REFERENCES

- Abderhalden, E. & Abderhalden, R. (1938). *Pflüg. Arch. ges. Physiol.* **240**, 746.
 Abderhalden, E. & Abderhalden, R. (1939). *Pflüg. Arch. ges. Physiol.* **242**, 508.
 Di Maggio, G. (1946). *Boll. Soc. ital. Biol. sper.* **22**, 1081.
 Knight, B. C. J. G. (1937*a*). *Biochem. J.* **31**, 731.
 Knight, B. C. J. G. (1937*b*). *Biochem. J.* **31**, 966.
 Lwoff, A. (1943). *L'évolution physiologique*, p. 32. Paris: Hermann.
 Robbins, W. J., Bartley, M. A., Hogan, A. G. & Richardson, L. R. (1937). *Proc. nat. Acad. Sci., Wash.*, **23**, 388.

The Heterogeneity of the Glycerophosphate Fraction of Rat Liver

BY JUNE OLLEY AND M. BLEWETT

Medical Research Council, Human Nutrition Research Unit, M.R.C. Laboratories, Holly Hill, Hampstead, London, N.W. 3

(Received 28 December 1949)

Zilversmit, Entenman & Chaikoff (1948) found that in the liver of the dog, after injection of ^{32}P , a fraction rich in glycerophosphate had specific activity-time curves (S.A./ t) which indicated that glycerophosphate is a precursor of lecithin. Popják & Muir (1949) obtained similar evidence in support of this view from comparison of the S.A./ t curves of a 'glycerophosphate' fraction and those of total phospholipin in rat liver. These conclusions were based on the criteria proposed by Zilversmit, Entenman & Fishler (1943) for the relationships of the S.A./ t curves of a precursor of the ultimate compound.

The validity of these conclusions is dependent on the composition of the 'glycerophosphate' fraction. Several methods have been described for the extraction of 'glycerophosphate' from trichloroacetic acid (TCA) extracts of liver, some of which are outlined in Fig. 1.

Lepage (1944) fractionated TCA extracts of rat liver by the methods of Leva & Rapoport (1943) and Lepage & Umbreit (1943). He found that 58 % of the phosphoric esters present in the 'glycerophosphate' fraction and reacting with periodate could be accounted for as esters of known properties other than glycerophosphate, i.e. pentose-5-phosphate,

Fig. 1. Separation of 'glycerophosphate' by the methods of Zilversmit *et al.* (1948), Lepage (1944) and Kaplan & Greenberg (1944).

* S.A. = specific activity (% injected ^{32}P /mg. P).

glucose-6-phosphate and fructose-6-phosphate. Zilversmit *et al.* (1948) and Kaplan & Greenberg (1944) used the procedure of Kurssanov (1938) consisting of a 3 hr. hydrolysis with 10% (w/v) sodium hydroxide to separate glucose-6-phosphate and glycerophosphate. The 'glycerophosphate' fraction separated by the Kaplan & Greenberg (1944) procedure gave a negative test for pentose.

Methods have now been developed to test the purity of the 'glycerophosphate' fraction obtained by the various procedures. These were based on the following principles: (1) α -glycerophosphate is oxidized by periodate to phosphoglycolaldehyde; this ester is hydrolysed with liberation of inorganic phosphate in conditions in which glycerophosphate is stable, i.e. with 2N-sulphuric acid for 1 hr. at 100°. Phosphorylated aldehydes with similar hydrolysis characteristics will be formed from other compounds containing adjacent hydroxyl groups, but the rate of reaction with periodate is likely to be different. (2) Interconversion of α - and β -glycerophosphate takes place in acid or alkaline solution as shown:

The conversion of the β -compound to the α -form by sulphuric acid in the presence of periodate is complete (Burmaster, 1946).

The 'glycerophosphate' fraction was separated by various procedures from the liver of animals injected with ^{32}P . The s.a. of the inorganic phosphate liberated after hydrolysis with acid following oxidation with periodate was then estimated. In order to test the homogeneity of the fraction, the following were varied: (a) interconversion of α - and β -glycerophosphate or substituted glycerophosphate, (b) the time allowed for oxidation with periodate, (c) the time of hydrolysis with acid of the phosphorylated aldehydes. By this means it was hoped to determine the minimum number of phosphoric esters present in the 'glycerophosphate' fraction.

Compounds with adjacent hydroxyl groups reacting with periodate are subsequently referred to as α -periodate-reacting phosphoric esters (α -PE), and compounds which react with periodate only after conversion from the β - to the α -form as β -periodate-reacting phosphoric esters (β -PE).

METHODS

Experimental animals. Male albino rats of the Wistar strain weighing 150 g. were maintained for 8 days on a diet previously described (Blewett, Campbell & Olley, 1949). Food and water were removed from the animals 4 hr. before intraperitoneal injections of ^{32}P . Each animal received 50 μC . of ^{32}P as $\text{Na}_2\text{H}^{32}\text{PO}_4$ in 0.5 ml. water and was killed by

decapitation 2 hr. later. At this time the s.a. of liver inorganic P is maximal and the s.a. values of the fractions with higher turnover rates will not have decreased towards the values of the slower fractions. Differences therefore in s.a. of phosphate esters should be most easily detected at this time.

