## DISCUSSION

As both eyes in an animal receive the same blood supply a difference between the manganese contents of a given tissue from the two eyes is not to be expected. Such differences were, however, observed, and in one or two cases were probably real and not due to experimental error. For the majority of the tissues the mean values for each of the two animals showed greater differences. In no case where a different value is given (Table 3) for the two oxen did the values for the two eyes from each overlap.

This apparent difference between individual oxen cannot be used to explain the poor agreement between the values obtained in this laboratory and those of Tauber & Krause. With the exceptions of the sclera and conjunctiva the values obtained by the American workers are several times larger. The values for the conjunctiva given in the present paper are to be considered with caution as there is some doubt as to whether conjunctiva alone was included in the samples.

In regard to which of the fresh tissues contain the greatest concentrations of manganese there is fair agreement with the American results if the sclera and conjunctiva be omitted.

The retina appeared to be one of the richest tissues with respect to manganese and, as this tissue was also the site of the chemical visual processes, fractionation was attempted. When dialysis was applied to retinal homogenate more manganese was recovered in the non-dialysable material than was present (by calculation) in the original homogenate. No explanation of this can be offered since rigorous precautions against contamination were taken. However, it appears from the experiment that little of the manganese in the retina is dialysable.

The manganese in retina appeared to be attached to the retinal tissue and the latter was therefore fractionated, using a well defined technique in use in this laboratory, to give preparations containing most of the rods, the photoreceptors of scotopic vision. There appeared to be a smaller concentration of manganese in the rods than in the remainder of the retina. Hence there is so far no evidence that the manganese present in the retina is selectively or specificially concerned with visual biochemistry. The functions of manganese present in the eye tissues may well be displayed at other sites in the body (see Fore & Morton, 1952*b*, on rabbit tissues).

## SUMMARY

1. The manganese contents of the tissues of single ox eyes have been determined.

2. The results have been compared with those of Tauber & Krause (1943) obtained using large samples from several hundred ox eyes.

3. Preliminary experiments on the fractionation of retinas have indicated that manganese in this tissue is non-dialysable and not specifically concerned in the visual process.

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The inception of this work and the early experiments owed much to Dr A. L. Stubbs, then holding an Imperial Chemical Industries Fellowship at the University of Liverpool.

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# The Determination and Distribution of Phosphocreatine in Animal Tissues

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Phosphocreatine has been described for over 20 years and methods for its determination in tissues generally depend upon the estimation of the phosphorus moiety which can be split off readily in acid solution. A much-used technique is that described by LePage & Umbreit (1945), whereby the inorganic P is determined by the method of Fiske & Subbarow (1925) after its precipitation from an alkaline solution as a barium or calcium salt. The difference between this value and that determined by the same method prior to precipitation, is assumed to give the phosphocreatine P. This method, which lacks the sensitivity desirable if low phosphocreatine concentrations are present, has the further disadvantage that its accuracy depends on the quantitative precipitation of inorganic P under the recom-

mended conditions (cf. Ennor & Stocken, 1948*a*) and upon the assumption that phosphocreatine is the only organophosphate hydrolysed by acid molybdate. There is, however, no evidence for this assumption, and the work of Weil-Malherbe & Green (1951) shows that other organophosphates may, in fact, be hydrolysed.

Another technique for the determination of phosphocreatine depends on the estimation by means of the Jaffe reaction of the creatinine resulting from a 20 min. autoclaving of the sample in acid solution (McIlwain, Buchel & Cheshire, 1951).

A much more direct and rapid method, possessing a high degree of sensitivity, is to determine the creatine moiety as such. For this purpose the method described by Ennor & Stocken (1948*b*) is suitable, since it allows the determination of creatine in an alkaline medium in which phosphocreatine is perfectly stable. The creatine bound as phosphocreatine can then be determined after acid hydrolysis. The use of this method for the determination of phosphocreatine in tissue extracts and some analytical results on the distribution of phosphocreatine in various tissues will be described. In addition, a comparison has been made of the values obtained by this method, and the commonly used technique of determining the acid-molybdate-labile P.

