The Distribution of Acid-soluble Phosphates in the Fatty Liver

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Although increased attention has recently been paid to the function of the acid-soluble phosphates in liver metabolism, the bulk of the investigations has been concerned with changes in the distribution of organic phosphates brought about by variations in diet (Flock, Bollman & Mann, 1936*a*; Kaplan & Greenberg, 1944*b*; Rapoport, Leva & Guest, 1943 *a*, *b*). The effect of injections of glucose (Kaplan & Greenberg, 1944*a*), insulin (Cori & Goltz, 1925; Nelson, Rapoport, Guest & Mirsky, 1942; Kaplan & Greenberg, 1943, 1944*a*) and various enzyme inhibitors has also been studied. Little work, however, appears to have been done on the acid-soluble phosphates of fatty livers, although Flock *et al.* (1936*a*), working with dogs maintained on a high fat diet and thus having fatty livers, have stated that if the diet is given for 3 weeks or longer there is a decrease of inorganic phosphate to 60% of the normal value. The same authors (Flock *et al.* 1936*b*) also subjected dogs to carbon tetrachloride anaesthesia for 1 hr. on four successive days, but found that the distribution of the various acid-soluble-phosphate fractions from the livers was unaffected. The distribution of acid-soluble phosphates from fatty livers has recently become of added interest in view of the importance of adenylic acid in the oxidation of butyrate (Munoz & Leloir, 1943).

It has already been shown that the oxygen consumption and the acetoacetic acid production of liver slices from guinea pigs treated with carbon tetrachloride is considerably in excess of normal (Ennor, 1942). This together with the fact that adenosine triphosphate (*ATP*) is essential for the oxidation of fatty acids (Lehninger, 1945 *a*, *b*) suggested the likelihood of a correlation between the adenosine polyphosphate (*APP*) content and the increased metabolism of these fatty livers. Experiments designed to test this possibility have been carried out and are described in this communication. Preliminary results were reported earlier (Ennor & Stocken, 1947).

EXPERIMENTAL

Expression of results

All the above workers have expressed their results on the basis of wet weight of tissue, but, since it has been shown that the liver is subject to wide variations in water, fat and glycogen content (cf. Higgins, Berkson & Flock, 1932, 1933), it is clear that reference to a weight which includes these substances is open to criticism. Flock *et al.* (1936*a*) commented on this, but were unable to show that calculation on the basis of dry, fat-free weight altered their conclusions. Since variations in the acid-soluble organic phosphates of liver reflect variations in the metabolism of carbohydrate, protein and fat, and thus in the activities of the various enzymes concerned, it seems desirable to express all results in a more satisfactory manner. Expression on the basis of percentage N or nucleoprotein N would largely overcome these objections, and the figures so obtained could be more readily capable of interpretation from the standpoint of metabolic activity. Nucleoprotein N would appear to be the choice, but, because of the technical difficulties associated with its estimation when a large number of other determinations have to be done, all the results quoted in this communication have been expressed on the basis of total liver N. However, for the purpose of comparison with the results of other workers, some of our results are also quoted on the basis of wet liver weight. It is realized that the presence of labile protein in the liver will perhaps complicate the picture, but it is thought that the error introduced will be small, particularly as the mobile protein stores are largely depleted following a 24 hr. fast.

Methods

Phosphorus estimation. Inorganic phosphate was determined by the method of Berenblum & Chain (1938) and total P by the same method after ashing with HNO_3 and H_2SO_4.

Nitrogen. Duplicate samples (*c.* 0·1 g.) of unfrozen liver were ashed with the digestion mixture described by Campbell & Hanna (1937), and after suitable dilution samples were distilled in the apparatus of Markham (1942). The distillate was collected in a boric acid buffer used by Conway (1942) and titrated with 0·01 N-H_2SO_4.

Fat. In this paper 'fat' refers to the material extractable from the liver by means of a 70/30 (v/v) ethanol-diethyl ether mixture.

Creatine. The method used was that of Eggleton, Elsden & Gough (1943).

Pentose. This was estimated according to the method of Meijbaum (1939), but as suggested by Stone (1943) a solution of barium adenosine triphosphate was used as a reference standard.

Treatment of animals. Male guinea pigs, of *c.* 300 g. body weight, fed on a liberal diet of bran, cabbage and swede, were used throughout. Treated animals were given, on successive days, three subcutaneous injections each of 0·2–0·3 ml. of a mixture containing equal volumes of carbon tetrachloride and arachis oil. Free access was allowed to water, but food was withheld from both treated and control animals for 24 hr. prior to the experiment. Treated animals were killed 24 or 48 hr. after the final injection.

