithin."[2] The glycollecithins possess neither positional- nor stereoisomers. The synthesis of two representative members of the glycollecithins, namely, stearoyl- and palmitoylglycollecithin, is described and their infrared spectra and other physical data are reported. The stearoyl- and palmitoylglycollecithins were found to be highly soluble in water and to possess a strong hemolytic activity. The new phosphatides because of their structural similarity to the glycerollecithins should prove of interest as substrates upon which to conduct systematic zymological and other biochemical and physiological studies. A general procedure is described for the synthesis of bis-(glycol)-phosphatidic acids which, in the form of their phenyl esters, are obtained also as by-products in the synthesis of the glycollecithins.

The naturally occurring phosphatides have been identified as esters of either glycerol, sphingosine or inositol. The isolation of propylene glycol phosphate from sea urchin eggs[3] and from cattle brain[4] seems to indicate the presence of a fourth polyhydric alcohol in phosphatides, namely, propylene glycol. Others, without doubt, will be found in time. Theoretically it is possible to visualize an almost unlimited number of phosphatide analogs by varying the polyhydric component. Although no experimental evidence has been reported suggesting the natural existence of ethylene glycol-containing phosphatides, it occurred to the author that phosphatides of this type, because of their structural simplicity, would be of interest to both the chemist and biochemist. The synthesis of glycol analogs of lecithin, cephalin and phosphatidyl serine is being undertaken in this Laboratory. The present communication, the first in this series, describes the preparation of glycollecithins[5] and of bis-(glycol)-phosphatidic acids.

The synthesis of the glycollecithins follows in general the procedure developed in this Laboratory for the synthesis of the α-lecithins[6] except that monoacyl glycols are used as starting materials. Since the naturally occurring glycerollecithins contain mainly fatty acids with 16 and 18 carbon atoms, preference has been given to the synthesis of palmitoyl and stearoyl glycollecithin. The required starting materials, namely, monopalmitoyl and monostearoyl glycol until now have been obtained by heating mixtures of either glycol and fatty acid,[7,8] or of glycol, fatty acid, camphor-β-sulfonic acid and phenol[9] to 180° for several hours and separating the monoacyl and diacyl glycols by fractional crystallization. Both procedures, however, have the disadvantage of yielding many

(1) An account of this work was included in a lecture presented before the American Chemical Society at its 75th Anniversary meeting, New York, N. Y., September, 1951.

(2) The prefix "glycol" should not be confused with the prefix "glyco" in glycolipids. The latter prefix indicates the presence of a carbohydrate in the lipid molecule.

(3) O. Lindberg, *Ark. Kemi. Mineral. o. Geol.*, **A16**, No. 15, 1 (1943).

(4) O. Lindberg, *ibid.*, **A23**, 1 (1946).

(5) In naming the new phosphatides the names of their glycerol analogs are retained but modified by the prefix glycol.

(6) E. Baer and M. Kates, THIS JOURNAL, **72**, 942 (1950).

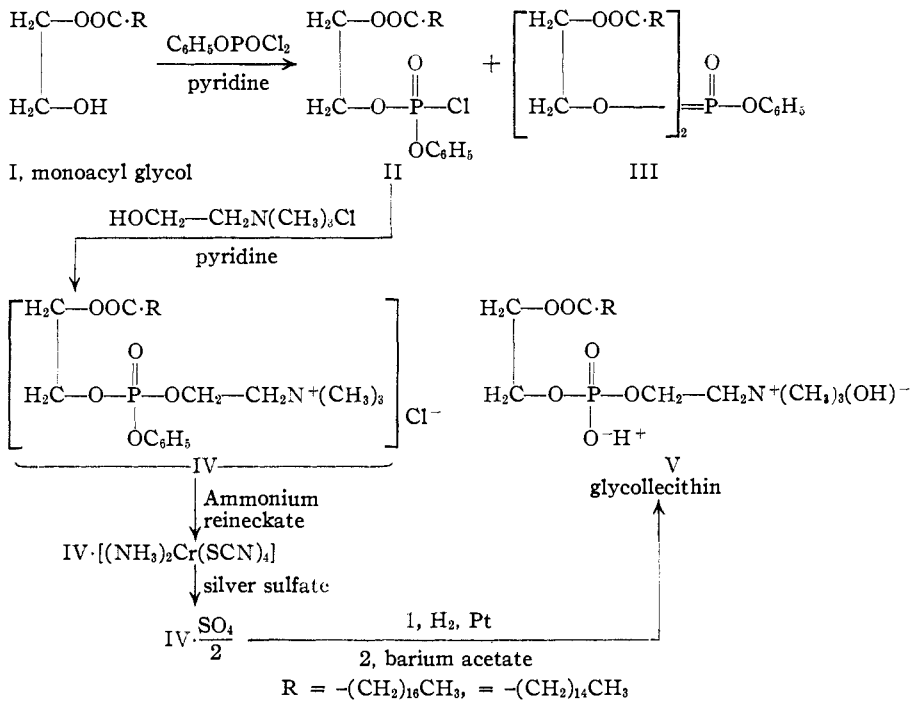
(7) R. F. Ruttan and J. R. Roebuck, *Trans. Roy. Soc. Can., III*, [3] **9**, 1 (1915).

(8) I. Bellucci, *Chem. Z.*, **35**, 669 (1911).

(9) T. P. Hilditch and J. G. Rigg, *J. Chem. Soc.*, 1774 (1935).

times more diester than monoester. Monostearoyl glycol has been obtained also by tritylation of glycol, acylation of the monotrityl glycol and removal of the trityl group by catalytic hydrogenolysis.[10] This method is tedious and not always results in a pure product (see note 25). Pure monopalmitoyl and monostearoyl glycols can be obtained much more directly and in good yields by the acylation of ethylene glycol with palmitoyl or stearoyl chloride and pyridine, provided that the reaction is carried out in a suitable mixture of solvents at low temperature and in the presence of a large excess of glycol.