Extraction of inorganic phosphate. The method involved only the estimation of inorganic P liberated at different stages of the reactions. In single rat livers it was necessary to determine the s.a. of quantities as small as 5–10 μg . P. Precipitation of phosphate with magnesia mixtures is not quantitative at this level and strong ammoniacal solutions hydrolyse phosphoric esters other than those hydrolysed in the reaction under investigation. A method was therefore developed for removing inorganic P in the presence of other phosphoric esters without precipitation. A standard radioactive phosphate solution (5 ml.) was placed in a 10 ml. graduated stoppered cylinder. To this solution were added 1 ml. 33% (v/v) H_2SO_4 , 1 ml. 10% (w/v) ammonium molybdate and 1 ml. of a solution containing 20% (w/v) Na_2SO_3 and 0.5% quinol. The solution was made up to 10 ml. and P estimated colorimetrically after 30 min. The solution (8 ml.) was then extracted in a separating funnel for 2 min. with *iso*-butanol (5 ml.). The reduced phosphomolybdate passed into the butanol layer. After standing for 30 min. to allow for drainage the aqueous layer was separated. This was followed by a further draining period of 10 min. to ensure complete removal of the aqueous phase. The butanol layer was washed into a graduated cylinder with ethanol and the volume noted. The radioactivity of a 5 ml. sample was determined using a G.T. 11 tube set up as an immersion counter. Nine samples of phosphate solutions containing from 2 to 100 μg . P gave a mean recovery of 98.0%, the standard error of the percentage deviation being 2.0%.

Determination of the amounts and specific activities of the periodate-reacting phosphoric esters. The amounts and s.a.'s of the total α -PE and β -PE were determined by the following method: three 5 ml. samples of neutralized TCA extract of rat liver containing from 5 to 50 μg . α -PE-P were pipetted into graduated stoppered cylinders. To sample 1, 0.5 ml. of 0.1M-KIO₄ in 3N-H₂SO₄ was added and the solution allowed to stand for 1 hr. Excess periodate was then destroyed with 1 ml. of 4% sodium metabisulphite. 1 ml. 33% (v/v) H_2SO_4 was added and the mixture heated at 100° for 1 hr. Sample 2 had the same treatment as sample 1 except that periodate reagent was omitted. To sample 3, 0.5 ml. 0.1M-KIO₄ in 3N-H₂SO₄ and 1 ml. 33% H_2SO_4 were added and the mixture heated at 100° for 1 hr. Excess periodate was destroyed with 1 ml. of 4% metabisulphite. Inorganic P was estimated, extracted, and the radioactivity determined as already described. The amount and radioactivity of P liberated by acid hydrolysis only was thus obtained from sample 2. Values for sample 1–2 gave the total α -PE and those for sample 3–1 the total β -PE.

Determination of changes in specific activity during periodate oxidation and sulphuric acid hydrolysis. If several compounds of different s.a.'s in an extract react at different rates with periodate, the s.a. of the P liberated during time intervals of 0– t , t_1 – t_2 etc. will have different values. If these time intervals are sufficiently short an indication of the number of compounds present should be obtained. To get a series of specific activities at intervals during the course of the periodate oxidation samples of equal volume and composition of a neutralized TCA extract of liver were pipetted into 10 ml. graduated stoppered cylinders. Equal samples were neces-

sary to avoid the effect of varying concentration on the rate of reaction. The solutions were made to 5 ml. Portions were treated as for sample 1, but the periodate reagent was destroyed after different times. One portion was treated as for sample 2. The amount and radioactivity of the P liberated were obtained in the manner already described. In another experiment samples were oxidized with periodate for 1 hr. and the hydrolysis with H_2SO_4 of the products of periodate oxidation similarly used to obtain a series of S.A.'s. Two samples were taken for each time interval. One portion was treated as for sample 1 and the other as for sample 2. The samples were heated at 100° for various times and the acid-hydrolysable P and radioactivity measurements were subtracted from the corresponding values of the sample treated with periodate.

Preparation of radioactive α -glycerophosphate. Radioactive α -glycerophosphate was synthesized by the method of King & Pyman (1914). H_3PO_4 (1.58 g.) was added to 19 ml. of a solution containing 100 $\mu\text{c.}$ of ^{32}P as H_3PO_4 . NaOH (1.72 g.) was then added, the solution cooled, and 1.58 g. of α -monochlorhydrin (British Drug Houses Ltd.) freshly distilled at 14 mm. and 121° , added. The solution was left for several days at room temperature, the glycerophosphate extracted as described and sodium glycerophosphate prepared by double decomposition. A 41% yield was obtained.

EXPERIMENTAL AND RESULTS

The 'glycerophosphate' fraction of Zilversmit et al. (1948)

TCA extracts of rat livers were treated by the method of Zilversmit *et al.* (1948) for extraction of the 'glycerophosphate' fraction. Inorganic phosphate was precipitated with barium chloride (Ennor & Stocken, 1948). There was no appreciable loss of added radioactive α -glycerophosphate in the barium precipitate. The amounts and radioactivities of total acid-hydrolysable α - and β -periodate-reacting phosphoric ester-P were measured. The results are shown in Table 1.