## EXPERIMENTAL

*Removal of tissues.* The animals used were well fed, and in order to reduce possible losses of phosphocreatine due to anoxia, all tissues were removed under surgical anaesthesia induced by the intraperitoneal injection of nembutal. The kidney, spleen and testis were exposed, with minimal handling, and a loose ligature placed around the vascular supply. The ligature was then tightened and simultaneously the vessels between the ligature and the organ were severed. The organ was then dropped into liquid $O_2$. Loose ligatures were placed around both ends of the gastrocnemius, and as these were tightened the muscle was simultaneously excised and dropped into liquid $O_2$. The abdominal incision was then temporarily closed, an incision made in the neck and the trachea exposed and opened. A tracheal cannula was inserted and connected to a respiration pump. Samples of brain were then obtained, either by the technique described by Kerr (1935), or by the rapid excision of a portion of the unfrozen brain which was then dropped into liquid $O_2$. The abdomen was then quickly re-opened and the incision extended into the thoracic cavity in such a way as to expose the heart. A lobe of liver was then excised, the heart removed and the two samples dropped into liquid $O_2$. The maximum time which elapsed between the removal of an organ or tissue from its natural environment and its immersion in liquid $O_2$ was 2 sec.

*Preparation of tissue extracts.* Trichloroacetic acid extracts of all tissues, with the exception of skeletal muscle, were prepared as described by Ennor & Stocken (1948*a*). In the case of skeletal muscle, frozen samples were dropped into the bowl of a tared Nalco type homogenizer, which was then re-weighed. Two volumes of 10% (w/v) trichloroacetic acid were then added and the tissue homogenized. The suspension was then centrifuged and the residue, after removal of the supernatant, re-extracted with an additional volume of 5% (w/v) trichloroacetic acid. The supernatants were then combined, adjusted to pH 7·0–7·3 by the addition of 5 N-NaOH and made to known volume. During these and subsequent operations the temperature of the extracts was kept at 0°.

*Determination of 'free' and 'bound' creatine.* In this paper, 'free' creatine refers to creatine determined directly on a sample of a neutralized extract by the method described by Ennor & Stocken (1948*b*), using the reagents and concentrations described by Eggleton, Elsden & Gough (1943). Since phosphocreatine has no free amino group it is non-reactive and is, moreover, perfectly stable under the alkaline conditions under which colour development occurs. 'Total' creatine is the sum of 'free' creatine and that which is released following acid hydrolysis. The difference between 'total' creatine and 'free' creatine gives the 'bound' creatine, which is assumed to have its origin in phosphocreatine.

For the determination of total creatine, a sample was treated under mild conditions (9 min. in 0·1 N-HCl at 65°) to hydrolyse the phosphocreatine with minimal formation of creatinine (cf. Barker, Ennor & Harcourt, 1950). The solution was then neutralized by the addition of the theoretical

Table 1. *Phosphocreatine distribution in normal animals*

(All values are expressed as mg./100 g. wet weight and are the means of duplicate determinations.)

| Animal | Spleen | Kidney | Liver | Cardiac muscle | Skeletal muscle | Brain | Testis |
|---|---|---|---|---|---|---|---|
| Rabbit 1 | 0·8 | 1·6 | 3·2 | 30·2 | 142 | — | — |
| 2 | 0·8 | 0 | 6·1 | 3·2 | 97 | — | — |
| 3 | 5·1 | 0 | 0 | 0 | 323 | 0 | — |
| 4 | 4·2 | 2·1 | 0 | 29·0 | — | 4·8 | — |
| Cat 1 | 8·5 | 6·1 | 17·0 | 54 | 222 | — | — |
| 2 | 7·2 | 19·4 | 24·5 | 82 | 342 | — | — |
| 3 | 5·2 | 3·7 | 14·8 | 9·7 | 156 | 41·8 | 10·5 |
| 4 | 3·2 | — | 0 | 14·4 | 194 | 22·6 | — |
| Guinea pig 1 | 4·0 | 1·6 | 0·3 | 60 | 157 | — | 14·9 |
| 2 | 4·4 | 5·3 | 1·8 | — | — | 0 | 5·8 |
| 3 | — | 0·4 | 1·0 | — | — | 34·1 | — |
| 4 | — | 2·3 | — | 0 | 100 | 42·7 | — |
| Dog | 5·2 | 4·4 | 1·8 | 45 | 251 | — | 5·6 |

amount of NaOH and cooled rapidly to room temperature by immersion in an ice bath. In practice, a 1·0 ml. sample was pipetted into a tube graduated at 10 ml., 2·0 ml. of water were added and the contents equilibrated at 65°. 1·0 ml. of 0·4 N-HCl was then added and, after 9 min., 1·0 ml. of 0·4 N-NaOH. After cooling, 1·0 ml. of a 0·05 M solution of sodium *p*-chloromercuribenzoate was added, followed by the diacetyl and 1-naphthol. The volume was adjusted to 10·0 ml. by the addition of water and the contents mixed and allowed to stand in the dark for 15 min. for colour development. The colour intensities were measured at the same time interval as the standards which contained 10–40 $\mu$g. creatine. All determinations of free and total creatine were carried out on the day of preparation of the extract.