The phosphorylation of the monoacyl glycols and the esterification of their phosphorylation products II with choline chloride was carried out in the beginning by following closely our procedure for the synthesis of the glycerollecithin phenyl esters[6]; that is, the chloroform solution of the monoacyl glycol was added to a chloroform solution of the monophenylphosphoryl dichloride and pyridine (step I), and the reaction product II was esterified with choline chloride (step II). This procedure, however, yielded mainly the bis-(glycol)-phosphatidic acid phenyl esters III and only a few per cent. of the desired glycollecithin phenyl esters IV. After testing a number of variations of the first phosphorylation step it was found that if the pyridine was added to a mixture of the monoacyl glycol and phenylphosphoryl dichloride the glycollecithin phenyl esters could be isolated as reineckates in yields of 23.5% for the palmitoyl and 29.3% for the stearoyl compound. Although these yields are not too satisfactory and are somewhat below those for the corresponding intermediates in the synthesis of the α-lecithins, the fact that the monoacyl glycols are far more readily accessible than the enantiomeric α,β-diglycerides makes the preparation of the glycollecithins comparatively easy. The synthesis, briefly, is as follows (Reaction Scheme I): Monopalmitoyl or monostearoyl glycol I, dissolved in a mixture of chloroform and petroleum ether, is phosphorylated by means of phenylphosphoryl dichloride and pyridine. The phosphorylation product, a mixture of the bis-(glycol)-phosphatidic acid phenyl ester III and the acylglycolphenylphosphoryl chloride II, after being freed from petroleum ether and redissolved in anhydrous chloroform, is treated with choline chloride in the presence of a large excess of pyridine, and the glycollecithin phenyl ester IV is isolated in the form of its reinecke salt. Conversion of the reineckate of IV to the sulfate of IV by means of silver sulfate, removal of the protective phenyl group by catalytic hydrogenolysis, and replacement of the sulfate ions with hydroxyl ions by means of barium acetate finally yields the glycollecithins V. The palmitoyl glycollecithin (PGL) and stearoyl glycollecithin (SGL) were obtained in over-all yields of 21 and 27%, respectively. On recrystallization from chloroform and ether they formed narrow prisms measuring sometimes several millimeters in length. The palmitoyl- and stearoyl-glycollecithins possess sintering–melting patterns similar to those of the corresponding glycerollecithins. On heating they sinter at approximately 80° and with increasing temperature gradually form translucent masses which suddenly coalesce with the formation of a meniscus at 242–243° (PGL) and 239–240° (SGL), respectively.



REACTION SCHEME I

The palmitoyl- and stearoylglycollecithins like the corresponding glycerollecithins are readily soluble in chloroform, methanol or ethanol and insoluble in acetone or ether. Unlike their glycerol analogs, however, they are highly soluble in water. The isolation of water-soluble phosphatides, primarily from vegetable sources, has been reported by various investigators.[11]

These substances appear to be complex *glycerol*-phosphatides with lecithins or cephalins as the nuclei, and seem to owe their solubility in water to

(10) P. E. Verkade, F. D. Tollenaar and T. A. P. Posthumus, *Rec. trav. chim.*, **61**, 373 (1942).

(11) B. Hansteen-Cranner, *Planta*, **2**, 438 (1926); V. Grafe, *Biochem. Z.*, **159**, 444 (1925); *Naturwissenschaften*, **15**, 513 (1927); V. Grafe and V. Horvat, *Biochem. Z.*, **159**, 449 (1925); V. Grafe and H. Magistris, *ibid.*, **162**, 366 (1925); H. Magistris and P. Schäffer, *ibid.*, **214**, 401 (1929); Gutstein, *ibid.*, **207**, 177 (1929); M. Eisler and Z. Gulaesy, *Zentr. Bakt. Parasitenk. Abt. I*, **117**, 500 (1930); B. Bleyer and W. Diemair, *Biochem. Z.*, **238**, 197 (1931).

the presence of hydrophilic constituents of the complex. The full identity of these controversial substances, however, has not yet been established.

The glycollecithins possess a strongly acidic and a strongly basic group and thus should be neutral substances. Indeed, carbon dioxide-free aqueous solutions of the glycollecithins were found to have *p*H-values of 6.9–7.0.[12] It is of interest to note that these values closely approach the value of 6.7 which has been reported by Chain and Kemp[13] as the isoelectric point of lecithin and was obtained by measuring the electrophoretic mobility of lecithin with changing *p*H.

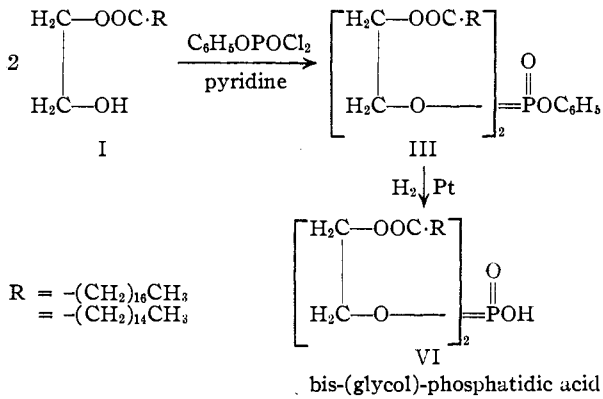
On combustion the crystalline glycollecithins gave carbon values which agree closely with those required by theory for the open structure shown in formula V and thus obviously are not inner-anhydrides or endo-salts. In more recent years it has become customary to write the lecithin structure in the form of a zwitterion, omitting the hydroxyl and hydrogen ions. It is important, however, to realize that such a structural formula does not agree with the analytical values obtained for either the crystalline glycerol- or glycollecithins. The structure of the glycerol- and glycollecithins in the *solid state* therefore should be expressed by formulas showing both the hydroxyl and hydrogen ions.[14]

The glycollecithins resemble the glycerollecithins in their ability to form alcohol-insoluble cadmium chloride addition compounds containing two moles of lecithin per three moles of cadmium chloride, but differ in that they form water-insoluble reineckates. The composition of these reineckates, however, is unusual since the ratio of glycollecithin to reineckate is 2:1.

The glycerollecithins, as is well known, are excellent emulsifiers. When used, however, in the preparation of fat emulsions for parenteral application they have the serious shortcoming of forming in time strongly hemolytic degradation products (lysolecithins). It was hoped that the glycollecithins which should give non-toxic degradation products might be used as substitutes for the glycerollecithins. Tests with stearoyl and palmitoyl glycollecithin revealed, however, that they possess a strong hemolytic activity of their own, a property which might have been expected considering their close structural similarity to the lysolecithins.

