XXII. GLYCEROPHOSPHORIC ACID.

II. THE GLYCEROPHOSPHORIC ACID OF THE NATURALLY OCCURRING PHOSPHATIDES.

By JAMES JAMIESON RAE¹.

From the Department of Medical Research of the University of Toronto.

(Received November 14th, 1933.)

THE naturally occurring phosphatides are usually represented as compounds of fatty acids and a base with the α - (unsymmetrical) variety of glycerophosphate (*e.g.* lecithin I), but the possibility has long been recognised that the β -variety (lecithin II) may also occur.

Glycerophosphoric acid can be obtained by hydrolysing the phosphatides with baryta. From this acid, as it occurs in egg-lecithin, liver-lecithin, brain-lecithin, kephalin and calcium phosphatide, barium salts were obtained and these were investigated as to the relative amounts of the α - and β -isomerides present, using an enzymic method. Some objections as to the applicability of this method were removed.

Willstätter and Lüdecke [1904] found that the glycerophosphoric acid prepared from egg-lecithin was optically active, and they therefore concluded that it was the α -form. This observation may prove the presence of the α -form but does not exclude the possibility of the β -isomeride being present. Tutin and Hahn [1906] maintained that both the α - and β -forms were present. Grimbart and Bailly [1915] introduced a simple chemical test for differentiating the two isomerides which was based on the Denigès reaction, and using this test they showed that lecithin was a mixture of both varieties. Levene and Rolf [1919], using a highly purified egg-lecithin, obtained a glycerophosphate which had an optical rotation of -0.74° . Willstätter and Lüdecke's value was -1.71° . Both groups of workers assert that the magnitude of the rotation depends very much on the mode of preparation.

Karrer and Salomon [1926] have shown, using a double salt precipitation method, that both varieties occur in lecithin. Owing to the confusion in this field and to the fact that Kay and Lee [1931] had recently brought forward a quantitative method for estimating the relative amounts of the α - and β -isomerides present in a mixture, it was thought advisable to prepare several phosphatides and estimate the relative amounts of the two isomerides in the glycerophosphates prepared from them.

¹ An abstract from a thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy in the University of Toronto.

EXPERIMENTAL.

Preparation of the phosphatides.

Lecithin was prepared from liver by a modification of Levene's [1921] method. 43 pounds of fresh ox-liver were minced and dried *in vacuo* at 37°. The material was finely ground and extracted with large volumes of alcohol. The extract was reduced in volume and allowed to stand overnight at 0°. This precipitated the cerebrosides. To the filtrate was added a saturated solution of cadmium chloride in hot ethyl alcohol until precipitation was complete. The crude lecithin cadmium chloride was repeatedly washed with ether to remove the kephalin. The purified salt was suspended in chloroform and decomposed with ammonia gas. After distilling off the chloroform the residue was dissolved in a minimum volume of ether and precipitated by adding it to a large volume of acetone. The yield was 69.8 g. Commercial egg-lecithin was purified by treating the material with cadmium chloride and recovering the lecithin in the same manner as that described above.

Brain-lecithin and brain-kephalin were prepared in the following way. 60 pounds of fresh ox-brain were freed from adhering tissue, minced and extracted with large volumes of acetone to dry the material and to free it from neutral fat. The brain was extracted with ether and the extract reduced to a small volume and allowed to stand at 0° for 15 hours. The supernatant liquid, now free from cerebrosides, was poured into alcohol. The alcohol-soluble part contained the lecithin. This was separated from the solution in a manner analogous to that described in the preparation of liver-lecithin. The kephalin was dissolved in ether and again allowed to stand at 0°, during which time more cerebrosides separated out. This process was repeated until no further precipitation occurred on standing. The liquid was added to alcohol and the kephalin precipitated. The yield was 455 g.

Chibnall and Channon [1927, 1, 2, 3] showed that the ether-soluble substances of cabbage-leaf cytoplasm contain little, if any, phospholipin in the commonly accepted use of the term, the major part of the lipin being present as the calcium salt of a diglyceridephosphoric acid. The calcium salt of phosphatidic acid was prepared in the following way. Four pounds of dried cabbage were exhaustively extracted with ether in a modified Soxhlet apparatus. The extract was evaporated to dryness, taken up in a small volume of ether and precipitated by adding to four volumes of acetone. The part insoluble in hot acetone gave the following results on analysis: P 4.00, 3.92 %; Ca 5.50, 5.45 %; Ca/P = 1.07. Calculated for Ca distearyl-glycerophosphoric acid: P 4.18 %; Ca 5.39 %; Ca/P = 1.

Preparation of barium glycerophosphate from the phosphatides.