Preparation of extracts. The animals were stunned by a blow on the head and the neck vessels severed. The liver was rapidly removed and the major portion dropped into liquid air; the remainder was analyzed for N, total P and fat. After a preliminary grinding in a mortar chilled with liquid air, duplicate samples of 2–3 g. of the frozen liver were homogenized with 4 ml. of ice-cold 10% (w/v) trichloroacetic acid (*TCA*). The homogenizer was similar to that described by Potter & Elvehjem (1936) but was provided with a stainless-steel pestle. The homogenate was diluted with 20 ml. of 5% (w/v) *TCA* and, after centrifugation, the residue was re-extracted with a further 20 ml. of the 5% acid. Since this procedure served to extract not less than 97% of the acid-soluble phosphates, further extraction was considered unnecessary. The combined extracts were made up to a total volume of 50 ml. with 5% *TCA*, filtered if necessary, and analyzed for inorganic and total P.

RESULTS

There is a marked difference in the total-P contents of the fresh livers from the treated and the control animals (Table 1). A similar difference is also apparent when the total- and organic-P contents of the *TCA* extracts are considered. Thus, when the figures are expressed on the basis of the wet weight of the liver, the values for the total P and total and organic acid-soluble P are higher in the control group than in the treated. Expression on the basis of the N content of the liver, however, leads to a reversal of this result.

Because of the facts that *ATP* is essential for the oxidation of fat, that there is a well recognized association between oxidation and phosphorylation, and that there is an increase in the rate of O_2 uptake in fatty livers (cf. Meier & Thoenes, 1933; Ennor, 1942), it might be anticipated that there would be an increase in the high energy phosphate levels in such livers. Attention was, therefore, in the first instance, focused on the compounds hydrolyzable in 7 min. in N-acid at 100°. A separation of *APP* from other easily hydrolyzable compounds, such as acetyl phosphate and phosphocreatine, and from many organic phosphates, is possible by barium precipitation and this was carried out on the *TCA* extracts. Several methods have been described for the preliminary separation, but that described by Le Page & Umbreit (1945) is the most convenient. This procedure has the advantage over the alternative one, in which solid baryta is used, in that it is much quicker, but it should be noted that the use of NaOH for the neutralization of *TCA* extracts results in the incomplete precipitation of inorganic phosphate at pH 8·2. Kosterlitz & Ritchie (1943) have commented on this, although it is not clear what concentration of Ba^{++} was present in their extracts. Under our conditions adjustment to pH 9·5, as recommended by these workers, results in a decrease of the solubility of barium phosphate to less than 1 μg. of P/ml. as compared with approximately 2·5 μg. of P/ml. at pH 8·2. In the bulk of our experiments, however, this latter pH has been chosen since the faint pink end point of phenolphthalein is easily reproducible. Further, the small amounts of inorganic phosphate which appear in the mother liquor do not introduce any complication.

Table 1. *Nitrogen, phosphorus and fat analyses of livers and liver extracts from control guinea pigs and from guinea pigs treated with carbon tetrachloride*

Fresh liver				*TCA* extract			
		Total P		Total P		Organic P	
Fat (%)	N (%)	(mg./g. N)	(%)	(mg./g. N)	(mg./100 g.)	(mg./g. N)	(mg./100 g.)
				Control animals			
—	3·51	94	0·33	24·7	86·6	17·9	62·6
6·6	3·58	91	0·33	26·5	95·0	20·6	73·9
6·6	3·73	92	0·34	27·4	102·0	21·7	80·7
—	3·54	98	0·35	29·2	102·0	22·8	79·4
7·8	3·63	95	0·34	27·4	99·4	20·6	74·8
6·0	3·06	—	—	27·6	84·6	20·4	62·4
—	3·78	93	0·35	28·3	107·0	22·5	85·4
5·4	3·65	—	—	25·5	93·0	19·1	69·6
9·0	3·57	—	—	27·0	96·5	20·3	72·6
	Mean 3·56	**94**	**0·34**	**27·1**	**96·2**	**20·7**	**73·5**
				Animals treated with carbon tetrachloride			
16·2	1·84	122	0·23	36·8	67·8	25·4	47·0
12·5	2·20	134	0·29	30·4	66·9	21·9	48·2
10·6	2·46	100	0·24	33·0	82·0	24·4	60·5
13·0	2·30	107	0·24	36·1	83·0	29·0	66·7
14·6	2·20	—	—	35·1	77·3	27·4	60·4
14·5	1·95	—	—	39·8	77·6	31·9	62·2
	Mean 2·16	**115**	**0·25**	**35·2**	**75·6**	**26·7**	**57·5**

Stone (1943) has found that the amount of *ATP* which is precipitable from *TCA* extracts of brain tissue depends on the concentration of $Ca(OH)_2$. Experiments to determine the amount of *ATP* precipitable by barium under our conditions indicated that there was no significant difference in the amounts precipitated when the concentration of Ba^{++} ranged from M/8 to M/30 and that the amount of *ATP* which remained in the mother liquor was less than 0·2 μg. P/ml. The final procedure adopted for the separation was, therefore, as follows.