A widely used antigen in the serodiagnosis of syphilis consists of beef-heart lecithin, cardiolipin and cholesterol. The first two antigen components are prepared by isolation from natural sources and are difficult to obtain with a constant degree of purity. This necessitates the determination of the serological activity of each batch. Investigations by Rosenberg,[15] Kline,[16] and Allen and Tonks[17] have shown that the beef-heart lecithin component can be replaced by synthetic lecithins (L-α-dimyristoyl- and L-α-dipalmitoyllecithin).[6] The synthetic lecithins as pure compounds have the advantage of possessing constant and predictable serological activities. From a theoretical as well as a practical point of view it was of interest to ascertain whether the glycollecithins possess similar serological activities. This investigation is being carried out in the Department of National Health and Welfare (Ottawa) under the direction of Dr. R. H. Allen and the results will be reported independently elsewhere.

Attempts are also being made to find synthetic substitutes for cardiolipin. An investigation by Allen and Tonks of the α-bisphosphatidic acids synthesized in this Laboratory[18] has shown that the tetramyristoyl bis-(L-α-glyceryl)-phosphoric acid in the presence of beef-heart lecithin indeed possesses a cardiolipin-like activity.[17] It was therefore of interest to determine whether the bis-(glycol)-phosphatidic acids which in the form of their phenyl esters are formed in considerable amounts as by-products in the synthesis of the glycollecithins, would also possess a cardiolipin-like activity and if so to what extent. To facilitate this investigation a procedure was developed by means of which the bis-(glycol)-phosphatidic acids are obtainable as the main products. The synthesis is as follows (Reaction Scheme II). One mole of phenylphosphoryl dichloride is treated with two moles of the monoacyl glycol in the presence of two moles of pyridine. The phenyl ester III which is obtained in an excellent yield, on being freed of its protective phenyl group by catalytic hydrogenolysis, yields the bis-(glycol)-phosphatidic acid VI. Both the distearoyl and dipalmitoyl bis-(glycol)-phosphatidic acids were prepared and were obtained as white, crystalline substances with sharp melting points.



REACTION SCHEME II

Experimental

Monoacyl Glycols.—In a 2-l. three-necked flask equipped with an oil-sealed, motor-driven stirrer and two dropping funnels, one of which was provided below the stopcock with an air-outlet protected by a calcium chloride tube, were placed 124.0 g. (2.0 moles) of anhydrous ethylene glycol, 31 ml. (0.4 mole) of anhydrous dimethylformamide[19] and 320

(12) The measurements were carried out in a Beckman *p*H meter (Model 6) keeping the electrode chamber filled with nitrogen. If air was permitted to enter the chamber, the *p*H gradually dropped to 6.4. The original values could be restored by bubbling nitrogen through the solution.

(13) E. Chain and I. Kemp, *Biochem. J.*, **28**, 2052 (1934).

(14) See also E. Baer, THIS JOURNAL, **75**, 621 (1953).

(15) A. A. Rosenberg, *J. Venereal Disease, Inform.*, **30**, 194 (1949).

(16) B. S. Kline, *Am. J. Syphilis, Gonorrhea, and Venereal Diseases*, **34**, 460 (1951).

(17) D. B. Tonks and R. H. Allen, *Science*, **118**, 55 (1953).

(18) E. Baer, *J. Biol. Chem.*, **198**, 853 (1952).

(19) The dimethylformamide had been dried with anhydrous calcium sulfate (Drierite).

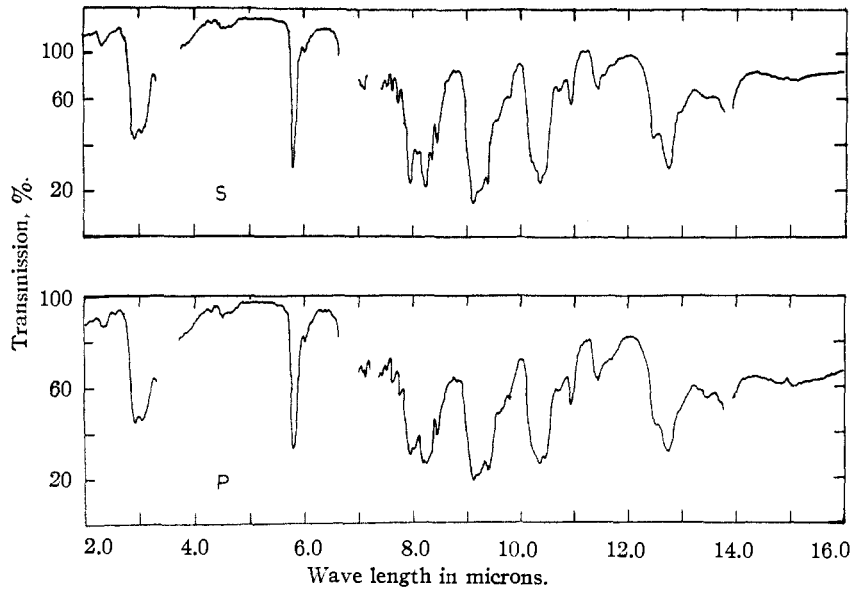


Fig. 1.—Baird Associates Model B double-beam spectrophotometer. The oil mulls of stearoyl glycollecithin (S) and palmitoyl glycollecithin (P) were squeezed to thin films between salt plates without spacers.

ml. of anhydrous and ethanol-free chloroform.[20] The contents of the flask were cooled to −15° in a bath of chipped ice and sodium chloride, the stirrer was set in motion and the solutions of 17.8 ml. (0.22 mole) of anhydrous pyridine[21] in 80 ml. of chloroform[20] and of 60.6 g. (0.2 mole) of freshly distilled stearoyl chloride or of 55.0 g. (0.2 mole) of palmitoyl chloride in 400 ml. of chloroform,[20] respectively, were added in the course of 15 minutes. The flow of both solutions was adjusted so that equimolar amounts of pyridine and acyl chloride entered the reaction vessel.[22]

After the addition of both solutions the cold-bath was removed and the stirring continued for 45 minutes. The colorless reaction mixture was then permitted to stand until two layers had formed. The layers were separated, and the lower one was washed in succession with 180 ml. of 1 *N* sulfuric acid and two 180-ml. portions of water. The chloroform solution, after freeing from suspended water by filtration, was brought to dryness *in vacuo* at a bath temperature of 30–35°. To the pure white solid, which consisted of a mixture of either mono- and distearoyl glycol (S) or mono- and dipalmitoyl glycol (P), was added 520 ml. (S) or 250 ml. (P) of 99% ethanol and the suspension, after placing in a water-bath at 40° (S) or 30° (P), respectively, was stirred vigorously for a period of 15 minutes. The small amount of undissolved material, consisting mainly of the diester, was centrifuged off and to the decanted solution, after cooling to room temperature, was added 25 ml. (S) or 15 ml. (P) of water. The precipitate, again mainly diester, was removed by centrifugation and to the decanted solution was added with stirring in the course of 10 minutes 240 ml. (S) or 200 ml. (P) of water.[23] After standing for one hour the mixture was separated by centrifugation and the solid material, monostearoyl glycol (MSG) or monopalmitoyl glycol (MPG), was freed of most of its moisture either by spreading on porous clay plates or by pressing between smooth filter paper. The substance was then dried *in vacuo* over phosphorus pentoxide to constant weight.

