THE IDENTIFICATION OF PHOSPHOCREATINE IN LIVER

by

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It is generally believed[9,13,14] that phosphocreatine is absent from the liver although a few claims[11,12] have been made for its presence. Such claims have been based upon the difference between the apparent inorganic phosphate and that precipitable by calcium in alkaline solution. Many factors, however, influence the precipitability of calcium phosphate and such a method provides no certainty that the phosphate so measured by difference is bound to creatine as phosphocreatine.

The most desirable proof of the presence of phosphocreatine in any tissue is given by separation of the compound in a crystalline form suitable for chemical characterization. In the case of the guinea pig liver the maximum concentration so far encountered is 20 mg%[2] and this only in fatty livers arising as a result of carbon tetrachloride intoxication. Such concentrations do not permit the economical separation of phosphocreatine in amounts large enough to allow of elementary analysis. For this reason attempts have been made to characterize the compound by other techniques and it is the purpose of this communication to detail these and to provide proof of the presence of phosphocreatine in the liver.

EXPERIMENTAL

Methods

Phosphorus Estimation

Inorganic P was determined as described by ENNOR AND STOCKEN[7]. Molybdate-labile P was determined by a modification of the method of BERENBLUM AND CHAIN[3] after allowing the aliquot to remain in acid-molybdate for 30 min at 30° before extraction with *iso*butanol.

Creatine Estimation

This was determined by the method of EGGLETON, ELSDEN AND GOUGH[4] as modified by ENNOR AND STOCKEN[6]. In all cases 1.4 mg of Na *p*-chlormercuribenzoate was added to the aliquots before the addition of the reagents.

Creatinine Estimation

This was determined by the alkaline picrate method.

Animals

Male guinea pigs of *c.* 500 g body weight, fed on a liberal diet of fresh lucerne and oats were

References p. 277.

used throughout. Treated animals were given carbon tetrachloride injections as described by ENNOR AND STOCKEN[5].

Preparation of Extracts

For a few quantitative experiments the technique described by ENNOR AND STOCKEN[5] was followed to the stage when the trichloracetic acid (TCA) extracts were combined. The pooled extracts were then brought to p_H 7.0 by the addition of 5 *N* NaOH and made to 50 ml.

In experiments employing paper partition chromatography and necessitating higher concentrations of labile material the following procedure was adopted.

Three carbon tetrachloride-treated animals were killed and the livers rapidly removed and dropped into liquid air. The livers (*c.* 70 g) were finely powdered in an earthenware mortar previously chilled to the temperature of liquid air. The powdered material was then transferred to a Waring Blendor. 150 ml of ice-cold 10% (w/v) TCA was added and when the mass of liver and TCA had thawed sufficiently it was homogenized for 2 min. The protein was then separated by centrifugation and re-extracted in the blendor with a further 100 ml of 5% (w/v) TCA. The two supernatants were then combined, 2 ml of 2 *M* $BaCl_2$ added and sufficient 5 *N* NaOH added to bring the p_H to 9.2. The mixture was allowed to stand for 30 min in an ice bath to ensure precipitation of the Ba salts and was then centrifuged. The precipitate was discarded and four volumes of ice-cold ethanol (previously adjusted to p_H 9.2) were added to the supernatant which was then allowed to stand overnight at —10°. The water-soluble, 80% ethanol-insoluble Ba salts were then separated by centrifugation and the mother liquor discarded. The Ba salts were washed twice with ethanol and once with diethyl ether and dried in vacuo over $CaCl_2$. The dry material was dissolved in the minimum volume of *N* HCl at 0°. Any insoluble material was centrifuged out and 5 *N* NaOH added to the clear solution to bring the p_H to 4–5 to reduce the risk of decomposition of labile material. 5% (w/v) Na_2SO_4 was added to precipitate the Ba, the end point being judged by the use of Na rhodizonate as an external indicator. The $BaSO_4$ was removed by centrifugation and the supernatant brought to p_H 7.0 by the addition of *N* NaOH. The solution was freeze-dried and the residue dissolved in the minimum volume of water. The resultant solution of Na salts was stored at —10° until required.

RESULTS

P: Creatine Ratio

The P:creatine ratio has been determined in both TCA extracts and aqueous solutions of the Na salts. Because of the stability of phosphocreatine in alkaline solution the modification[6] of the method of EGGLETON, ELSDEN AND GOUGH[4] is well suited to the measurement of creatine in the presence of phosphocreatine. Thus, an initial measurement of creatine gives "free creatine" and an additional estimation after hydrolysis gives "total creatine". "Bound creatine" is then obtained by difference.

In practice the hydrolysis was carried out under as mild conditions as possible in order to reduce the amount of creatinine formed. An appropriate aliquot was added to a 10 ml graduated tube and sufficient water to bring the volume to 3.0 ml. The tube and contents were then equilibrated in a water bath at 65° and, at zero time, 1.0 ml of 0.4 *N* HCl was added. Hydrolysis was carried out for 9 min at which time 1.0 ml of 0.4 *N* NaOH was added and the tube and contents then brought rapidly to room temperature by immersion in an ice bath. The subsequent procedure was as described earlier[6].