To 30 ml. of the *TCA* extract was added 1 ml. of 20% (w/v) $BaCl_2$ and sufficient 5 N-NaOH to bring the pH to 8·2 with phenolphthalein as internal indicator. The solution was allowed to stand for 20 min. to ensure complete flocculation and then centrifuged. The supernatant fluid was made up to 50 ml.: the precipitate was dissolved in the minimum volume of 0·1 N-HCl and made up to 25 ml. with water. All manipulations were carried out at 0°. Except where stated the barium was not removed from the various fractions prepared from the *TCA* extracts, since $BaSO_4$ does not interfere with the method used for the estimation of P.

It is clear that there is a marked increase in the 7 min.-hydrolyzable compounds present in the barium-insoluble fraction of *TCA* extracts of the fatty liver. Moreover, this increase is demonstrable whether expressed on the basis of wet weight or N, and in general may be correlated with the fat content (Table 2).

analytical methods have been criticized by Kalckar (1945), although it is not clear just how his criticism can be applied to the results obtained by these workers. A more satisfactory method for the identification of the compound responsible for the liberation of the phosphate under the conditions of hydrolysis lies in the determination of the N, P and pentose ratio. By this method Le Page & Umbreit (1945) and Le Page (1946) found that in the livers of rats the ratio of *ADP* to *ATP* is about 17 : 1. On the other hand Rapoport (1945), using myosin and a specific nucleotidase, produced evidence indicating a ratio of 2 : 1 in the fed, and 5 : 1 in the starved, liver.

We have also determined the ratios of N : 7 min.-labile P : pentose in the reprecipitated barium insoluble fractions. The normal liver gave a ratio of 5·0 : 1·3 : 0·9 and the fatty liver 5·0 : 1·3 : 1·0, and since *ADP* requires a ratio of 5·0 : 1·0 : 1·0 it might be concluded that a considerable proportion of the P released is derived from *ADP*. This conclusion, however, was not confirmed when the adenosine triphosphatase (*ATP*-ase) method (Rapoport & Nelson, 1945) for the determination of *ATP* was employed. From the N, P and pentose determinations a molar ratio of *ATP* to *ADP* ranging from 1 : 1 to 1 : 7 was indicated, whereas the ratios as determined by the myosin *ATP*-ase ranged from > 90 : 1 to 2 : 1. Because of the high degree of substrate specificity of the *ATP*-ase it would seem that

Table 2. *Amounts of phosphorus released in* 7 *min.* (P_7) *at* 100° *in* N-HCl *from the barium-insoluble fractions of TCA extracts prepared from livers of control guinea pigs and of guinea pigs treated with carbon tetrachloride*

Control animals				Animals treated with carbon tetrachloride			
Fat (%)	N (%)	P_7 (mg./100 g.)	P_7 (mg./g. N)	Fat (%)	N (%)	P_7 (mg./100 g.)	P_7 (mg./g. N)
—	3·89	4·8	1·2	23·0	2·15	9·8	4·5
—	3·58	6·4	1·7	10·8	2·53	5·5	2·1
—	3·63	3·9	1·1	15·9	1·86	6·9	3·7
—	3·25	3·3	1·0	13·0	1·77	5·3	3·0
—	3·57	3·8	1·1	16·2	1·84	6·1	3·3
9·8	3·48	4·7	1·4	12·5	2·20	6·2	2·8
—	3·51	5·7	1·6	13·4	2·79	5·9	2·1
6·6	3·58	1·9	0·6	14·5	1·95	8·8	4·5
6·6	3·73	4·5	1·1	10·6	2·46	6·9	2·9
6·0	3·06	6·3	2·0	13·0	2·30	8·0	3·6
5·4	3·65	7·4	2·0	14·6	2·20	8·5	3·9
9·0	3·57	7·5	2·1	—	—	—	—
8·8	3·93	8·3	2·1	—	—	—	—
		Mean 5·3	1·4			7·1	3·3

Most workers have been unconcerned with the origin of the inorganic phosphate released from this fraction in 7 min. at 100° in N-acid and many have, in fact, referred to it as *ATP*. In muscle extracts this phosphate certainly arises from *ATP*, but the picture in liver is somewhat different, as Kalckar (1945) has pointed out. Kaplan & Greenberg (1944*c*) concluded that the compound is *ATP*, but their these figures are the more reliable. The explanation of these differences lies in the possibility of contamination of the fraction with some N compound, the presence of which will greatly affect the ratio of labile P to N.