Monostearoyl Glycol.[24]—The MSG weighing 54.0 g. (82.2% of theory, calcd. for stearoyl chloride) and melting from 58.5–60.0° (with slight sintering at 57.5°) was recrystallized by dissolving in 400 ml. of warm petroleum ether (b.p. 80–100°) and cooling the solution to +10°; recovery 97.7%; over-all yield of MSG 52.8 g. (80.3%); m.p. 59.5–60.5°, reported m.p. 58.5°[7] and 60–61°.[25]

Anal. Calcd. for $C_{20}H_{40}O_3$ (328.5): C, 73.12; H, 12.28; stearic acid, 86.60. Found[26]: C, 73.15, 73.28, 73.16; H, 11.95, 12.27, 12.11; stearic acid, 86.37.

Monopalmitoyl Glycol.—The MPG, weighing 44.3 g. (73.9% of theory, calcd. for palmitoyl chloride) and melting from 51.5–52.5° (with slight sintering at 50.0°), was recrystallized by dissolving in 150 ml. of warm petroleum ether (b.p. 80–100°) and cooling the filtered solution to +10°; recovery 41.5 g. (93.0%); over-all yield of MPG 69.4%; m.p. 52–53°, reported m.p. 51.5°.[7]

Anal. Calcd. for $C_{18}H_{36}O_3$ (300.47): C, 71.94; H, 12.08; palmitic acid, 85.35. Found: C, 72.11; H, 11.91; palmitic acid, 85.46.

Glycollecithins

Glycollecithin Phenyl Esters. (a) Phosphorylation.—In a 500-ml. three-necked flask equipped with an oil-sealed and motor-driven stirrer, calcium chloride tube and dropping funnel were placed 50.0 mmoles of the monoacyl glycol (16.4 g. of the monostearoyl—or 15.0 g. of the monopalmitoyl glycol), 50.0 mmoles (10.5 g.) of freshly fractionated phenylphosphoryl dichloride, 55 ml. of ethanol-free and anhydrous chloroform[20] and 150 ml. of petroleum ether (b.p. 60–80°).[27] The flask was immersed in a water-bath of 20° and a solution of 55.0 mmoles (4.4 g.) of anhydrous pyridine in 75 ml. of petroleum ether (b.p. 60–80°) was added dropwise to the vigorously stirred phosphorylation mixture over a period of three hours. Fifteen minutes after the last of the pyridine had been added the chloroform and petroleum ether were distilled off under reduced pressure with the exclusion of moisture at a bath temperature of 25–30°. To the solution of the residue in 140 ml. of ethanol-free chloroform[20] were added 110 mmoles (8.8 g.) of pyridine and 65.0 mmoles (9.1 g.) of finely powdered and thoroughly dried choline chloride, and the mixture was stirred with the exclusion of moisture until most of the choline chloride had disappeared (3 days).

(b) Isolation of the Glycollecithin Phenyl Esters as Reineckates.—The reaction mixture was filtered and brought to dryness under reduced pressure (raising the bath temperature gradually to 40°). The residue was thoroughly triturated with 150 ml. of petroleum ether (b.p. 35–60°) and the mixture was separated by centrifugation. The trituration of the solid with petroleum ether was repeated twice using 150 ml. of solvent each time.[28] The residue was

(20) Prepared for immediate use by distillation from phosphorus pentoxide.

(21) Pyridine of good commercial grade was refluxed over barium oxide and distilled with the exclusion of moisture.

(22) It was found best to add the pyridine and the acyl chloride separately. A chloroform solution containing both substances assumes rapidly a strong yellow color which subsequently has to be removed by treatment with charcoal.

(23) The addition of greater amounts of water leads to the precipitation of impurities and should be avoided.

(24) According to Ruttan and Roebuck[7] both monostearoyl and monopalmitoyl glycol are soluble in alcohol to a considerable extent; 100 g. of absol. ethanol dissolves 4.17 g. or 10.61 g. of MSG at 25 and 29°, and 10.67 g. or 24.08 g. of MPG at 16 and 25°, respectively. A commercial preparation of alleged ethylene glycol monostearate, in spite of its reasonably good melting point (m.p. 57–60°, authentic glycol monostearate, m.p. 59.5–60.5°, glycol distearate, m.p. 75°), was found to contain very little of the alcohol-soluble monostearate. Caution, therefore, should be exercised in using commercial preparations of the monoacyl glycols.

(25) Ref. 10. For the purpose of comparison the monostearoyl glycol was prepared using Verkade's three-step procedure. However, in spite of repeated recrystallization of the MSG from petroleum ether its m.p. could not be raised above 59.5°. The test with concd. sulfuric acid showed that it tenaciously retained traces of trityl compounds.

(26) Analytical values of three independent preparations.

(27) Distilled over phosphorus pentoxide.

(28) The petroleum ether extracts contain considerable amounts of the distearoyl and dipalmitoyl bis-(glycol)-phosphatidic acid phenyl esters. A direct synthesis of these substances is described in the latter part of this paper.

then dissolved in 180 ml. of 99% ethanol and the solution, in the course of five minutes, was run into a vigorously stirred solution of 30.0 g. of ammonium reineckate[29] and 30.0 g. of anhydrous sodium carbonate in 1350 ml. of distilled water. The precipitate was collected with suction on a buchner funnel, washed with water, followed by alcohol and dried thoroughly *in vacuo* over phosphorus pentoxide. To obtain the stearoyl or palmitoyl glycollecithin phenyl ester reineckate, the mixture of reineckates was triturated at room temperature with 200 ml. of anhydrous ethyl acetate and the fine suspension was separated by centrifugation. The extraction of the reineckate mixture with ethyl acetate was repeated three times more at room temperature followed by a final extraction with boiling ethyl acetate (water-bath), using each time 100 ml. of the solvent.[30] The combined clear extracts were concentrated under reduced pressure to a volume of 110 ml. and to the concentrate was added gradually 180 ml. of petroleum ether (b.p. 35–60°). The precipitate was centrifuged off and triturated with 100 ml. of 99% ethanol.[31] The mixture was separated by centrifugation, the reineckate was treated once more with 50 ml. of 99% ethanol and then dried *in vacuo* (0.02 mm.) over phosphorus pentoxide. The reineckates of the stearoyl- and palmitoylglycollecithin phenyl esters weighed 13.0 g. (29.3%) and 10.3 g. (23.5%) and melted from 143–144° and 141–142°, respectively. Both reineckates were pure enough at this stage for further processing.

