XXI. GLYCEROPHOSPHORIC ACID.

I. THE RECOGNITION AND SEPARATION OF α - AND β -GLYCEROPHOSPHATES.

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SYNTHETIC glycerophosphates have been known for many years and have been prepared in several ways. The acid may exist in two isomeric forms, α and β . Carré [1903] showed that glycerol when esterified with phosphoric acid at 105° yielded almost entirely monoglycerylphosphoric acid. At higher temperatures further condensation took place. Later Power and Tutin [1905] prepared synthetic glycerylphosphates by a similar method and also obtained the mono-derivatives. Poulenc Frères [1907] showed that a well-crystallised sodium glycerylphosphate could be prepared by heating monosodium phosphate with two molecular proportions of glycerol and hydrolysing the resulting di-ester with sodium hydroxide. All these methods yielded mixtures of α - and β -glycerophosphates.

There are three principal methods for preparing pure α - and β -glycerophosphates. (1) King and Pyman [1914] mixed pure α -monochlorohydrin and tri-sodium phosphate, and on working up the product obtained pure α -glycerophosphate. Pure β -glycerophosphate was obtained from α -dichlorohydrin and phosphoryl chloride. (2) Bailly [1915] prepared pure α -glycerophosphate from epichlorohydrin. (3) Fischer and Pfähler [1920] prepared pure α -glycerophosphate from acetone-glycerol, phosphorus oxychloride and quinoline.

The following methods have been advanced to distinguish between the α - and β -isomerides.

1. Grimbert and Bailly [1915] found that the α -ester on careful oxidation (so as to avoid hydrolysis) yields a compound containing a ketone group, which can be identified by the Denigès reaction. The β -form on the other hand does not give this reaction.

2. Karrer and Salomon [1926] described a method which depends on the fact that barium β -glycerophosphate forms with barium nitrate a difficultly soluble double salt which precipitates on standing. The α -form does not give this double salt. Kay [1931] has criticised Karrer and Salomon's results on the ground that they used supersaturated solutions from which a salt would crystallise out on standing without the addition of barium nitrate. We have, however, put this method to an important use to be described later.

3. Since Levene and Yawagama [1920] had found that the rate of hydrolysis of the phosphoric esters of the sugars with sulphuric acid varied with the position of the phosphate group, it was thought advisable to see if the α - and β -forms of

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glycerophosphate would show this peculiarity. To test this point solutions of the α - and β -barium glycerophosphates were hydrolysed with *N* sulphuric acid at 100°. *k* was calculated for a unimolecular reaction. The average values for one experiment were for the α , $k=0.00281$, and for the β , $k=0.00266$. From these results it is evident that the rates of hydrolysis of the two isomerides with strong acids are not appreciably different.

4. *The enzymic hydrolysis method* for distinguishing between the two isomerides depends upon the fact that both animal and plant phosphatases hydrolyse the two forms at markedly different rates. Kay [1926] has shown that mammalian kidney phosphatase attacks the β - more rapidly than the α -isomeride. Earlier workers had stated that the α - and β -forms were attacked at the same rate. A plant phosphatase was investigated by a group of Japanese workers [Akamatsu, 1923; Inouye, 1928; Kobayashi, 1927; Asakawa, 1929], who showed its presence in takadiastase. It has been found by Kay and Lee [1931] that with most mammalian phosphatases and with takaphosphatase the β - is attacked more rapidly than the α -form. This fact has been made the basis for their method of distinguishing between the two isomerides. With phosphatase from *Propionibacterium jensenii* [Werkman and Kendall, 1931] and with blood-phosphatase, as shown by Roche [1931], the α - is attacked more rapidly than the β -form.

In the present investigation a study has been made of the rates of enzymic hydrolysis of pure β - and of a specially purified α -glycerophosphate. Purification of the α - from small amounts of the β -isomeride has been accomplished both by the use of Karrer's double salt method and by partial enzymic hydrolysis of the impure material. Since the β -isomeride is hydrolysed more rapidly than the α -form, the former is removed and although there is some loss of the α -form, it is obtained pure towards the end of the reaction.