Using this technique, which has been shown by Barker & Ennor (1951) to result in excellent recoveries of both added creatine and phosphocreatine, analyses have been carried out on the phosphocreatine contents of various tissues from a number of different species of laboratory animals.

## RESULTS

The results show that phosphocreatine was present in all tissues examined (Table 1), although there was a considerable variation in the absolute amount. Skeletal muscle, as has been found by other workers, contained much greater amounts than any other tissue. The variation in phosphocreatine content of cardiac muscle was much greater than would have been expected on the basis of analogy with skeletal muscle. No explanation is available for the occasional very low values for phosphocreatine, but it is doubtful if they are due to any rapid degradation of the compound, since the heart was, in all cases, beating vigorously when dropped into liquid $O_2$.

It is difficult to compare these results with those of other workers because, almost without exception, it has been the custom to determine molybdate-labile P rather than the creatine moiety of phosphocreatine (cf., however, McIlwain *et al.* 1951). Further comment on such procedures is made below, but because of the variation in phosphocreatine content in different organs it was considered of interest to compare the free and total creatine contents and to determine the percentage of the total creatine which is bound as phosphocreatine. This has been done in Table 2.

The concentration of total creatine found in skeletal muscle is much greater than in any other tissue. On the other hand, the proportion which is phosphorylated, whilst greater and showing much less variation in muscle than in any other tissue, is not as great as would be anticipated on the basis of the difference in phosphocreatine levels. If one neglects the values less than 10 % the figures suggest that, irrespective of the tissue, the proportion of creatine phosphorylated is about 20 % of the total amount present.

Table 2. *Distribution of free and total creatine in normal animals*

(All figures are the means of duplicate determinations. $F$ = mg. free creatine/100 g. wet wt.; $T$ = mg. total creatine/100 g. wet wt.; % $B$ = % of creatine bound as phosphocreatine.)

| Animal | | Spleen | | | Kidney | | | Liver | | | Cardiac muscle | | | Skeletal muscle | | | Brain | | | Testis | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $F$ | $T$ | $B$ (%) | $F$ | $T$ | $B$ (%) | $F$ | $T$ | $B$ (%) | $F$ | $T$ | $B$ (%) | $F$ | $T$ | $B$ (%) | $F$ | $T$ | $B$ (%) | $F$ | $T$ | $B$ (%) |
| Rabbit 1 | | 4·7 | 5·2 | 9·6 | 4·1 | 5·1 | 2·0 | 4·2 | 6·2 | 32 | 93 | 112 | 17·0 | 322 | 410 | 21·5 | — | — | — | — | — | — |
| | 2 | 5·9 | 6·4 | 7·8 | 4·6 | 4·6 | 0 | 10·4 | 14·2 | 27 | 103 | 105 | 1·9 | 322 | 382 | 15·7 | — | — | — | — | — | — |
| | 3 | 9·0 | 12·2 | 26 | 29·3 | 29·3 | 0 | 21·4 | 21·4 | 0 | 131 | 131 | 0 | 239 | 440 | 23 | 101 | 101 | 0 | — | — | — |
| | 4 | 7·6 | 10·2 | 26 | 11·3 | 12·6 | 10·3 | 15·5 | 15·5 | 0 | 114 | 132 | 13·6 | — | — | — | 101 | 104 | 2·9 | — | — | — |
| Cat 1 | | 8·9 | 14·2 | 37·2 | 11·8 | 15·6 | 24·4 | 41·1 | 51·6 | 20·4 | 169 | 203 | 16·8 | 324 | 462 | 30 | — | — | — | — | — | — |
| | 2 | 8·4 | 12·9 | 35 | 28·2 | 40·3 | 30 | 39·9 | 55·2 | 27·8 | 149 | 200 | 25·5 | 219 | 432 | 26 | — | — | — | — | — | — |
| | 3 | 7·2 | 10·4 | 30 | 12·1 | 14·4 | 16 | 35·6 | 44·8 | 20·5 | 105 | 110·7 | 5·2 | 97 | 193·6 | 50 | 104 | 129·6 | 19·8 | 69 | 75·5 | 9·4 |
| | 4 | 5·9 | 9·0 | 35 | — | — | — | 33·5 | 33·5 | 0 | 182 | 191·3 | 4·9 | 195 | 315 | 38 | 115 | 128·7 | 10·6 | — | — | — |
| Guinea pig 1 | | — | — | — | 14·2 | 15·6 | 9·0 | — | — | — | 48·7 | 48·7 | 0 | 216 | 278 | 22 | 71·0 | 97·5 | 27 | — | — | — |
| | 2 | 7·4 | 10·1 | 26 | 16·5 | 19·8 | 16·6 | 5·5 | 6·6 | 16·7 | — | — | — | — | — | — | 121 | 121 | 0 | 32 | 35·6 | 10·1 |
| | 3 | — | — | — | 8·1 | 8·4 | 2·4 | 8·3 | 9·3 | 10·7 | — | — | — | — | — | — | 60·0 | 80 | 25 | — | — | — |
| | 4 | 10·4 | 12·9 | 19·4 | 7·6 | 8·6 | 23·3 | 4·4 | 4·6 | 4·3 | 80·7 | 118 | 31·5 | 341 | 439 | 22·3 | — | — | — | 27·7 | 37·0 | 25·0 |
| Dog | | 9·4 | 12·6 | 25·4 | 11·6 | 14·3 | 18·9 | 2·8 | 3·9 | 28 | 240 | 268 | 10·3 | 214 | 370 | 42 | — | — | — | 174 | 183 | 5·5 |