It can be calculated from this table that 28% of the PE-P was in the β -form. The β -esters had an extremely low S.A. As it seemed possible that they were formed during the sodium hydroxide hydrolysis by the decomposition of a liver component with a slow rate of renewal, the ester fraction giving soluble barium salts obtained from the TCA liver extracts was examined before and after hydrolysis. A sample was hydrolysed with sodium hydroxide at 100° and treated with magnesia mixture; to another equal sample all the reagents were added, but it was not hydrolysed with sodium hydroxide. The fractions were then analysed and the changes produced by hydrolysis are shown in Table 2.

It can be seen that the amounts of residual P were almost unchanged. The loss of α -PE was greater than the total loss in two out of the three experiments. This could only be accounted for by a gain in β -PE-P and therefore there must have been a conversion of the α -compounds to the β -form by sodium hydroxide. The S.A. of the β -PE fraction after alkaline hydrolysis was very low compared with those of either the α -PE or β -PE fractions before hydrolysis (Table 2). If it is accepted that the β -PE fraction originates from the α -PE fraction, it follows that the latter is heterogeneous and contains material of very low S.A. We also investigated the S.A.'s of the α -compounds after various reaction times with periodate and sulphuric acid as described above.

Leva & Rapoport (1943) found that the reaction of glycerophosphate with periodate was completed in 40 min. and the hydrolysis of the phosphoglycolaldehyde in 1 hr. A periodate reaction rate curve was obtained for a sample of radioactive sodium α -glycerophosphate containing 27 $\mu\text{g.}$ P in an aqueous solution and in the presence of inactive TCA tissue extract containing 20 $\mu\text{g.}$ P. The reaction in the presence of tissue extract was followed by radioactivity determinations on the P extracted by isobutanol. The reaction was complete

Table 1. *Analysis of esters forming soluble barium salts obtained from TCA extracts of rat liver after 3 hr. hydrolysis with 10% NaOH*

	Total P	H_2SO_4 - hydrolysable P + inorganic P not precipitated by magnesia mixture	α -Periodate reacting ester-P	β -Periodate reacting ester-P	$\alpha + \beta$ -Periodate reacting ester-P	Residual P*
Percentage composition (10 animals)	100	18.46	39.4	15.59	—	26.55
S.E.		1.49	2.20	0.74		2.60
Specific activity 2 hr. after ^{32}P injection (12 animals)	1.15	—	1.29	0.25†	0.995	—
S.E.	0.058		0.082		0.062	

* Residual P is P resistant to hydrolysis by acid and alkali before and after treatment with periodate.

† The specific activity of the β -periodate reacting phosphate esters was computed from the mean specific activities of the α - and $\alpha + \beta$ -periodate reacting phosphate esters. The low activity of this fraction made the subtraction of individual radioactivity measurements of the α -fraction from the $\alpha + \beta$ -fraction inaccurate.

Table 2. Effect of NaOH hydrolysis on the specific activities and amounts of phosphoric esters forming soluble barium salts of TCA extract of rat liver

Rat no.	Amounts (mg. P/100 g. fresh liver)										Specific activities 2 hr. after ³² P injection			
	Acid-hydrolysable P + inorganic P not precipitated by magnesia mixture					β-Periodate reacting ester P					α-Periodate reacting ester P			
	Total P after treatment with magnesia mixture					Residual P*					β-Periodate reacting ester P			
	Before hydrolysis					Before hydrolysis					Before hydrolysis			
	Change on hydrolysis	Before hydrolysis	Change on hydrolysis	Before hydrolysis	Change on hydrolysis	Change on hydrolysis	Before hydrolysis	Change on hydrolysis	Before hydrolysis	Change on hydrolysis	Change on hydrolysis	Before hydrolysis	Change on hydrolysis	Before hydrolysis
293	30.4	3.98	15.0	7.50	1.48	2.82	3.88	0.948	10.5	0.175	1.11	1.28	1.84	0.23
294	27.5	5.68	13.1	4.80	1.75	1.56	4.48	1.59	8.07	0.830	1.07	1.19	1.28	
299	28.7	3.10	16.6	5.45	0.945	3.20	4.96	1.30	6.18	0.480	0.81	0.98	1.46	

*† See footnotes to Table 1.

between 40 min. and 1 hr. for pure glycerophosphate and in 4 min. in the presence of tissue extract. Catalysis of periodate oxidation must therefore occur in the presence of TCA tissue extracts. Small time intervals had therefore to be chosen to detect changes in s.a. during periodate oxidation.

Evidence as to the number of compounds present in the mixture may be deduced from the number of inflexions in the specific activity-reaction time (s.a./Rt) curve of the phosphate liberated. A progressive increase or decrease of s.a. could be due to

Table 3. Changes in specific activity of α-periodate reacting phosphoric esters during the periodate oxidation of rat liver extracts

(The extracts were prepared by the method of Zilversmit *et al.* (1948). The fractions from rats 296 and 297 were hydrolysed for 3 hr. with 10% NaOH. The fraction from rat 299 was not hydrolysed with NaOH. Further results are shown in Fig. 2. All samples were hydrolysed with 2N-H₂SO₄ for 1 hr. at 100°.)