Stearoylglycollecithin Phenyl Ester Reineckate.—The reineckate was obtained in an analytically pure state by reprecipitation from ethyl acetate (7.5 ml. per 1 g. of reineckate) with petroleum ether (b.p. 35–60°, 10 ml. per 1 g. of reineckate), triturating the precipitate twice with 99% ethanol (two 10-ml. portions per 1 g. of original reineckate) and drying *in vacuo* (0.1 mm.) over phosphorus pentoxide; recovery 95%, m.p. 144.5–145°.

Anal. Calcd. for $[C_{31}H_{57}O_6NP][(NH_3)_2Cr(SCN)_4]$ (889.1): C, 47.28; H, 7.14; N, 11.03; P, 3.48. Found: C, 47.23, 47.52; H, 7.47, 7.15; N (Dumas), 11.04; P, 3.42.[32]

Palmitoylglycollecithin Phenyl Ester Reineckate.—The reineckate was purified as described for the stearoyl compound using 7.5 ml. of ethyl acetate, 10 ml. of petroleum ether (b.p. 35–60°) and two volumes of 10 ml. of 99% ethanol per 1 g. of reineckate; recovery 96.3%, m.p. 141–142°.

Anal. Calcd. for $[C_{29}H_{53}O_6NP][(NH_3)_2Cr(SCN)_4]$ (861.1): C, 46.03; H, 6.91; N, 11.38; P, 3.60. Found: C, 45.87; H, 7.03; N, 11.60 (Kjeldahl); P, 3.49.

At room temperature the stearoyl- and palmitoylglycollecithin phenyl ester reineckates are readily soluble in acetone, ethyl acetate, dioxane or dimethylformamide, only slightly soluble in methanol and insoluble in ether, petroleum ether, 99% ethanol, benzene, chloroform or tetrachloromethane. They are also insoluble in boiling ether, chloroform or tetrachloromethane, but are readily soluble in boiling 99% ethanol.

Conversion of the Glycollecithin Phenyl Ester Reineckates to the Sulfates.—To the vigorously stirred solution of 1.0 mmole of the glycollecithin phenyl ester reineckate (8.89 g. or 8.61 g. of the stearoyl or palmitoyl compound, respectively) in 175 ml. of a mixture of acetone and ethyl alcohol (1:1) was added rapidly a hot solution of 0.5 mmole (1.56 g.) of silver sulfate in 135 ml. of distilled water, and the mixture was stirred for 10 minutes. At the end of this time the silver reineckate was centrifuged off, washed in succession with 20 ml. of water and 50 ml. of ethanol and the combined solutions, after the addition of 5 ml. of octyl alcohol, were brought to dryness under reduced pressure at a bath temperature of 35 to 45°.[33] The residue was kept *in vacuo* at 45° until it had become solid. The solid material was suspended in 80 ml. of acetone, filtered with suction and washed with 40 ml. of acetone. The white stearoyl- and palmitoylglycollecithin phenyl ester sulfates, after drying *in vacuo* at room temperature weighed 6.11 g. (96%) and 5.67 g. (93.2%), respectively. The sulfates at this stage were pure enough for further processing. For analytical purposes they were purified by dissolving in 99% ethanol (70 ml.), clearing the solutions by centrifugation, removing the alcohol *in vacuo*, taking up the residues in acetone (100 ml.) and filtering the mixtures with suction. The stearoyl- and palmitoylglycollecithin phenyl ester sulfates, after drying *in vacuo*, weighed 5.44 g. (89.0%) and 5.3 g. (93.4%), respectively.

Stearoylglycollecithin Phenyl Ester Sulfate.—*Anal.* Calcd. for $[C_{31}H_{57}O_6NP]_2SO_4 \cdot 2H_2O$ (1273.5): C, 58.47; H, 9.35; N, 2.19; P, 4.86. Found: C, 58.47, 58.52, 58.59; H, 9.45, 9.44, 9.38; N (Kjeldahl) 2.12, (Dumas) 2.18; P, 4.82.

Palmitoylglycollecithin Phenyl Ester Sulfate.—*Anal.* Calcd. for $[C_{29}H_{53}O_6NP]_2SO_4 \cdot 2H_2O$ (1217.4): C, 57.24; H, 9.11; N, 2.30; P, 5.09. Found: C, 57.15; H, 9.12; N, (Kjeldahl) 2.18, 2.38; P, 5.01.

The glycollecithin phenyl ester sulfates lose their water of crystallization *in vacuo* at 100° forming a glass-like mass on cooling to room temperature: 263.2 mg. of the stearoylglycollecithin phenyl ester sulfate (dihydrate) gave off 6.1 mg. of water; calculated for 2 moles of water, 7.4 mg.

Anal. Calcd. for $[C_{31}H_{57}O_6NP]_2SO_4$ (1237.6): P, 5.01; N, 2.26. Found: P, 4.94; N (Kjeldahl), 2.29.

The stearoyl- and palmitoylglycollecithin phenyl ester sulfates are readily soluble in chloroform, ethanol or water, and insoluble in acetone or ether.