In the expectation that the α -glycerophosphate might consist of two optical enantiomorphs with different rates of enzymic hydrolysis, an attempt was made to effect a separation of the one enantiomorph from the other by means of the partial enzymic hydrolysis of purified α -glycerophosphate. An attempt was also made to resolve the α -form by means of fractional crystallisation of several of its alkaloidal salts. None of the fractions obtained by the method of partial hydrolysis or by fractional crystallisation appeared to have any significant degree of optical rotation. In a second communication [Rae, 1934], the results are presented of a study of the relative amounts of the α - and β -isomerides in the glycerophosphates derived from the naturally occurring phosphatides.

EXPERIMENTAL.

Materials. The crystalline salt of sodium β -glycerophosphate, obtained from Boot's Pure Drug Company, appeared to consist almost entirely of the β -isomeride. It gave only a very slight Denigès reaction, and no sample of β -glycerophosphate has been obtained or prepared in this laboratory which has a higher rate of enzymic hydrolysis. On the other hand, several samples of the α -isomeride, which have been obtained from time to time, have appeared to contain different and widely variable amounts of the β -isomeride. Because of the discrepancies in the various experiments in which the reputedly pure α -compound was used, it was thought advisable to prepare some of the material from authentic α -monochlorohydrin by King and Pyman's [1914] method. For this purpose Eastman Kodak monochlorohydrin was purified by fractional distillation under reduced pressure until a fraction of constant boiling-point was obtained. 50 g. of this material were condensed with phosphoric acid, and 49 g. of calcium α -glycerophosphate were obtained, 52 % of the theoretical yield.

The results of several experiments with reputed α -glycerophosphate were communicated to Dr F. L. Pyman, of Boot's Pure Drug Company, who very kindly prepared a special batch of the α -salt, which appeared practically identical with that prepared in Toronto.

Methods of estimating rates of enzymic hydrolysis.

(1) *By animal phosphatase.*

Enzyme. Phosphatase occurs abundantly in liver, kidney, intestine and bone. For extraction the fresh tissue is ground up with 10–20 times its weight of chloroform water and allowed to stand for 48 hours at room temperature. It is then filtered through cotton-wool or coarse filter-paper and is ready for use.

Substrates. Approximately $M/100$ aqueous solutions of sodium glycerophosphate were used. These were prepared from the barium salts by adding an equivalent amount of sodium sulphate. The various substrates to be compared were analysed for phosphorus colorimetrically [King, 1932] and diluted to the same concentration.

Buffers. The optimum p_H for mammalian phosphatase acting on glycerophosphates is between 8.9 and 9.2. The following buffers were used in different experiments.

- (i) Sørensen's glycine buffer, p_H range 8.53–12.90 [Clark, 1928].
- (ii) Palitzsch's borate buffer, p_H range 6.77–9.24 [Clark, 1928].
- (iii) Michaelis's veronal buffer, p_H range 6.8–9.6 [Michaelis, 1930].

Method. The hydrolyses are carried out as follows. A series of tubes at a range of p_H covering the optimum is prepared (4 for each p_H), using for each 5 ml. substrate (adjusted to the desired p_H), 5 ml. buffer and 1 ml. of the enzyme solution. To two of each set of 4 tubes (the controls) are immediately added 2 ml. of 25 % trichloroacetic acid; after 10 minutes the contents are filtered. The other 2 tubes are placed in a water-bath at 37.5° for exactly 2 hours. These are then removed, 2 ml. of 25 % trichloroacetic acid added and the contents filtered after

Fig. 1.

Fig. 2.

Fig. 1. Rates of hydrolysis by takaphosphatase of pure α -; 75 % α -, 25 % β -; 50 % α -, 50 % β ; 25 % α -, 75 % β -; and pure sodium β -glycerophosphates. Phthalate buffers used.