Rat no.	Time interval of periodate oxidation	P liberated (μg.)	Specific activity 2 hr. after ³² P injection
296	0-8 sec.	7.06	1.08
	8-17 sec.	3.22	1.09
	17-48 sec.	6.32	0.81
	48-80 sec.	2.00	1.60
	80 sec.-2 min.	1.51	2.01
	2-10 min.	7.54	0.98
	10-15 min.	0	—
297	0-10 sec.	6.12	1.12
	10-22 sec.	3.06	0.36
	22-54 sec.	3.56	0.63
	54 sec.-4 min.	6.08	0.80
299	0-7 sec.	11.90	1.29
	7-16 sec.	3.40	0.53
	16-59 sec.	5.05	0.91
	59 sec.-4 min.	11.00	0.69
	4-15 min.	12.60	0.57

Table 4. Changes in specific activity during the H₂SO₄ hydrolysis of compounds formed by periodate oxidation of α-phosphoric esters of rat liver extracts

(The extracts were prepared by the method of Zilversmit *et al.* (1948) and hydrolysed for 3 hr. with 10% NaOH. Periodate oxidation was carried out for 1 hr.)

Rat no.	Time interval for hydrolysis with 2N-H ₂ SO ₄ at 100° (min.)	P liberated (μg.)	Specific activity 2 hr. after ³² P injection
310	0-5	13.70	0.81
	5-10	16.15	1.29
	10-20	12.40	1.57
	20-30	11.70	0.45
	30-40	21.10	0.27
	40-60	3.02	0.83
311	0-4	2.76	2.41
	4-7	2.66	1.42
	7-20	11.58	0.95
	20-30	1.20	2.80
	30-40	0	—

increasingly higher proportions of one particular compound reacting during any time interval, whereas an inversion of the $S.A./Rt$ curve must have represented the presence of a different compound predominating in the reaction at that time. Some data are shown in Tables 3 and 4 and Fig. 2. The soluble barium salts of esters in the TCA extract of the liver of rat 296, for example, must have contained at least four α -PE fractions after sodium hydroxide hydrolysis. There was a wide range of specific activities which were more marked after sulphuric acid hydrolysis of the periodate oxidation products, e.g. the specific activities for rat 310 varied from 0.26 to 1.57.

The water-insoluble, ethanol-insoluble barium salts from the trichloroacetic acid extract of rat liver

TCA extracts of rat livers were fractionated by the method of Kaplan & Greenberg (1944) and by the method of Lepage (1944). Precipitation of the insoluble barium salts of phosphates in the Kaplan & Greenberg (1944) fractionation was effected with barium chloride and sodium hydroxide. α - and β -PE-P were determined. 4.84 mg./100 g. fresh liver (s.e. 0.47; number of animals, $n=11$) of $\alpha + \beta$ -PE was found by the Kaplan & Greenberg (1944) procedure, approximately 90% being in the α -form. The total $\alpha + \beta$ -PE was 6.25 mg./100 g. (s.e. 0.57, $n=7$) by the Lepage (1944) procedure. Table 5 shows the results, for five animals, obtained with both methods on the same extract.

Table 5. Comparison of the 'glycerophosphate' fraction prepared by two methods

($\alpha + \beta$ -Periodate-reacting phosphoric esters in ethanol-insoluble fraction of TCA extract of rat liver.)

Rat no.	mg. P/100 g. fresh liver	
	Kaplan & Greenberg (1944) separation	Lepage (1944) separation
347	3.57	4.48
348	6.20	8.25
362	5.55	5.55
363	3.48	4.45
364	3.40	7.50

The higher results obtained by the Lepage (1944) method were found to be due to the difference in pH at which the ethanol precipitation was carried out. The Kaplan & Greenberg (1944) ethanol precipitation is done in the presence of 0.6 ml. *N*-acetic acid, the Lepage (1944) precipitation at pH 8.2. The extracts from two rats were treated by the Kaplan & Greenberg (1944) procedure and the ethanolic supernatant then brought to pH 8.2 with sodium hydroxide. A heavy precipitation of the salts used in the separation occurred together with 0.78 mg./100 g. and 1.24 mg./100 g. $\alpha + \beta$ -PE-P; this phosphate could not be glycerophosphate, if according to

Kaplan & Greenberg (1944) a 97% recovery of added α -glycerophosphate occurs in the ethanol precipitate—an observation which was confirmed by us on two rats. The fraction prepared by the method of Kaplan & Greenberg (1944) was therefore more likely to have a higher percentage of glycerophosphate than that prepared by the Lepage procedure.

The 'glycerophosphate' fraction of Kaplan & Greenberg (1944)

The *S.A.* values of the 'glycerophosphate' fractions prepared by this method were followed during the course of the periodate reaction. The ethanol precipitate was hydrolysed with 10% (w/v) sodium hydroxide and the liberated phosphate removed with magnesia mixture. The hydrolysate was found to contain 4.72 mg./100 g. (s.e. 0.33, $n=7$) of α -PE-P which accounted for approximately 45% of the total fraction. The *S.A.* values of the α -PE-P were significantly higher in this fraction (1.71, s.e. 0.096, $n=7$) than those of the α -PE-P separated by the method of Zilversmit *et al.* (1948) (see Table 1). Comparison with Table 2 also shows that the amounts were significantly reduced. Table 6 shows that these phosphoric esters prepared by the Kaplan & Greenberg method were also a mixture of at least three compounds, but that the very low *S.A.*'s occurring during certain time intervals in the periodate oxidation of fractions obtained by the method of Zilversmit (Table 3) are not found in the case of the phosphoric esters separated by Kaplan & Greenberg's procedure.