Removal of the Phenyl Group by Catalytic Hydrogenolysis.—A solution of 5.0 mmoles of the stearoyl- or palmitoylglycollecithin phenyl ester sulfate (6.37 g. or 6.08 g., respectively) in 100 ml. of 99% ethanol, together with 1.34 g. of platinic oxide (Adams catalyst)[34] was shaken vigorously at room temperature in an all-glass hydrogenation vessel (750-ml. capacity) in an atmosphere of hydrogen at an initial pressure of 50 cm. of water, until the absorption of hydrogen had ceased. The reductive cleavage usually was finished in 15–20 minutes and consumed slightly more hydrogen (21.2 mmoles) than required by theory (20.0 mmoles). After displacing the hydrogen by nitrogen and, if necessary, adding chloroform to redissolve the lecithin, the catalyst was removed by centrifugation and washed with 20 ml. of alcohol. To the combined alcoholic solutions was added with stirring a solution of 1.4 g. of barium acetate ($1H_2O$) in 10 ml. of water and the stirring was continued for five minutes. At the end of this time the barium sulfate was removed by centrifugation, the water-clear solution was brought to dryness *in vacuo* at a bath temperature of 30–35° and the residue was treated with 50 ml. of acetone. The solid material was centrifuged off, washed with acetone and dried *in vacuo* (0.01 mm.) over phosphorus pentoxide and sodium hydroxide. The stearoyl glycollecithin (SGL) or palmitoyl glycollecithin (PGl) were obtained in almost theoretical yields: 4.96 g. (97%) and 4.73 g. (98%), respectively. In general, the glycollecithins were clearly soluble in water. If a test showed that they contained water-insoluble impurities they were purified by centrifuging their saturated aqueous solutions until they were clear and pouring the supernatant solutions with stirring into 30–40 times their volume of acetone. The glycollecithins were centrifuged off, washed several times with acetone and dried *in vacuo*. The glycollecithins (4.96 g. SGL, 4.73 g. PGL) were obtained in crystalline form and in an analytically pure state by dissolving the crude material in chloroform (96 ml. USP grade), adding ether (48 ml. USP grade), centrifuging the solutions while still warm[35] and keeping the decanted supernatant solutions in a closed vessel at room temperature (20–25°) until spontaneous crystallization had set in.

(29) The ammonium reineckate was prepared as described in "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 555.

(30) With increasing number of extractions the separation of the reineckate–ethyl acetate mixture by centrifugation becomes more and more difficult.

(31) If the treatment of the reineckates with ethanol is omitted they form on drying a dark-red glass-like substance.

(32) The colorimetric determination of phosphorus in the presence of chromium was carried out as described by Baer and Kates, THIS JOURNAL, **70**, 1397, footnote 26 (1948).

(33) Since the glycollecithin phenyl ester sulfate solution has a strong tendency to froth during distillation, a large distilling flask (1-l.) was used and the solution was added dropwise, taking care that the flask contained not more than a few milliliters of liquid at any time.

(34) The catalyst was prepared as described in "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1948, p. 467, except that potassium nitrate was used (A. H. Cook and R. P. Linstead, *J. Chem. Soc.*, 952 (1924)) as is customary in our laboratory.

(35) If the lecithin precipitates before the solution has become clear it is redissolved by warming slightly and the centrifugation is continued.

After the first crystals had formed 8 ml. of ether was added and the addition of 8-ml. portions of ether was repeated at intervals of 15 to 30 minutes until a total of 48 ml. of ether had been added. The glycollecithins were deposited in well formed narrow prisms measuring several millimeters in length. After standing overnight the mixtures were filtered with suction and the crystals were dried *in vacuo* over phosphorus pentoxide. The SGL and PGL were recovered in yields of 87% (4.31 g.) and 82% (3.88 g.), respectively. Both glycollecithins were free of potassium, a contaminant of Adams catalyst.

Stearoylglycollecithin.—The substance started to sinter at approximately 70° and formed translucent droplets adhering to the walls of the test-tube at approximately 80°. On further heating (10° per min. up to 210° and 4° per min. from there on) the substance coalesced suddenly with the formation of a meniscus at 239–240°.[36]

Anal. Calcd. for $C_{25}H_{54}O_7NP$ (511.7): C, 58.67; H, 10.63; P, 6.06; N, 2.73. Found: C, 58.86; H, 10.47; P, 6.12; N (Kjeldahl), 2.68, 2.83.

Palmitoylglycollecithin.—The substance started to sinter at 68°, formed translucent droplets at approximately 80°, and coalesced suddenly at 242–243°.

Anal. Calcd. for $C_{23}H_{50}O_7NP$ (483.6): C, 57.12; H, 10.42; P, 6.41; N, 2.89. Found: C, 57.22; H, 10.50; P, 6.58; N (Kjeldahl), 2.88, 2.92.

At room temperature (25°) the stearoyl- and palmitoylglycollecithins are readily soluble in 95 or 99% ethanol, methanol, chloroform or dioxane, slightly soluble in tetrachloromethane, and insoluble in acetone, ether, petroleum ether, ethyl acetate or benzene. Both glycollecithins are soluble in water to a considerable extent; 100 ml. of water (26°) take up approximately 28–30 g. of stearoyl glycollecithin or 47–49 g. of palmitoyl glycollecithin. The glycollecithins can be recovered in good yields (up to 90%) by pouring their saturated aqueous solutions into 30–40 times their volume of acetone.

The X-ray powder diffraction pattern of stearoyl glycollecithin (recrystallized twice from chloroform–ether) was taken using copper Kα radiation (λ 1.54050) obtained from a Phillips copper-target X-ray tube and filtered through a piece of nickel foil to remove background radiation. The intensities of the diffraction rings were estimated visually on an arbitrary scale and are quoted in parentheses after the crystal spacings: 7.69 Å. (2), 7.08 (2), 6.32 (2), 5.82 (2), 4.98 (10), 4.53 (10), 4.33 (7), 3.83 (10), 3.53 (1), 3.23 (4), 3.06 (1), 2.78 (1), 2.63 (2), 2.48 (2), 2.30 (1), 2.14 (3), 1.93 (3).

The stearoyl- and palmitoylglycollecithins possess a strong hemolytic activity. Twenty micrograms of either one of these two substances causes in 10 minutes the complete hemolysis of 0.08 ml. of washed and centrifuged human erythrocytes suspended in 5 ml. of a 0.9% saline solution (dil. 1:250,000). Comparative tests with aqueous suspensions of L-α-distearoyl-, L-α-dipalmitoyl- and L-α-dimyristoyllecithins showed that these lecithins are without hemolytic activity.