Fig. 2. Rates of hydrolysis by mammalian phosphatase of pure α - and β -glycerophosphates. Glycine buffers used.

10 minutes. 10 ml. of filtrate from each tube are then analysed for inorganic phosphorus by a colorimetric method. The increase in inorganic phosphorus, expressed in mg. P per 10 ml. of reaction mixture, represents the amount of hydrolysis. In no case was the amount of hydrolysis a large fraction of the total substrate present.

(2) *By plant phosphatase.*

Enzyme. 0.5 ml. of 2 % takadiastase solution freshly prepared.

Buffers. The optimum p_H for plant phosphatase is 3.0–5.0.

(i) Clark and Lub's phthalate buffer, p_H range 2.2–6.2 [Clark, 1928].

(ii) Michaelis's [1931] acetate-veronal buffer, p_H range 2.62–9.64.

Substrates. These were the same as with mammalian phosphatase except that the p_H was adjusted to 4.2 before adding the buffer or the enzyme. This was the mean p_H of the several buffer mixtures used, and the final p_H of the mixtures did not differ appreciably from that of the buffer added in any case. The other details were the same as for the mammalian phosphatase.

Figs. 1 and 2 show the type of results obtained with pure α - and β -glycerophosphates using mammalian phosphatase and takaphosphatase.

Separation of the α -isomeride from the β -isomeride by enzymic means.

If pure α - or β -glycerophosphate is hydrolysed enzymically and fractions are removed from the reaction mixture as the hydrolysis proceeds, these fractions should show no difference in their rates of enzymic hydrolysis; but if a mixture of α - and β -glycerophosphates be used there should be a progressive change in the rates of hydrolysis of the fractions removed, since the β -form is hydrolysed faster than the α -isomeride. A large amount of sodium glycerophosphate was hydrolysed by the enzyme and portions of the reaction mixture were removed at the start and at approximately 25, 50 and 75 % hydrolysis. These fractions were freed from inorganic phosphorus by adding the calculated amount of magnesia

Fig. 3.

Fig. 4.

Fig. 3. Rates of hydrolysis by takaphosphatase of fractions (1, 2, 3, 4) obtained by hydrolysing reputed sodium α -glycerophosphate (source 1) with takaphosphatase.

Fig. 4. Rates of hydrolysis by takaphosphatase of fractions (1, 2, 3, 4) obtained by hydrolysing reputed sodium α -glycerophosphate (source 2) with takaphosphatase.

mixture without the ammonia. The solutions were then made pink to phenolphthalein by adding ammonia and the inorganic phosphate precipitated was centrifuged off. To the centrifugate was added an excess of normal lead acetate which precipitated the lead glycerophosphate. This was decomposed with hydrogen sulphide, and the solution, freed from this reagent, was made alkaline to phenolphthalein with baryta.

The barium glycerophosphate was precipitated by adding the filtrate to four volumes of alcohol. When two samples of reputed α -glycerophosphate, obtained from two different sources, were treated in this way and the several fractions compared for rates of enzymic hydrolysis, the results shown in Figs. 3 and 4 were obtained. These findings indicate that the α -salt as supplied to us contained some of the β -isomeride as an impurity.

Separation by double salt precipitation with barium nitrate.

An attempt was therefore made to purify commercial calcium α -glycerophosphate, after conversion into the barium salt, by a modification of Karrer and Salomon's [1926] method for differentiating the α - from the β -form. 30 g. of the barium salt dissolved in 300 ml. of water and 24 g. of barium nitrate dissolved in 300 ml. of water were mixed and allowed to stand in the refrigerator. The solution was reduced in volume until a precipitate had formed. After filtering, the filtrate was added to alcohol and the precipitated barium glycerophosphate was dissolved

Fig. 5. Rates of hydrolysis by takaphosphatase of reputed sodium α -glycerophosphate before and after treatment with barium nitrate; (1) before treatment, (2) one treatment, (3) two treatments. α - refers to authentic α -acid.

in water and reprecipitated. This process was repeated to remove all the barium nitrate. The salt was again treated with the required amount of barium nitrate, and after standing, the barium glycerophosphate was again separated. The original salt and the salt after one, and after two, treatments were then compared as to their rates of enzymic hydrolysis. The results are given in Fig. 5. Judging by the rates of enzymic hydrolysis, one treatment with barium nitrate is sufficient to free the α -salt from the contaminating β -salt.