Table 6. Changes in specific activity of α -periodate reacting phosphoric esters during the periodate oxidation of rat-liver extracts

(The ethanol precipitates were prepared by the method of Kaplan & Greenberg (1944) and hydrolysed for 3 hr. with 10% NaOH. Further results are shown in Fig. 2.)

Rat no.	Time interval of periodate oxidation	P liberated (μ g.)	Specific activity 2 hr. after 32 P injection
330	0-8 sec.	13.3	0.81
	8 sec.-1 min.	13.5	1.68
	1-4 min.	8.5	2.13
	4 min.-1 hr.	2.3	1.15
313	0-30 sec.	14.3	1.19
	30 sec.-1 min.	1.6	2.50
	1-2 min.	5.2	1.41
	2-15 min.	5.8	1.36

An investigation of the ethanol-soluble fraction of the water-soluble barium salts separated by Kaplan & Greenberg's (1944) procedure showed a reason for this discrepancy. This fraction had a low *S.A.* at 2 hr. (Table 7). The ethanol was removed *in vacuo* and the remaining extract hydrolysed with 10% sodium hydroxide for 3 hr., neutralized and the inorganic

Table 7. *Analysis of the ethanol supernatant of a TCA extract of rat liver*

(The fraction was prepared by the method of Kaplan & Greenberg (1944) 2 hr. after injection of ^{32}P . The values are given $\pm$ s.e. (no. of animals in parentheses). The β -periodate reacting ester P after NaOH hydrolysis was $50.67\% \pm 2.32$ (10) of the total periodate reacting ester P. Glycerolphosphorylcholine is converted to 55% β -glycerol esters after 3 hr. hydrolysis with 1N-NaOH (Baer & Kates, 1948). The amounts and specific activities of the P resistant to periodate oxidation were estimated by difference.)

Treatment	P fraction	mg. P/100 g. fresh liver	Specific activity
Unhydrolysed	Total P	13.50 $\pm$ 0.63 (7)	0.371 $\pm$ 0.053 (6)
	P resistant to periodate oxidation	5.64	0.68
3 hr. hydrolysis with 10% NaOH	Total P	8.23 $\pm$ 0.58 (3)	—
	$\alpha + \beta$ -PE	7.86 $\pm$ 1.58 (7)	0.149 $\pm$ 0.029 (6)
	α -PE	3.94 $\pm$ 0.77 (7)	0.212 $\pm$ 0.039 (6)
	β -PE	3.93 $\pm$ 0.85 (7)	0.079 $\pm$ 0.017 (6)

Table 8. *Effect of NaOH hydrolysis on the solubility of the ethanol-soluble fraction of TCA extracts of rat liver*

(The ethanol-soluble fraction was separated by the method of Kaplan & Greenberg (1944). After 3 hr. hydrolysis with 10% NaOH the Ba salts of the α - and β -periodate reacting phosphoric esters were water-soluble, but were precipitated by ethanol. Small quantities of periodate-resistant phosphoric ester P were also precipitated after NaOH hydrolysis.)

Rat no.		mg. P/100 g. fresh liver			
		Unhydrolysed	NaOH hydrolysed		
		$\alpha + \beta$ -Periodate reacting P	Total P	α -Periodate reacting P	β -Periodate reacting P
330	Total fraction	6.45	9.15	3.23	3.22
330	Ethanol-insoluble Ba salt	0.176	6.22	2.42	3.20
332	Total fraction	7.11	8.38	3.26	3.85
332	Ethanol-insoluble Ba salt	0.169	7.00	3.26	3.00

P precipitated with magnesia mixture. Estimation of α - and β -PE showed that there were large quantities of these compounds in the supernatant (Table 7) and that the β -compounds had a very low s.a. Estimation of the α - and β -PE-P, in samples from two rats, before and after sodium hydroxide hydrolysis showed that the low activity β -ester was formed during the hydrolysis by conversion of the α -form; e.g. 350 μg . of α -PE-P of s.a. 0.22 yielded on hydrolysis 212 μg . of α -PE-P of s.a. 0.33 and 133 μg . of β -PE-P of s.a. 0.049. There were therefore at least two alkali-resistant esters present in this fraction which would be included in the 'glycerophosphate' fraction separated by the Zilversmit *et al.* (1948) procedure.

Table 8 shows the change in solubility of the esters in this most soluble fraction after hydrolysis for 3 hr. with 10% sodium hydroxide.

The ethanol was removed *in vacuo* and samples of the remaining extract, before and after hydrolysis for 3 hr. with 10% NaOH, were treated with 2 ml. of 50% barium acetate and a small amount of inorganic phosphate and adjusted to pH 8.2. The solution was left at 0° for 1 hr., centrifuged and the supernatant treated with 4 vol. of ethanol. The ethanol precipitate was dissolved in TCA and treated with H_2SO_4 to remove Ba. α - and β -PE-P were then determined.