Glycollecithin Reineckates.—A clear solution of 0.5 mmole of the stearoyl- or palmitoylglycollecithin (0.256 or 0.242 g., respectively) in 10 ml. of dist. water was added with vigorous stirring to a solution of 1.5 mmoles (0.50 g.) of ammonium reineckate in 30 ml. of water to which 1.5 ml. of 1 *N* hydrochloric acid has been added. The precipitate was collected with suction on a buchner funnel, washed with water until the filtrate was colorless and dried *in vacuo* over phosphorus pentoxide. The reineckates of stearoyl- and palmitoylglycollecithin weighed 0.303 and 0.313 g. (91.4 and 98.7% of the theory calculated on the basis of two moles of glycollecithin per one mole of reinecke ion, respectively). For analysis they were recrystallized from methanol.

Stearoylglycollecithin Reineckate.—*Anal.* Calcd. for $[C_{25}H_{53}O_6NP][(NH_3)_2Cr(SCN)_4]$ (813.09): C, 42.84; H, 7.31; N, 12.06; P, 3.81; and for $[C_{25}H_{54}O_7NP, C_{25}H_{53}O_6NP][(NH_3)_2Cr(CSN)_4]$ (1324.7): C, 48.96; H, 8.59; N, 8.46; P, 4.68. $[C_{25}H_{53}O_6NP, C_{25}H_{52}O_6NP][(NH_3)_2Cr(CSN)_4]$ (1306.4): C, 49.63; H, 8.56; N, 8.58; P, 4.74. Found: C, 49.08; H, 8.44; N, 8.57; P, 4.72.

Palmitoylglycollecithin Reineckate.—*Anal.* Calcd. for $[C_{23}H_{49}O_6NP][(NH_3)_2Cr(SCN)_4]$ (785.04): C, 41.31; H, 7.06; N, 12.49; P, 3.95; and for $[C_{23}H_{50}O_7NP, C_{23}H_{49}O_6NP][(NH_3)_2Cr(CSN)_4]$ (1268.6): C, 47.34; H, 8.34; N, 8.84; P, 4.89. $[C_{23}H_{49}O_6NP, C_{23}H_{48}O_6NP][(NH_3)_2Cr(CSN)_4]$ (1250.6): C, 48.02; H, 8.30; N, 8.96; P, 4.96. Found: C, 47.45; H, 8.53; N, 8.72; P, 4.95, 4.86.

At room temperature (20–25°) the stearoyl- and palmitoylglycollecithin reineckates are insoluble in ether, ethyl acetate, chloroform or benzene, and slightly soluble in methanol or acetone. Both substances are, however, readily soluble in warm methanol, ethanol or acetone.

The Cadmium Chloride Compounds of Stearoyl- and Palmitoylglycollecithin.—A solution of 0.51 g. (50% excess) of cadmium chloride ($2.5H_2O$) in 0.6 ml. of water and 12 ml. of 99% ethanol was added gradually and with stirring to a solution of either 0.512 g. of stearoylglycollecithin or 0.484 g. of palmitoylglycollecithin in 17 ml. of 99% ethanol. The precipitate was centrifuged off, washed in succession with 99% ethanol and ether, and dried in a high vacuum at room temperature. The stearoyl- and palmitoylglycollecithin cadmium chloride compounds weighed 0.747 g. (95%) and 0.753 g. (99%), respectively.

Stearoylglycollecithin Cadmium Chloride.—*Anal.* Calcd for $[C_{25}H_{54}O_7NP]_2[CdCl_2]_3$[37] (1573.5): C, 38.17; H, 6.92; N, 1.78; P, 3.94. Found: C, 38.10; H, 6.92; N (Kjeldahl), 1.79; P, 3.89.

Palmitoylglycollecithin Cadmium Chloride.—*Anal.* Calcd. for $[C_{23}H_{50}O_7NP]_2[CdCl_2]_3$ (1517.4): C, 36.41; H, 6.64; N, 1.85; P, 4.08. Found: C, 36.48; H, 6.53; N, 1.81; P, 4.04.

The glycollecithin cadmium chloride compounds are readily soluble in water and can be reprecipitated from their concentrated aqueous solutions by the addition of ethanol without changing the molecular ratios of glycollecithin and cadmium chloride.

Bis-(glycol)-phosphatidic Acids[38]

Bis-(glycol)-phosphatidic Acid Phenyl Esters.—To the stirred solution of either 3.28 g. (10 mmoles) of monostearoyl glycol or 3.00 g. (10 mmoles) of monopalmitoyl glycol in 30 ml. of anhydrous and ethanol-free chloroform[20] were added under anhydrous conditions from two separate dropping funnels and at an equal rate of flow in the course of one hour 1.06 g. (5 mmoles) of phenylphosphoryl dichloride and 0.95 ml. (12 mmoles) of anhydrous pyridine each dissolved in 15 ml. of chloroform. After standing at room temperature for 24 hours the solution was brought to dryness under reduced pressure (bath 25–35°) and the residue was extracted successively with four 50-ml. portions of boiling petroleum ether (b.p. 35–60°). The combined extracts were cleared by centrifugation and brought to dryness under reduced pressure (bath 35–40°). To remove the last traces of petroleum ether and pyridine the finely powdered bis-(glycol)-phosphatidic acid phenyl esters were kept *in vacuo* (0.02 mm.) over phosphorus pentoxide for 24 hours.

Distearoyl Bis-(glycol)-phosphoric Acid Phenyl Ester.—The crude product weighed 3.59 g. (90.3% of theory); calcd. P, 3.89. Found P, 4.07. For purification the phenyl ester (3.59 g.) was dissolved in 140 ml. of warm acetone, the solution was cooled to +8°, filtered with suction and the material was dried *in vacuo* over phosphorus pentoxide. The recovered phenyl ester weighed 2.51 g. (70%), m.p. 59–60°.

Anal. Calcd. for $C_{46}H_{83}O_8P$ (795.1): C, 69.48; H, 10.52; P, 3.89. Found: C, 69.72; H, 10.30; P, 3.90.

Dipalmitoyl Bis-(glycol)-phosphoric Acid Phenyl Ester.—The crude phenyl ester weighing 3.35 g. (90.5% of theory) was purified by dissolving in 80 ml. of acetone, cooling the solution gradually to 0°, collecting the solid with suction on a buchner funnel and drying the phenyl ester *in vacuo* over phosphorus pentoxide. The recovered phenyl ester weighed 2.50 g. (75.7%) and melted from 51.5–52.5°.

Anal. Calcd. for $C_{42}H_{75}O_8P$ (739.0): C, 68.25; H, 10.23; P, 4.19. Found: C, 68.66; H, 10.14; P, 4.28.

(36) The melting point determinations were carried out in sealed-off capillary tubes using an electrically heated bath of *n*-butyl phthalate and short-stem thermometers with a range of 50°.