Attempts to separate α -glycerophosphate into optical enantiomorphs.

The method used above for estimating the relative amounts of α - and β -isomerides in a mixture of the two is open to a serious objection. Since the α -form has an asymmetric carbon atom in its formula, it might be expected that *d*-, *l*-, racemic and *dl*-, forms might all exist, and that these various forms might be hydrolysed at different rates. If this were so, what is considered from

the graph to be a mixture of the α - and β -isomerides might in fact contain no β -form but might be only the *d*- or *l*- form of the α -salt being hydrolysed at a faster rate than the authentic racemic α -salt. To settle this problem an attempt was made to resolve the synthetic α -form into its optical antipodes. Some earlier workers have suggested that the naturally occurring α -form was optically active. Levene and Rolf [1919] found slight activity in glycerophosphates from natural sources. The amount of rotation depended to a large extent on the method and the amount of purification used. Karrer and Benz [1926, 1, 2; 1927], after carefully separating the α - and β -forms from natural sources, obtained no rotation for either salt. They consider that earlier workers who found activity were probably observing the rotation of some impurity. Karrer and Benz, although they failed to obtain a separation with either strychnine or quinine, still believe that the α -form is optically active, because they prepared a dimethyl ether of the dimethyl ester of the α -salt which showed some slight activity. Abderhalden and Eichwald [1918] endeavoured to prepare the α -salt in its active form by acting on *d*-monobromohydrin dissolved in pyridine with phosphorus oxychloride in the cold. After a lengthy procedure, they separated the glycerophosphoric acid as the lithium salt. The analysis gave values too low in phosphorus and too high in carbon. The observed rotation was $+0.48^\circ$, giving a specific rotation of $+6.26^\circ$. In none of these attempts has conclusive evidence been given for the resolution of the α -salt.

The following attempts have been made to resolve the α -salt into its optical enantiomorphs.

(a) *By enzymic means.* Takaphosphatase was the enzyme used. The method was similar to that described above when the purification of the commercial α -salt was discussed. The three or four fractions obtained were analysed for phosphorus and barium, and their optical activity (Hg green) and their rates of enzymic hydrolysis were compared, using both mammalian and takaphosphatase. The results obtained when purified α -glycerophosphate or the synthetic product

Fig. 6.

Fig. 7.

Fig. 6. Rates of hydrolysis by takaphosphatase of fractions obtained by hydrolysing with takaphosphatase sodium α -glycerophosphate purified by means of barium nitrate. The solid line is given by an authentic α -salt.

Fig. 7. Rates of hydrolysis by takaphosphatase of fractions obtained by hydrolysing with takaphosphatase sodium α -glycerophosphate prepared synthetically by the Pyman method. The solid line is given by an authentic α -salt.

was used are shown in Figs. 6 and 7. These graphs indicate that if any resolution has occurred, it is without effect on the rates of enzymic hydrolysis. The optical rotations were all less than the experimental error.

Propionibacterium jensenii contains a phosphatase which hydrolyses glycerophosphates at an optimum p_H of 7.0. To test the effect of this enzyme a large amount of these bacteria was obtained. By following a similar treatment to that described above for takaphosphatase, fractions were obtained at various percentages of hydrolysis, and these were compared as to their optical activity and enzymic activity. There was no significant difference between the fractions.