It can be seen that hydrolysis converted a large percentage of the periodate-reacting phosphoric esters with barium salts soluble in ethanol into esters with water-soluble, but ethanol-insoluble, salts.

The properties of the low-activity fraction of rat liver containing the esters with barium salts soluble in ethanol are similar to those of α -glycerolphosphorylcholine, which has been extracted from beef pancreas by Schmidt, Hershman & Thannhauser (1945) and King & Aloisi (1945) and prepared synthetically by Baer & Kates (1948). Thus, the barium, lead and mercury salts are soluble in ethanol; it is only very slowly hydrolysed by N-sulphuric acid, but reacts rapidly with periodate; the formation on alkaline hydrolysis of ethanol-insoluble barium salts, which, on treatment with acid and alkali, equilibrate intermolecularly to give a mixture of α - and β -forms, parallels the formation of α - and β -glycerophosphates and choline from glycerolphosphorylcholine on alkaline hydrolysis.

*Comparison of 'glycerophosphate' fractions separated by the methods of Kaplan & Greenberg (1944) and of Zilversmit *et al.* (1948)*

In Fig. 2 the changes in s.a. during the oxidation of the α -phosphoric esters are compared. *P*, *Q*, *R* and *S* are the time intervals during which each of four esters in the fraction separated by the Zilversmit procedure were exerting a maximum influence on the s.a. of the phosphorus liberated in the periodate oxidation. *X*, *Y* and *Z* are similar time intervals for the α -phosphoric esters separated by the Kaplan & Greenberg procedure. The difference between these two sets of results may be due to the absence in the

latter of the low-activity ethanol-soluble α -ester (Table 7) producing in the first set the low activity represented by *Q*.

Fig. 2. Changes in specific activity of phosphoric aldehydes caused by the different rates of periodate oxidation of α -periodate-reacting phosphoric esters. Six representative diagrams taken from results for eleven rats. *P*, *Q*, *R* and *S* represent the measured time intervals during which each of four α -PE separated by the Zilversmit *et al.* (1948) procedure were exerting a maximum effect on the s.a. in the periodate oxidation. *X*, *Y* and *Z* are similar time intervals for α -PE separated by the Kaplan & Greenberg (1944) procedure.

Further inflexions might have been revealed if determinations had been made at smaller time intervals. It should be noted that no inflexion in the s.a./*Rt* curve would occur if two compounds in a fraction had similar reaction rates or produced changes of s.a. in the same direction. Duplicate s.a./*Rt* curves on the same liver extract showed good agreement.

DISCUSSION

Significance of the heterogeneity of the 'glycerophosphate' fraction

The 'glycerophosphate' fraction of rat liver prepared by the method of Zilversmit *et al.* (1948) has been found to be a mixture of at least four phosphoric esters reacting with periodate and contained in addition a compound or compounds resistant to acid and alkaline hydrolysis and to periodate oxidation. In this method the sugar phosphates are hydrolysed by alkali. The methods of Kaplan & Greenberg (1944) and Lepage (1944) involve a precipitation of the 'glycerophosphate' fraction with ethanol. The ethanol precipitate and ethanol-soluble fraction are then treated with alkali to hydrolyse sugar phos-

phates. At least three phosphoric esters reacting with periodate are found in the ethanol precipitate and at least two in the ethanol-soluble fraction.

The 'glycerophosphate' fraction prepared by the method of Zilversmit *et al.* (1948) therefore includes the ethanol-soluble and ethanol-insoluble compounds. These had widely differing specific activities. Ethanol-soluble barium salts of phosphoric esters from TCA extracts of rat liver have been reported by other workers. Lepage & Umbreit (1945) found 16.0 mg. P/100 g. fresh rat liver in the fraction of TCA extracts with ethanol-soluble barium salts and Rapoport, Leva & Guest (1943) give figures ranging from 3 to 9 mg./100 g. depending on the dietary treatment. Flock, Bollman & Mann (1936) found approximately 16 mg. ethanol-soluble P/100 g. fresh liver for dogs on lean meat and high fat diets. The TCA extract of dog liver obtained by Zilversmit *et al.* (1948) may have been as complex as the rat liver extracts investigated by us. It would appear therefore that the s.a./*t* curve of the complex 'glycerophosphate' of Zilversmit *et al.* (1948) cannot be used as evidence that glycerophosphate is in fact a precursor of lecithin. The marked increases in the specific activity of their alkali-stable fraction after administration of choline may be due to an increase in general liver phosphorylations and not to an increase in turnover of a lecithin precursor. An increase in the s.a. of lecithin was in fact found by these workers to be accompanied by an increase in the s.a. of both alkali-stable and alkali-labile phosphorus.