The phosphatide was dissolved in a suitable solvent and emulsified by adding it slowly to an equal volume of hot water. This emulsion was then added to a hot baryta solution and boiled for one hour on the water-bath. The fatty acid formed barium soaps, and these were filtered off and washed with hot water. The filtrate and washings were treated with carbon dioxide to remove excess barium. The filtrate was reduced in volume and a slight excess of normal lead acetate was added. The lead glycerophosphate was washed, suspended in water and decomposed with hydrogen sulphide. The solution was freed from hydrogen sulphide and made neutral to phenolphthalein by the addition of baryta. It was then added to four volumes of alcohol, and the precipitated barium glycerophosphate was washed with alcohol and ether and dried *in vacuo* over sulphuric acid. In this

manner barium glycerophosphates were prepared from egg-, liver-, and brain-lecithin, brain-kephalin and calcium phosphatide.

These salts were analysed for barium, using a micro-gravimetric method. This method makes use of the filter stick (Stäbchen) and the residue is weighed as barium sulphate. Another method used was the titrimetric benzidine method described by King [1932, 1]. The phosphorus was estimated colorimetrically, using King's [1932, 2] method.

Estimation of relative amounts of α - and β -isomerides in glycerophosphates from natural sources.

Both animal and plant phosphatases hydrolyse α - and β -glycerophosphates at different rates. It was decided, therefore, using Kay and Lee's [1931] enzymic method, to determine the relative amounts of the α - and β -isomerides of glycerophosphoric acid occurring in the natural phosphatides. Solutions of authentic

Fig. 1.

Fig. 2.

Fig. 1. Rates of hydrolysis by mammalian phosphatase of pure α - and β -glycerophosphates and of the glycerophosphate from egg-lecithin. Glycine buffer used.

Fig. 2. Rates of hydrolysis by mammalian phosphatase of pure α - and β -glycerophosphates and of the glycerophosphate from liver-lecithin. Veronal buffer used.

α - and β -salts, and of the unknown glycerophosphate were made up approximately $M/100$, analysed for phosphorus and diluted to exactly the same strength. Rates of hydrolysis were measured by the method described in the previous paper [Rae *et al.*, 1934]. From these curves it may be concluded that the glycerophosphate from egg-lecithin (Fig. 1) is a mixture of α - and β -forms in which the β -form predominates. Liver-lecithin (Figs. 2 and 6) is a mixture of about equal proportions of α - and β -forms. Brain-lecithin (Fig. 3), brain-kephalin (Fig. 4), and calcium phosphatide (Fig. 5) contain predominantly the α -form. The study of rates of enzymic hydrolysis thus furnishes a simple method for determining the relative amounts of the α - and β -isomerides occurring in the natural phosphatides.

The objection that since the α -form contains an asymmetric carbon, what is considered a mixture of α - and β -forms may be only one of the optical isomerides

of the α -form, has been dealt with in a previous paper [Rae *et al.*, 1934], where it has been shown that in several attempts to resolve the α -form, in no case were fractions obtained that exhibited any difference in their rates of enzymic hydrolysis.

Fig. 3.

Fig. 4.

Fig. 3. Rates of hydrolysis by takaphosphatase of pure α - and β -glycerophosphates and of the glycerophosphate from brain-lecithin. Phthalate buffer used.

Fig. 4. Rates of hydrolysis by mammalian phosphatase of pure α - and β -glycerophosphates and of the glycerophosphate from brain-kephalin. Glycine buffer used.

Fig. 5.

Fig. 6.

Fig. 5. Rates of hydrolysis by mammalian phosphatase of pure α - and β -glycerophosphates and of the glycerophosphate from calcium diglyceridephosphoric acid from cabbage. All at pH 8.9.

Fig. 6. Rates of hydrolysis by takaphosphatase of pure α - and β -glycerophosphates and of the glycerophosphate from liver-lecithin. Phthalate buffer used.

Another objection is that a wandering of the phosphate group from the α - to the β -position or *vice versa* may occur during the hydrolysis of the phosphatide with baryta. Thus a true picture of the relative amounts of the two isomerides present would not be obtained. To test this, samples of both α - and β -glycero-

phosphates were boiled for 2 hours with the customary amount of baryta. These salts were then compared enzymically and no change in the characteristic rate of hydrolysis was observed in either of them.

These results clearly indicate that the glycerophosphoric acid of the natural phosphatides consists of both the α - and β -isomerides. The formulae of these phosphatides should be written to represent the probability of the occurrence of the symmetrical β -variety, and not always as the asymmetrical α -type.

SUMMARY.

The relative amounts of α - and β -glycerophosphates occurring in several natural phosphatides have been determined by an enzymic method. Egg-lecithin contains a glycerophosphoric acid which is predominantly the β -form; liver-lecithin contains about equal amounts of the α - and β -forms; whereas brain-lecithin, brain-kephalin and calcium phosphatide contain predominantly the α -form.

My thanks are due to Dr E. J. King, who directed this research, and to Dr H. D. Kay, under whose direction some of the enzyme work was carried out.

This research was carried out under the tenure of a fellowship in the Department of Medical Research in the University of Toronto.

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