(37) The same ratio of lecithin and cadmium chloride (2:3) has been observed in the cadmium chloride compounds of several saturated α-lecithins[6] and the amorphous cadmium chloride compound of glycerylphosphorylcholine.[32]

(38) The synthesis of mono-(glycol)-phosphatidic acids has been accomplished recently by P. E. Verkade and J. H. Uhlenbroek (*Proc. Koninkl. Nederland, Akad. Wetenschap., LV* (B), 110 (1952)) by using the method of Baer for the synthesis of glycerolphosphatidic acids (*J. Biol. Chem.*, **189**, 235 (1951)).

Catalytic Hydrogenolysis.—A solution of 4.77 g. of the distearoyl or 4.43 g. of the dipalmitoyl bis-(glycol)-phosphoric acid phenyl ester (6 mmoles) in a mixture of 95 ml. of chloroform and 20 ml. of 99% ethanol to which had been added 0.91 g. (4 mmoles) of platinic oxide (Adams catalyst) was shaken vigorously in an all-glass hydrogenation vessel in an atmosphere of hydrogen at an initial pressure of 40–50 cm. of water until the absorption of hydrogen ceased. In approximately one hour the theoretical amount of hydrogen (24 mmoles) had been consumed. After replacing the hydrogen with nitrogen and removing the catalyst, the solvents were distilled off under reduced pressure at a bath temperature of 30–35°. The bis-(glycol)-phosphatidic acid was washed with three 50-ml. portions of dilute acetic acid (10%) and was dried *in vacuo* (0.01 mm.) over solid sodium hydroxide. Both phosphatidic acids were free of potassium.

Distearoyl-bis-(glycol)-phosphoric Acid.—For purification the crude distearoyl-(glycol)-phosphoric acid weighing 4.09 g. (95% of theory) was triturated with 35 ml. of 99% ethanol, the mixture was separated by centrifugation and the solid was recrystallized from 140 ml. of boiling 99% ethanol. The bis-(glycol)-phosphatidic acid was recovered in a yield of 75% (3.07 g.) and melted from 92.5–93.5°. At room temperature the distearoyl bis-(glycol)-phosphoric acid is insoluble in ether or acetone, very slightly soluble in methanol, ethanol, ethyl acetate, tetrachloromethane, petroleum ether or benzene, but readily soluble in chloroform. The substance is also readily soluble in warm methanol, ethanol, acetone, ethyl acetate, tetrachloromethane, petroleum ether (b.p. 100–120°) and benzene.

Anal. Calcd. for $C_{40}H_{79}O_8P$ (718.2): C, 66.89; H, 11.09; P, 4.46. Found: C, 67.09; H, 10.96; P, 4.33.

Dipalmitoyl-bis-(glycol)-phosphoric Acid.—For purification the crude dipalmitoyl-bis-(glycol)-phosphoric acid, weighing 3.94 g. (99% of theory), was triturated with 35 ml. of 99% ethanol and the mixture was separated by centrifugation. The precipitate was recrystallized from 140 ml. of warm 99% ethanol; recovery 3.01 g. (76.5%), m.p. 89.0–90.5°. At room temperature the dipalmitoyl bis-(glycol)-phosphoric acid is practically insoluble in methanol, ethanol, acetone, ethyl acetate or petroleum ether, slightly soluble in benzene or tetrachloromethane, and readily soluble in chloroform. It is also readily soluble in hot methanol, ethanol, acetone, ethyl acetate, tetrachloromethane and petroleum ether (b.p. 100–120°).

Anal. Calcd. for $C_{36}H_{71}O_8P$ (662.13): C, 65.30; H, 10.81; P, 4.68. Found: C, 65.46; H, 10.77; P, 4.71.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

Enzymic Synthesis of Peptide Bonds. VI. The Influence of Residue Type on Papain-catalyzed Reactions of Some Benzoylamino Acids with Some Amino Acid Anilides[1,2]

BY SIDNEY W. FOX, MILTON WINITZ AND CORNELIUS W. PETTINGA

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Each of thirteen benzoylamino acids has been submitted to reaction with glycinanilide in the presence of papain. Only one, benzoylglycine, participated in a synthesis leading to a larger peptide. The failure of benzoylaminoisobutyric acid to react was explainable on the basis of steric hindrance. Benzoyltryptophan has been shown earlier to react in another *p*H range. Benzoylglutamic acid and benzoyltyrosine fail to react at *p*H's in which the corresponding reactions with aniline had previously been shown to proceed rapidly. All eight other reactions proved to be transacylations yielding glycine-free products. On reacting with each of four amino acid anilides, benzoylglycine yielded benzoylglycylamino acid anilide. When benzoylalanine was employed instead of benzoylglycine there resulted two syntheses and two transacylations. The acylamino acid and the amino acid anilide thus each contribute to selectivity in synthesis. The specificities observed when the carboxoid or aminoid component is systematically varied contrasts, at the two amino acid residue level, with the broad preferences observed in reactions of benzoylamino acid with aniline. From these results it is apparent how a single protease participating in peptide bond synthesis may favor unique synthetic reactions, and reject or divert others. To emphasize that the substrate contributes to this specificity to a degree comparable to the influence of the enzyme, these phenomena are referred to as *zymosequential specificity*. These observations suggest the possibility that, in protein synthesis, each peptide intermediate becomes part of the protease to give, in effect, a new enzyme at each step.

In any consideration of mechanisms of protein synthesis, adequate explanation of replication of highly specific protein structures must be paramount. One mechanism, among those which have been postulated, suggests that the enzymes which are known to catalyze proteolysis, also mediate protein synthesis.[3] Any pathway which invokes the agency of proteases is more worthy of consideration if it also explains the variability of this replication from one tissue to another and from one species to another. Experimentally valid instances of the type of model peptide bond synthesis which correspond to the latter, have been described.[4] In these comparisons the enzyme preparation was the sole initial variable.

This paper presents evidence for influences which may be considered as a basis for uniquely limited replication of protein molecules.

Experimental Procedure

Reactants.—These have been described in previous papers.[4–8]

Enzyme Experiments.—The essential features have been described previously. The enzymes were from the same

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(2) Presented at the Twelfth International Congress of Pure and Applied Chemistry, New York City, September 12, 1951. The essential results are described in the Ph.D. theses of Cornelius W. Pettinga, 1949, and Milton Winitz, 1951.

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