There is evidence from this work that the 'glycerophosphate' fractions extracted by other procedures are also very heterogeneous. Taking rat 310 as a typical example, Table 6 shows that the ethanol precipitate separated by the method of Kaplan & Greenberg (1944) contained, after sodium hydroxide hydrolysis, a fraction of s.a. less than 0.8, another greater than 2.1 and one of intermediate s.a. Any of these fractions could have been glycerophosphate. Compounds of higher s.a. activity were precipitated when the sodium hydroxide hydrolysate of the ethanol precipitate was treated with three volumes of ethanol, methanol, diacetone alcohol or methyl cellosolve.

Bollman, Flock & Berkson (1948) and Campbell, Olley & Blewett (1949) found that calculations assuming liver inorganic phosphate to be the precursor of phospholipins gave a constant measure of turnover rate throughout the time interval studied. They therefore concluded that the s.a./*t* curve of the true precursor of the phospholipins must follow very closely that of the inorganic phosphate. The 'glycerophosphate' fraction of rat liver was too impure to apply the criteria of Bollman *et al.* (1948) and Campbell *et al.* (1949) for glycerophosphate as a precursor of phospholipins. However, with the diet

used throughout these experiments, inorganic phosphate at 2 hr. after ^{32}P injection had an average S.A. of 2.75. One fraction in the mixture separated by Kaplan & Greenberg's (1944) procedure appeared to have a S.A. at 2 hr. approaching this value. There may therefore be a compound present in the 'glycerophosphate' fraction of rat liver with a S.A./t curve as high as that of inorganic phosphate; this would give a constant measure of phospholipin turnover if it were taken as the precursor in the calculations.

The ethanol-soluble, alkali-stable, barium salts from trichloroacetic acid extracts of rat liver

The esters with ethanol-soluble barium salts in TCA extracts of rat liver have not been identified. Rapoport *et al.* (1943) state that this fraction probably contains choline sphingosine phosphate. However, sphingosine does not react with periodate (Carter, Glick, Norris & Phillips, 1942) and choline sphingosine phosphate would not, therefore, appear to account for the phosphate liberated by periodate oxidation and subsequent hydrolysis in our experiment. There were at least two periodate-reacting phosphoric esters in our fraction of ethanol-soluble barium salts. The low-activity phosphoric ester converted to the β -form on sodium hydroxide hydrolysis appeared to have the properties of α -glycerylphosphorylcholine. Glycerylphosphorylcholine has so far only been isolated from pancreas. Schmidt *et al.* (1945), however, observed that an ethanol-soluble, light petroleum-insoluble phosphorus fraction was present in other organs including liver. Aloisi & Buffa (1948) detected an increased oxygen uptake on the addition of α -glycerylphosphorylcholine to guinea pig-liver homogenates. No increase was observed with the β -isomer. After sodium hydroxide hydrolysis the α - and β -periodate-reacting phosphoric esters in the ethanol-soluble fraction of rat liver had widely different specific activities (Table 7). If the α - β -conversion by sodium hydroxide hydrolysis were not complete, the α -compound would have been a mixture of the β -compound and a compound of higher S.A. An investigation of the S.A. of the α -ester at different time intervals during the periodate

oxidation would elucidate this point. However, the S.A. of the β -compound could be taken as that of the suspected glycerylphosphorylcholine. The S.A. of total phospholipin at 2 hr. for rats fed on the same diet was found to be 0.38 (S.E. 0.028, $n=9$). Blewett *et al.* (1949) found for rats under identical conditions that the choline-containing and non-choline-containing phospholipin had the same activity at 6 hr. after ^{32}P injection. Were this β -periodate-reacting ester of S.A. 0.079 at 2 hr. after ^{32}P injection definitely proved to be α -glycerylphosphorylcholine, the possibility of this compound as a precursor of lecithin in the liver would be excluded. Schmidt *et al.* (1945) found that glycerylphosphorylcholine is split by alkaline phosphatase into its three constituents. The compound may therefore well be a metabolite in the degradation of lecithin.

The ethanol-soluble fraction from TCA extracts of rat liver has been found to contain several phosphorus compounds in quite large amounts. These are interesting because of their low specific activities and possible relationship to the phospholipins.

SUMMARY

1. A method has been developed by which the heterogeneity of a mixture may be established from the changes in specific activity during chemical treatments. This method has been applied to the 'glycerophosphate' fraction of rat liver.

2. The 'glycerophosphate' fraction of rat liver obtained by ethanol precipitation has been found to contain, after hydrolysis with sodium hydroxide, at least three periodate-reacting phosphoric esters of widely differing specific activities.

3. A compound which has many of the properties of α -glycerylphosphorylcholine has been found in a trichloroacetic acid extract of rat liver. This compound has a lower specific activity at 2 hr. than lecithin and may be a degradation product of the latter.

The authors would like to thank Prof. B. S. Platt for his interest in this work and also Mr I. G. Campbell for his helpful suggestions.