Having established that there was a marked increase in the 7 min.-hydrolyzable fraction, it was considered desirable to investigate the possibility

of changes in the concentration of compounds hydrolyzable at longer intervals. Hydrolysis curves were determined on samples of both the original *TCA* extracts and the barium-insoluble fraction. All hydrolyses were carried out in N-HCl at 100° for periods of 7, 15, 30, 60 and 180 min., and on removal from the bath the tubes were rapidly cooled in a freezing mixture and then analyzed for the inorganic phosphate released. Figs. 1 and 2 illustrate curves

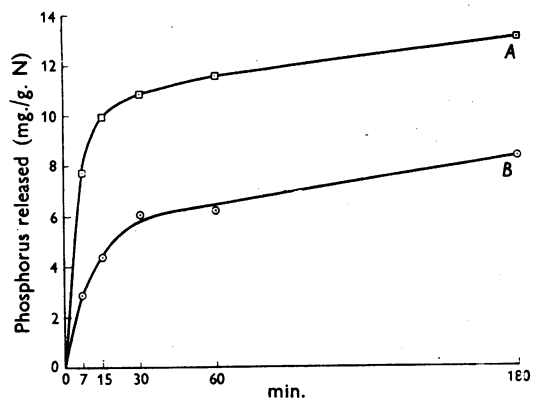

Fig. 1. Hydrolysis curves illustrating the amounts of inorganic P released at 100° in N-HCl for varying periods from *TCA* extracts of a fatty liver (*A*) and a normal liver (*B*). All points on the curves represent the means of at least two determinations.

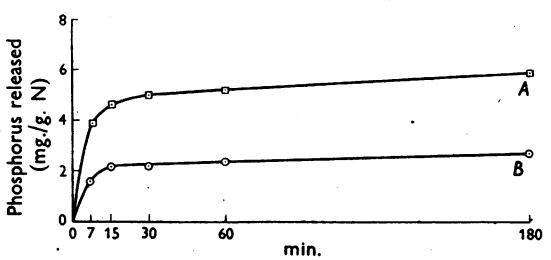

Fig. 2. Hydrolysis curves illustrating the amounts of inorganic P released at 100° in N-HCl for varying periods from the barium-precipitable fraction of *TCA* extracts, the hydrolysis curves of which are shown in Fig. 1. *A*, fatty liver, *B*, normal liver. All points represent the means of at least two determinations.

typical of those obtained from normal and fatty livers. It will be seen that the general shape of the curves is similar, but those illustrating the behaviour of the phosphate compounds in the fatty liver are on a higher level, due largely to the presence of increased amounts of compounds hydrolyzable in 7 min. This is more clearly seen from the data given in Table 3. At the 7–15 min. interval in the *TCA* extracts the difference in favour of the fatty liver is proportionally the same as in the 0–7 min. interval. For the remaining intervals no certain conclusions can be reached as to any differences between the normal and fatty liver.

The compounds which appear in the *TCA* extracts of liver include *ATP*, *ADP*, glucose-1-phosphate, phosphocreatine, phosphopyruvic acid, hexosediphosphate, fructose-6-phosphate, phosphoglyceric acid and possibly acetyl phosphate. Breakdown of these compounds, if present in our extracts, must therefore be responsible for the differences between the normal and the fatty liver in the 0–7 min. period. Since the first four compounds and acetyl phosphate are completely hydrolyzed in this interval, they are likely to be responsible for by far the greater part of the P released.

ATP, *ADP* and hexosediphosphate are precipitable by barium and it follows that the differences between the amounts of P released in 7 min. in the *TCA* extracts and in the barium-insoluble fraction will show whether the increased amount of *APP* is, in fact, entirely responsible for the difference noted in the 7 min.-hydrolyzable compounds present in the *TCA* extract. These differences, together with those at other intervals, are presented in Table 4, and in most instances were obtained by direct estimation in the barium-soluble fraction and not from the difference between the values for the *TCA* extracts and the barium-insoluble fraction.

These figures also show a marked difference between the normal and fatty livers, and it is clear that the increased amount of P released in 7 min. from *TCA* extracts of fatty livers (Table 2) is not entirely due to the differences which have been noted in the barium-precipitable fraction. Thus, in addition to a rise in the *APP* content, there is also an increase in readily hydrolyzable compounds in the barium soluble fraction. These differences persist to about the same degree over the 7–15 min. interval, whilst the amounts released over the 15–30 min. period are of the same order. Rather more P is apparently released between 30 and 60 min. with the fatty livers, but here, as in the next interval, the results are too variable to permit any definite conclusions. No attempt has been made, as yet, to identify the compounds responsible for any differences noted at the longer time intervals since interest was, in the main, centred on the more readily hydrolyzable compounds.

Most workers, who have been concerned with the estimation and the identification of labile P compounds, appear to have used either the method of Fiske & Subbarow (1925) or modifications thereof. In these methods the phosphomolybdate is reduced in aqueous solution and it is essential to adhere strictly to carefully standardized conditions in order to achieve consistent and dependable results (cf. Stone, 1943).