(b) *By the use of active alkaloids.* Brucine glycerophosphate was prepared by treating 10 g. of Ca α -glycerophosphate with sufficient *N* oxalic acid to form the free acid. The calcium oxalate was centrifuged off, and to the filtrate was added a slight excess of brucine dissolved in 50 % alcohol. The fractions which crystallised out were dissolved in 20 % alcohol, and a slight excess of barium acetate was added. The mixture was poured slowly into four volumes of alcohol and the barium glycerophosphate formed was filtered off. This salt was reprecipitated for purification. The fractions were analysed for phosphorus and compared as to rates of enzymic hydrolysis and optical activity. The results showed that, although there was a slight optical rotation in all fractions, there was no definite shift in values from one member of the series to the next. The results were with one or two exceptions within the experimental error. The resolution, if any, was without effect on the rates of enzymic hydrolysis.

A second experiment was done without converting the fractions into the barium salts. In this there was a slight shift in rotation, but since brucine itself has such a high negative rotation this might be due to varying traces of brucine.

Quinine glycerophosphate was made by mixing quinine sulphate with barium glycerophosphate. After filtering off the barium sulphate formed the mixture was allowed to crystallise spontaneously. The results from these fractions indicated that the optical rotations were not of sufficient magnitude to be considered significant. Quinine glycerophosphate was then prepared using the free alkaloid instead of the sulphate. Table I gives the results obtained.

Table I.

Fraction	Quinine glycerophosphate g.	P %	$[\alpha]_D$	Enzyme experiments mg. P hydrolysed	
				Mammalian phosphatase	Taka-phosphatase
1	10.00	4.40	-0.4°	0.238	0.129
2	14.00	3.36	-0.1	0.236	0.142
3	16.00	3.42	-0.1	0.245	0.148
4	9.00	3.45	+0.1	0.269	0.145
5	11.00	3.30	0.0	0.261	0.142
6	7.05	3.44	0.0	0.241	—
7	7.96	3.35	-0.4	0.252	—
8	7.00	3.30	-0.2	0.229	0.132

These results do not show any decided drift of optical activity in either direction. Another experiment was done without conversion into the barium salt. The fractions obtained were analysed for nitrogen and phosphorus and their solubilities determined. There was no significant difference in solubility.

From these four attempts it was concluded that there had been insufficient evidence to indicate any resolution of the α -glycerophosphate into its optical antipodes by means of the active alkaloids quinine and brucine.

There remained the possibility that a resolution had occurred but that the optical rotations of the fractions obtained were too small to measure. An attempt was therefore made to obtain an increase in rotation by the addition of tungstates

and molybdates as Gernez [1887; 1888; 1889, 1, 2] had shown to be the case with tartaric acid. When a molecular proportion of sodium molybdate was added to dilute solutions of tartaric acid and α -glycerophosphoric acid the following results were obtained.

Table II.

Salt used	g. dissolved in 15 ml.	Observed rotation before	Observed rotation after	Increase in rotation
<i>d</i> -Tartaric acid	0.150	+0.4°	+13.33°	33.3 times
α -Glycerophosphoric acid	0.172	0	0	0

These results were obtained with Dr Pyman's authentic synthetic α -salt and two fractions from enzymic hydrolysis of the synthetic α -salt. A similar result was obtained using sodium tungstate in place of sodium molybdate. When two molecular proportions of either sodium molybdate or tungstate were used the result was the same. Apparently the addition of these salts to the glycerophosphates does not enhance the rotation, if there is such, to a point where it is detectable.

SUMMARY.

Various methods for determining the relative amounts of α - and β -glycerophosphates are described, and the application of one of these to unknown glycerophosphates is given in detail.

An enzymic method for detecting small amounts of the β -isomeride in reputed α -salts is described and a method outlined for the purification of the α -salt.

An attempt has been made to resolve the α -salt into its optical isomerides using an enzymic method and the alkaloids brucine and quinine. No positive evidence of separation has been obtained.

The addition of molybdates and tungstates does not appear to increase the rotation of glycerophosphoric acid to a point where it can be read.

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