REFERENCES

- Aloisi, M. & Buffa, P. (1948). *Biochem. J.* **43**, 157.
Baer, E. & Kates, M. (1948). *J. biol. Chem.* **175**, 79.
Blewett, M., Campbell, I. G. & Olley, J. (1949). *Nature, Lond.*, **164**, 621.
Bollman, J. L., Flock, E. V. & Berkson, J. (1948). *Proc. Soc. exp. Biol., N.Y.*, **67**, 308.
Burmester, C. F. (1946). *J. biol. Chem.* **164**, 233.
Campbell, I. G., Olley, J. & Blewett, M. (1949). *Biochem. J.* **45**, 105.
Carter, A. E., Glick, F. J., Norris, W. P. & Phillips, G. E. (1942). *J. biol. Chem.* **142**, 449.
Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **42**, 549.
Flock, E., Bollman, J. L. & Mann, F. C. (1936). *J. biol. Chem.* **115**, 179.
Kaplan, N. O. & Greenberg, D. M. (1944). *J. biol. Chem.* **156**, 511.
King, E. J. & Aloisi, M. (1945). *Biochem. J.* **39**, 470.
King, H. & Pyman, F. L. (1914). *J. chem. Soc.* **105**, 1238.
Kurssanov, A. (1938). *Biochimia*, **3**, 467.
Lepage, G. A. (1944). *J. biol. Chem.* **152**, 593.

- Lepage, G. A. & Umbreit, W. W. (1943). *J. biol. Chem.* **147**, 263.
- Lepage, G. A. & Umbreit, W. W. (1945). In *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, by W. W. Umbreit, R. H. Burris & J. F. Stauffer. Minneapolis: Burgess Publishing Co.
- Leva, E. & Rapoport, S. (1943). *J. biol. Chem.* **149**, 47.
- Popják, G. & Muir, H. (1949). *Biochem. J.* **45**, xi.
- Rapoport, S., Leva, E. & Guest, G. M. (1943). *J. biol. Chem.* **149**, 57.
- Schmidt, G., Hershman, B. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 523.
- Zilversmit, D. B., Entenman, C. & Chaikoff, I. L. (1948). *J. biol. Chem.* **176**, 193.
- Zilversmit, D. B., Entenman, C. & Fishler, M. C. (1943). *J. gen. Physiol.* **26**, 325.

The Quantitative Reactions of Thiols and Disulphides with Silver Nitrate

By R. CECIL

Department of Biochemistry, University of Oxford

(Received 3 November 1949)

1. The Estimation of some Thiols and Disulphides

Most of the existing methods of estimating thiols depend on the quantitative oxidation of thiols to disulphide. Errors may result from the oxidation proceeding beyond this stage, or from other substances present being oxidized simultaneously. A further difficulty arises when the amount of oxidation is measured colorimetrically, as the readings have to be compared against that given by a standard solution of the thiol concerned. Since most thiols, with the exception of reduced glutathione (GSH), oxidize appreciably in the presence of air, their standard solutions must be checked simultaneously by another method if errors are to be avoided.

These remarks apply equally to the estimation of disulphides, since the available methods consist in reducing them in whole or in part to thiols and estimating the latter.

A method making use of the direct reaction of the thiol group with a heavy metal would have advantages over the oxidation methods. Kolthoff & Harris (1946) have devised an amperometric titration method for thiols with silver nitrate. Benesch & Benesch (1948) reduced the scale of the method and applied it to biologically important thiols. They also applied it to serum proteins, but in doing so they took no account of the reaction between silver ions and disulphide groups (see Part 2 of this paper), which may well have affected their results.

In the method to be described, which was developed independently, the same reaction, namely the formation of silver mercaptide, is used, but the amount of free silver ion present is measured potentiometrically using a silver electrode. The addition of an excess of silver nitrate and estimation of the remaining free silver ion by back titration with potassium bromide, using the silver electrode to determine the end point, was found to be more

convenient than a direct titration with silver nitrate.

This method has also been adapted to the estimation of disulphides. These are first split by the action of sodium sulphite (Clarke, 1932), and the thiol formed is then estimated.

EXPERIMENTAL

Apparatus

Since absolute measurements of electrode potentials are not required in this type of work it is permissible to use a calomel reference electrode together with a salt bridge. So long as the diffusion processes at the liquid junctions cause only small variations in the e.m.f. of the cell, the measurements made can be interpreted with sufficient accuracy in terms of the concentration of the ion, or the activity of its salt, to which the electrode is reversible.

The arrangement used (Fig. 1) was designed to allow electrical contact to be made between the calomel electrode and the solution being titrated, without contaminating the latter with chloride. A good tap at *A* is essential, as any leakage results in chloride siphoning over into the titration vessel. Sufficient grease can be applied to the tap to prevent any leakage while still retaining adequate conductivity.

The Ag electrodes consisted of thin Pt wire which was lightly plated with Ag just before use. They gave the theoretical slope of 58 mV./pAg unit at 18° over the range pAg 3 to pAg 9–10, where $pAg = -\log [Ag^+]$. The glass electrodes were made according to MacInnes & Dole (1929) from Corning 015 electrode glass. All values of pH quoted were measured with them. Both the glass and the Ag-plated platinum electrodes were fused on to glass side arms which were conveniently mounted in lumps of plasticine on brass blocks.

Potentials were measured with a standard Cambridge pH meter, used throughout as a millivolt-meter. Readings with the glass electrodes were reproducible to 1 mV. and with the Ag to 0.5 mV. When a glass electrode was being used, a Weston cell was introduced into the circuit in such a way that the glass electrode was always negative to the calomel electrode and so could be connected to the grid of the electrometer valve.