Table 3. *Amounts of phosphorus released at various time intervals in* N-HCl *at* 100° *from TCA extracts and barium-insoluble fractions prepared from livers of control guinea pigs and of guinea pigs treated with carbon tetrachloride*

TCA EXTRACT

Animals treated with carbon tetrachloride

0–7 min.		7–15 min.		15–30 min.		30–60 min.		60–180 min.		Total, 180 min.	
(mg./ g. N)	(mg./ 100 g.)	(mg./ g. N)	(mg./ 100 g.)	(mg./ g. N)	(mg./ 100 g.)	(mg./ g. N)	(mg./ 100 g.)	(mg./ g. N)	(mg./ 100 g.)	(mg./ g. N)	(mg./ 100 g.)
7·8	17·9	—	—	—	—	—	—	—	—	—	—
6·2	14·7	—	—	—	—	—	—	—	—	—	—
6·3	11·6	2·8	5·3	1·4	2·6	1·0	1·9	0·8	1·4	13·1	24·2
5·5	11·9	2·4	5·3	1·0	2·3	0·7	1·6	1·5	3·7	10·6	23·4
5·0	13·2	2·1	5·8	0·5	1·4	0·9	2·7	—	—	—	—
9·6	18·6	3·2	5·6	1·1	2·2	1·0	1·7	1·7	3·5	15·3	29·8
7·8	17·2	2·2	4·7	0·9	1·9	0·7	1·6	1·5	3·5	14·3	31·5
Mean **6·9**	**15·0**	**2·5**	**5·3**	**1·0**	**2·1**	**0·9**	**1·9**	**1·4**	**3·0**	**13·3**	**27·2**

Control animals

2·9	10·4	1·5	5·4	1·7	5·8	0·0	0·0	2·2	8·0	9·5	35·3
3·9	14·3	1·1	4·1	1·0	3·6	0·1	0·2	1·9	7·0	11·1	31·4
4·2	14·9	1·4	5·0	0·5	1·8	0·0	0·0	2·2	7·8	12·0	43·1
4·0	15·8	1·2	4·9	0·8	3·0	1·2	4·6	1·1	4·7	10·0	39·4
2·4	8·6	—	—	—	—	—	—	—	—	—	—
3·0	11·8	—	—	—	—	—	—	—	—	—	—
4·0	14·3	—	—	—	—	—	—	—	—	—	—
3·0	11·0	—	—	—	—	—	—	—	—	—	—
4·5	15·7	1·6	5·6	0·3	1·3	1·2	5·2	—	—	—	—
Mean **3·5**	**13·0**	**1·4**	**5·0**	—	—	—	—	—	—	—	—

BARIUM-INSOLUBLE FRACTION

Animals treated with carbon tetrachloride

2·6	8·0	—	—	—	—	—	—	—	—	—	—
2·9	6·9	—	—	—	—	—	—	—	—	—	—
3·3	6·1	0·6	1·4	0·6	1·0	0·2	0·3	0·5	1·0	3·1	5·6
2·8	6·2	0·7	1·5	0·2	0·3	0·2	0·6	0·5	0·8	2·9	6·6
2·1	5·9	0·8	2·4	0·4	1·0	0·0	0·0	—	—	—	—
4·5	8·8	1·4	2·6	0·5	1·0	0·5	0·9	1·0	2·0	1·6	3·2
3·9	8·5	0·7	1·7	0·4	0·8	0·2	0·3	0·7	1·6	3·0	6·6
Mean **3·2**	**7·2**	**0·8**	**1·9**	—	—	—	—	—	—	—	—

Control animals

1·6	5·6	0·6	1·9	0·0	0·0	0·1	0·3	0·4	1·4	1·8	6·3
2·0	7·4	0·5	1·9	0·4	1·6	0·0	0·0	0·9	3·3	1·8	6·4
2·1	7·5	0·6	2·0	0·3	1·1	0·0	0·0	0·4	1·4	2·8	9·8
2·1	8·3	0·6	2·3	0·1	0·2	0·7	2·6	0·2	0·8	1·8	7·1
Mean **1·9**	**7·2**	**0·6**	**2·0**	—	—	—	—	—	—	—	—

The presence of phosphocreatine in rat-liver extracts has been denied (Flock *et al.* 1936*a*) and affirmed (Le Page & Umbreit, 1945; Le Page, 1946), although in no case have any specific methods for its identification been employed. The failure of Flock *et al.* (1936*a*) to detect phosphocreatine may have been due to their use of mercury for the removal of glycogen from the extracts. This metal is known to produce rapid catalytic decomposition of phosphocreatine (Fiske & Subbarow, 1929). Since the latter is very labile in acid molybdate, it is customary to follow either the comparative colorimetric procedure employed by Fiske & Subbarow (1929), or to precipitate the inorganic phosphate and then obtain the phosphocreatine by difference. A somewhat similar procedure was adopted in the first instance for the determination of acetyl phosphate (Lipmann & Tuttle, 1944). In the method for the determination of P devised by Berenblum & Chain (1938), however, the acid molybdate-labile compounds, if present, are only in contact with the acid phase for 1 min. during the extraction of the phosphomolybdate complex by *iso*butanol. The aqueous layer is then discarded and it is thus only the P released in this short interval which adds to the 'true' inorganic phosphate present. According to Fiske & Subbarow (1929) 28·9 % of the phosphocreatine present is hydrolyzed in 3 min. at 18° in acid molybdate and Lipmann & Tuttle (1944) have shown that acetyl phosphate is 75 % decomposed in *c.* 2 min. at 23°.