Since phosphocreatine is usually determined by measurement of the P split off in acid molybdate, it is of interest to compare the results so obtained with those obtained by the present method. For this purpose, inorganic P was determined on one sample by the method described by Ennor & Stocken (1950). Another sample was then allowed to stand in acid molybdate at 30° for 30 min.; under these conditions, authentic phosphocreatine is completely hydrolysed. At the end of the hydrolysis period the resultant phosphomolybdate was extracted by the usual technique of Ennor & Stocken (1950). Additional samples were taken for the determination of bound creatine, as described above, and the results (Table 3) expressed in terms of P.

Table 3. *Comparison of values obtained for phosphocreatine measured as acid-molybdate-labile* P *and as bound creatine*

(All figures are expressed as mg. P/100 g. wet weight of tissue. Found = molybdate-labile P; theory = P equiv. to bound creatine, assuming this is bound as phosphocreatine.)

| | Kidney | | Liver | | Spleen | | Brain | | Testis | | Skeletal muscle | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Animal | Found | Theory | Found | Theory | Found | Theory | Found | Theory | Found | Theory | Found | Theory |
| Guinea pig 1 | 4·0 | 0·8 | 0·8 | 0·2 | — | — | 0 | 0 | — | — | — | — |
| 2 | 1·6 | 0·1 | 1·7 | 0·4 | — | — | — | — | — | — | — | — |
| 3 | 1·4 | 0·3 | 0 | 0·3 | — | — | 6·3 | 6·3 | — | — | — | — |
| Cat | — | — | 0 | 0 | 1·4 | 0·4 | 2·1 | 3·3 | — | — | 27·5 | 28·5 |
| Dog | 1·6 | 0·7 | 0 | 0·3 | 2·1 | 0·8 | — | — | 2·2 | 0·8 | 40·6 | 36·9 |
| Rabbit 1 | 1·1 | 0 | 0·7 | 0 | 1·6 | 0·4 | 1·0 | 0 | — | — | 27·6 | 22·5 |
| 2 | 1·2 | 0·1 | 1·5 | 0 | 1·4 | 0·3 | 1·8 | 0·4 | — | — | — | — |