Table 4. *Amounts of phosphorus (mg./g. N) released in the indicated intervals from compounds present in the barium-soluble fraction of TCA extracts of livers of control guinea pigs and of guinea pigs treated with carbon tetrachloride, and hydrolyzable in* N-HCl *at* 100°

0–7 min.	7–15 min.	15–30 min.	30–60 min.	60–180 min.	Total, 180 min.
Animals treated with carbon tetrachloride					
3·0	2·2	0·8	0·8	0·3	10·0
2·7	1·7	0·8	0·5	1·0	7·7
2·9	1·3	0·1	0·9	—	—
5·1	1·8	0·6	0·5	1·2	13·2
3·9	1·5	0·5	0·5	0·8	11·0
3·3	—	—	—	—	—
5·2	—	—	—	—	—
Mean 3·7	1·7	—	—	—	—
Control animals					
1·3	0·9	1·7	0·0	1·8	7·7
1·9	0·6	1·0	0·0	1·0	9·0
2·1	0·8	0·2	0·0	1·8	9·4
1·9	0·6	0·8	0·5	1·9	8·2
1·8	—	—	—	—	—
1·9	—	—	—	—	—
2·0	—	—	—	—	—
Mean 1·8	0·7	—	—	—	—

It is thus clear that these compounds will only be estimated in part as inorganic phosphate when the Berenblum & Chain (1938) method is employed, and the remainder would be estimated together with other labile compounds in the 7 min.-hydrolyzable fraction. The compounds which are likely to make a substantial contribution to the amount of P released in 7 min. in the barium-soluble fraction are phosphocreatine, glucose-1-phosphate, phosphopyruvic acid and possibly acetyl phosphate. It follows, therefore, that if these are present in increased amounts in the fatty liver, they would account in some measure for the increase in the 7 min.-hydrolyzable material seen in the barium-soluble salts derived from the *TCA* extracts of the fatty liver.

The presence of a molybdate-labile compound or compounds in the *TCA* extracts was indicated by increases in the amounts of P measurable after allowing the sample to remain in the acid molybdate for 30 min. at 30° before extraction of the phosphomolybdate complex with *iso*butanol. Under these conditions we have established that phosphocreatine is completely decomposed and that no P is split from *ADP* or *ATP*.

The results (Table 5) indicate that there exists a marked difference between the control and the treated series and that, moreover, the normal liver contains some acid molybdate-labile compound or compounds. Since the fatty livers from guinea pigs treated with carbon tetrachloride exhibit an increased acetoacetic acid production (Ennor, 1942), it may be presumed that increased amounts of fat are being oxidized. It would seem, therefore, that such livers should contain demonstrable amounts of acyl phosphates, if these are formed from the fatty acid chain as has been suggested by Lehninger (1945*b*). We have made repeated attempts to detect the presence of this compound by the hydroxylamine method described by Lipmann & Tuttle (1945). In no case has a positive reaction been obtained, even though extracts have been employed containing amounts of labile P corresponding to 60 μg. of acetyl phosphate/ml.

Table 5. *Acid molybdate-labile compounds present in TCA extracts of livers from control guinea pigs and from guinea pigs treated with carbon tetrachloride*

Control animals		Animals treated with carbon tetrachloride	
P (mg./g. N)	P (mg./100 g.)	P (mg./g. N)	P (mg./100 g.)
0·9	2·7	—	—
0·2	0·6	1·5	3·1
0·4	1·4	1·7	2·8
0·8	2·8	2·1	4·1
0·2	0·7	1·8	4·8
0·5	1·6	—	—
Mean 0·5	1·6	1·8	3·7

The possibility of the presence in the extracts of substances which might interfere with the reaction has been checked by the detection of added succinylhydroxamic acid corresponding to 25 μg. of acetylphosphate.

It appears, therefore, that the suggestion of Kaplan & Greenberg (1944*c*) that the acid molybdate-labile compound present in their extracts was acetyl phosphate is unlikely. It may also be noted that Kaplan & Greenberg based their suggestion upon the premise that phosphocreatine was not

present in the liver. This is in disagreement with the results of Le Page & Umbreit (1945), although these latter workers did not provide unequivocal proof that the labile compound was phosphocreatine. We have tried to obtain more certain evidence by correlating the labile P with the bound creatine. Such a correlation has been achieved with extracts from both normal and fatty livers, but because of the presence of inhibitors which interfere with the estimation of free creatine, the analytical methods are being investigated. It is hoped that the results will be published later. For the moment, however, from these unpublished experiments, it is considered that the acid molybdate-labile P arises for the most part from phosphocreatine, and certainly not from acetyl phosphate.

DISCUSSION

Since the technique adopted throughout these investigations differs, in some respects, from that of other workers it is desirable to point out these differences and to discuss any bearing they may have on the results. It is generally accepted that true, normal 'resting values' for lactic acid and *ATP* in brain and muscle will not be obtained unless the organs are removed under anaesthesia. We have not used any anaesthetic before removal of the liver and have found no unequivocal evidence in the literature that anaesthesia is necessary to obtain maximum *ATP* and *ADP* levels in this organ. Le Page (1946), however, has found some indication of a loss of rat-liver *ATP* when the whole animal was frozen without anaesthesia. To detect any changes in the absence of anaesthesia we have taken a group of six animals, three of which were treated with carbon tetrachloride. All were given an injection of nembutal (50 mg./kg. body wt.) and, when light surgical anaesthesia had been effected, the liver was rapidly removed and dropped into liquid air. In all cases the *TCA* extracts and the corresponding barium-insoluble fractions gave analytical figures which confirmed the previous findings; hence no decomposition of the labile phosphate detectable by our methods occurs in the liver of guinea pigs when the animals are killed without anaesthesia.

The estimation of the N : 7 min.-labile P : pentose ratios has been carried out by Le Page & Umbreit (1945) and Le Page (1946) to establish the identity of the adenosine polyphosphates in the barium-insoluble fraction. Our results, with similar methods, on guinea-pig liver, confirm the opinions of these workers and indicate that a considerable fraction of the 7 min.-labile P has its origin in *ADP*. This conclusion is not supported by results obtained with the *ATP*-ase method. These were only few in number since we were concerned initially with quantitative changes in those compounds possessing high-energy phosphate bonds (~ph; Lipmann, 1941). As far as the determination of the identity of the compounds is concerned, it seems more likely that dependable results will be achieved with the specific *ATP*-ase than with chemical methods, which will be subject to error from possible contamination with N or P compounds. It is worthy of note that Rapoport (1945), using the *ATP*-ase method, has found *ATP* in livers of rats in amounts much greater than those found by Le Page & Umbreit (1945) and Le Page (1946).

The changes in the distribution of the acid-soluble phosphates in the fatty liver of the guinea pig, in so far as we have investigated them, indicate a marked increase in *APP* and phosphocreatine. In the interpretation of these results the work of Kaplan & Greenberg (1944*b*) is of interest, as these workers, using fasted and fat-fed rats, claimed a decrease in ~ph levels, attributed to the appropriation of ~ph for the oxidation of fat without a replacement being effected through the utilization of acetoacetate in the liver. It is doubtful, however, if such a hypothesis is tenable, since it assumes the obligatory formation of acetoacetate as the end product of fatty acid oxidation. This is not necessarily so as is indicated by numerous experiments with anti-ketogenic substances. An explanation for the non-appearance of acetoacetate may be found in the association of the citric acid cycle with fatty acid oxidation (cf. Quastel & Wheatley, 1935). Positive evidence for this is found in the action of the enzyme citrogenase, present in the liver (Breusch, 1943), which catalyzes the reaction between acetoacetate and oxaloacetate to form citrate (Wieland & Rosenthal, 1943; Buchanan, Sakami, Gurin & Wilson, 1945 *a*, *b*).

It is therefore suggested that the increased amount of ~ph which has been noted in our experiments arises from energy derived from the citric cycle as a direct consequence of the increased amounts of fat which are being oxidized.

It has been suggested by Lehninger (1945*b*) that ~ph might be generated by the stepwise oxidation of fatty acids down to the stage of acetoacetate. Experiments with malonate suggest that this process is unlikely to result in increases in ~ph. Thus the well known *in vivo* and *in vitro* effect of malonate in increasing acetoacetate production is generally assumed to be due to the denial of the citric acid cycle as one avenue for the oxidation of fat. Moreover, malonate has been shown by Kaplan & Greenberg (1944*a*) to produce a decrease in ~ph in the rat liver. It would seem, therefore, that the increases in ~ph which we have observed are due not to the reactions involved in the degradation of the fat down to the acetoacetate level, but to the reactions which occur following the entry of the acetoacetate into the citric acid cycle.

The increased amount of *APP* which we have observed in the fatty livers of guinea pigs is of interest in view of the work of Lehninger (1945*b*) on the role of *ATP* and *ADP* in fatty acid oxidation. In considering the implication of these results it should be remembered that Lehninger showed that, in crude rat-liver homogenates, the activation produced by *ADP* was equal to that by *ATP* when both were present in equimolar concentrations. Under these conditions there is twice the amount of ~ph available in the *ATP*-catalyzed system as in that catalyzed by *ADP*. When the molar concentration of *ATP* was reduced to one half that of *ADP*, thus providing equal amounts of ~ph, *ATP* produced only 70% of the activation provided by *ADP*. It does not seem that this can be explained by the assumption that the diphosphate is raised to the triphosphate stage by phosphorylation, since the addition of adenylic acid, which presumably would be phosphorylated if this were so, was without effect. The only conclusion to be drawn from these results is that *ADP* is at least as important as *ATP* to the *in vivo* systems responsible for the oxidation of fatty acids.