It is clear that the molybdate-labile P found almost always exceeds that demanded by theory on the basis of the bound creatine. A closer agreement is found in the case of skeletal muscle, where the phosphocreatine content is relatively high. The method for the determination of bound creatine can be considered reliable since good recoveries of added authentic phosphocreatine were obtained by Barker & Ennor (1951). Moreover, no creatine-containing compound is known which reacts in the same way as phosphocreatine with α-naphthol and diacetyl both prior to and after mild hydrolysis. The method may then be expected to yield results which accurately represent the phosphocreatine content of a tissue. For the determination of the P moiety of phosphocreatine, using the criterion of lability in acid molybdate, no such claims can be made. Since the amount of P found generally greatly exceeds that expected on the basis of bound creatine, it must be concluded that organophosphates other than phosphocreatine are acid-molybdate-labile, and hence expression of results in terms of phosphocreatine will be erroneous. Lipmann (1941), commenting on the presence of acid-molybdate-labile phosphate in tissue, has pointed out that this does not constitute proof of the presence of phosphocreatine. Franks (1932) has made a similar observation. More recently, Weil-Malherbe & Green (1951) have shown that several organophosphates commonly occurring in tissues are acid-molybdate-labile.

## DISCUSSION

The method which has been described for the determination of phosphocreatine has several advantages over existing methods. Prime amongst these is the fact that determination of free creatine is carried out in an alkaline medium in which phosphocreatine is perfectly stable. Acid hydrolysis of another sample and subsequent determination of total creatine allows calculation of bound creatine and thus of phosphocreatine. If the suggested conditions of hydrolysis are adhered to, the small amount of creatinine which is formed (Barker & Ennor, 1951) may be neglected.

The method has the additional merit that a check can be made on the accuracy by which the P of phosphocreatine is determined. This is of some importance, particularly in the case of work involving radioactive P, where contamination by P from other compounds may produce gross errors in the determination of the specific activity. However, close agreement of bound creatine with molybdate-labile P would not necessarily constitute proof of the reliability of results when radioactive P is employed. Clearly, some specific enzymic procedure is to be preferred.

It has been shown that phosphocreatine has a much wider distribution than is generally supposed, and that it was present in all tissues examined. Of especial interest is the fact that, whilst the concentration varies greatly from tissue to tissue, the proportion of the total amount of creatine present in the phosphorylated form shows much less variation. This suggests that the amount of phosphocreatine

depends on the creatine content of the tissue. The belief that the presence of phosphocreatine is almost exclusively confined to muscular and nervous organs (Eggleton & Eggleton, 1929; Gerard & Tupikova, 1939) has given rise to the concept (Lipmann, 1941) that phosphocreatine has a connexion with the speed of action required by these organs. The occurrence of phosphocreatine in such organs as spleen, liver and kidney, on which no sudden demands for energy are made comparable to that placed upon, for example, skeletal muscle, suggests the desirability for some modification of this concept.

It would seem probable that phosphocreatine has a function other than that of acting as a reservoir of readily available phosphate-bond energy for the adenylic acid system and that it may contribute energy to endergonic reactions directly and without the mediation of the latter system.


## SUMMARY

1. A method is described for the determination of phosphocreatine in protein-free tissue extracts.
2. Phosphocreatine has been shown to occur in all tissues investigated and the proportion of the total creatine present which is phosphorylated is of the same order irrespective of the tissue concerned.
3. Measurement of the phosphocreatine content of tissue by determination of acid-molybdate-labile phosphorus has been shown to be subject to error.


We are indebted to Mr H. Barker for assistance in the preliminary part of this work and to Miss J. D'Alton for technical assistance. It is a pleasure to express our thanks to the Director-General of Health and to the Director of the Commonwealth Serum Laboratories for laboratory accommodation pending the transfer of this Department to Canberra.

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# Histamine Metabolism in the Human Placenta and in the Umbilical Cord Blood

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In a recent paper (Kapeller-Adler, 1951) a new simple microchemical method and a new unit have been proposed for the estimation of histaminase activity in biological media. In the present work, this new method, along with the biological estimation of histamine, has been applied to the study of histamine metabolism in human placenta and in umbilical cord blood. A significant decrease in histaminase activity has been observed in placentae of cases of severe toxaemia of pregnancy and a significant increase in placentae of women with twin pregnancy. The placental histamine content of women with severe toxaemia of pregnancy has been found to be greatly increased as compared with that of normal placentae. The results are related to previous observations made upon this subject with different methods. In view of contradictory findings concerning the action of histaminase on cadaverine (Kapeller-Adler, 1951), it was thought desirable to include observations on the effect of placental histaminase on this diamine in this paper.

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