In later work Lehninger (1945*a*) employed purified homogenates and showed that although *ATP* produced the customary activation, *ADP* was inactive. Lehninger interpreted this as indicating the specific nature of the activation produced by *ATP*. While this is undoubtedly so in the system employed, the earlier results (Lehninger, 1945*b*) cannot be discounted. They suggest that, whilst the study of purified liver homogenates will undoubtedly give valuable evidence as to the precise nature of the activators of a particular system, the findings are not necessarily applicable without modification to the oxidation of fatty acids in the intact liver occurring in association with the oxidation of other substrates.

On thermodynamic grounds, the phosphorylation of *ADP* to *ATP* requires no greater utilization of energy than that of creatine to phosphocreatine. But it is of interest that the fatty liver, whilst containing greater amounts of ~ph (*APP* and phosphocreatine), does not contain exclusively *ATP* if complete reliance be placed upon the determination of the N : 7 min.-labile P : pentose ratios. Some doubt as to the reliability of these analyses arises when one considers the increases in phosphocreatine which have been demonstrated, since it is difficult to conceive of ~ph being transferred to creatine without a prior transfer to saturation point of the adenylic acid system, and with the consequent appearance of *ATP*. Further experiments with the more specific *ATP*-ase methods should settle this point.

It is well known that phosphocreatine is capable of phosphorylating adenylic acid, and Lehninger (1945*b*) has shown that the activation of fatty acid oxidation is greater when both adenylic acid and phosphocreatine are present than with phosphocreatine alone. The phosphocreatine found in the normal liver may then be regarded as representing a readily available source of energy, which can be utilized through the adenylic acid system when the occasion arises, and its presence in increased amounts in the fatty liver is most probably the result of an overflow of ~ph which cannot be stored as *APP*.

The continued failure to detect the presence of acyl phosphates in the fatty liver is disappointing in view of the accumulated evidence which suggests the necessity of phosphorylation as an integral part of the fatty acid oxidation mechanism. That acyl phosphates may be oxidized in the absence of added *ADP* or *ATP* (Lehninger, 1944) is interesting but is not conclusive proof of the intermediate formation of these compounds, since they themselves are capable of phosphorylating adenylic acid with the formation of *ATP*.

SUMMARY

1. The change in the distribution of acid-soluble phosphates in the fatty livers of guinea pigs treated with carbon tetrachloride has been investigated.

2. In such livers there is an increase in the total acid-soluble organic phosphates.

3. This increase is considered as primarily due to increases in adenosine polyphosphates and phosphocreatine, and in general runs parallel to the fat content.

4. Acyl phosphates have not been detected in liver extracts.

5. The results are interpreted in the light of modern work on fatty acid oxidation.

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The Estimation of Creatine

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During the course of an investigation into the metabolism of fatty livers (Ennor & Stocken, 1948) it became desirable to identify, and subsequently to estimate, amounts of a very labile phosphorus compound present in the livers of normal guinea pigs and in others treated with carbon tetrachloride. Although phosphate liberated from organic phosphate compounds in acid molybdate is often considered to arise from phosphocreatine, there are other possibilities, and we endeavoured therefore to correlate the labile phosphorus with the creatine liberated following mild hydrolysis. It was decided to estimate the creatine as such rather than after conversion to creatinine, and an attempt was made to employ the method of Eggleton, Elsden & Gough (1943). These authors have pointed out that trichloroacetic acid (*TCA*) extracts of liver contain inhibitory substances which prevent the estimation of creatine by the Barritt (1936) modification of the Vosges & Proskauer (1898) reaction. The suggestion was also made that these inhibitors were the ω-amino aliphatic acids.

In the fractionation of *TCA* extracts of guinea-pig liver by means of barium (Le Page & Umbreit, 1945) it was found that both the inhibitor and phosphocreatine were precipitated in the ethanol-insoluble fraction. An examination of this fraction indicated that the inhibitory substance or substances was more likely to be glutathione than an amino-acid as suggested by Eggleton *et al.* (1943). Such a supposition, because of the wide distribution of the tripeptide, necessarily casts some doubt upon the validity of the application of the α-naphthol—diacetyl reaction to the estimation of creatine in tissues.

This communication is concerned with some experiments on the inhibition of the Barritt reaction by sulphydryl compounds and suggests a modification of the procedure of Eggleton *et al.* by means of which a more accurate determination of the creatine content of tissues may be made.