Labile phosphorus as determined represents the sum of the inorganic P and the P released in a 30 min hydrolysis period at 30° in acid-molybdate under the conditions of sulphuric acid and ammonium molybdate concentration suggested by BERENBLUM AND CHAIN[3]. Subtraction of inorganic P then gives *true* labile P.

BARKER, ENNOR AND HARCOURT[1] have shown that the nature of the ions present (apart from H^+) have an influence upon the products of the hydrolysis of phosphocreatine. Because of this effect and the assumption that phosphocreatine will hydrolyze to creatine and orthophosphoric acid some recovery experiments have been carried out, the results of which are set out in Table I.

References p. 277.

TABLE I

RECOVERY EXPERIMENT

Recoveries of added inorganic phosphate, creatine (Cr.) and phosphocreatine (Pc.) from a trichloroacetic acid extract of guinea pig liver. All figures represent the means of at least duplicate determinations.

μg P as KH_2PO_4			μg Cr.		
Added	Found		Added	Found	
	Inorganic P	Mol. Lab.		Free	Total
20	20.8	19.4	10	10.1	10.3

μg P as Pc.			μg Cr. as Pc.		
Added	Found		Added	Found	
	Inorganic P	Mol. Lab.		Free	Total
3.8	0	3.7	16.5	0	16.6

As would be anticipated from an earlier paper[7] recoveries of inorganic P and P bound as phosphocreatine are satisfactory. The creatine analyses show that free creatine may be recovered both before (101%) and after (103%) acid hydrolysis. It may therefore be assumed that under these conditions any creatine which is liberated during the course of the hydrolysis will not be converted to creatinine and thus escape measurement. In the case of added phosphocreatine approximately theoretical recoveries have been obtained of both the P and creatine moieties indicating that, under these conditions, phosphocreatine will split into creatine and phosphoric acid.

TABLE II

DETERMINATION OF BOUND P (Mol. labile P — Inorganic P) AND BOUND CREATINE (Total Cr — Free Cr) IN TCA EXTRACTS OF GUINEA PIG LIVER

All analyses are the means of at least duplicate determinations.

μg P/ml			μg Cr./ml		
Inorganic P	Mol. Lab. P	Bound P	Free Cr.	Total Cr.	Bound C
10.25	11.81	1.56	20.1	26.8	6.7

Molar Ratio P/Cr. = 1.56/31 ÷ 6.7/131
= 0.99

μg P/ml			μg Cr./ml		
Inorganic P	Mol. Lab. P	Bound P	Free Cr.	Total Cr.	Bound C
15.9	18.5	2.6	23.8	34.5	10.7

Molar Ratio P/Cr. = 2.6/31 ÷ 10.7/131
= 1.03

References p. 277.

This method has been applied to the measurement of molybdate labile P and bound creatine in guinea pig liver. The TCA extracts were subjected to no treatment other than neutralization to p_H 7.0 immediately after preparation. The results of such analyses on two extracts prepared from different animals both of which were treated with carbon tetrachloride are given in Table II. It is considered that the molar ratio which approximates 1.0 in both cases is indicative of the presence of phosphocreatine.

In some experiments however it has not proved possible to obtain a P:creatine ratio of unity when determinations of P and creatine have been carried out on the Ba soluble fraction of TCA extracts either before or after concentration. In these cases a considerable amount of P in excess of that demanded by theory on the basis of the "bound creatine" has been found. Addition of H_2SO_4 or HCl to neutral TCA extracts containing such excess P has resulted in a precipitate of material giving a positive biuret reaction and it is believed that the excess P arises from a protein like material not precipitable by TCA under the conditions of the extraction procedure. In these cases the extracts have been treated with copper acetate as described by FISKE AND SUBBAROW[8] for the purification of phosphocreatine from skeletal muscle. Analysis of the alkaline extract of the copper salts has invariably yielded a P:creatine ratio of 1.0.

Molybdate Hydrolysis Experiments

It has been shown by BARKER, ENNOR AND HARCOURT[1] that the products of the hydrolysis of phosphocreatine in acid solution in the presence of ammonium molybdate are primarily creatinine and phosphoric acid. Moreover it was possible, in the case of authentic phosphocreatine, to obtain a complete balance sheet and to account for all the creatine or phosphocreatine by measurement of both the creatine and creatinine following such hydrolysis. It was thought that such treatment would lend confirmatory evidence for the assumption that the molybdate-labile compound present in such livers as have been examined is indeed phosphocreatine. The results of such an experiment (Table III) indicate that this is the case.

TABLE III

ESTIMATION OF CREATINE (Cr.) AND CREATININE (Crine.) IN TCA EXTRACT OF GUINEA PIG LIVER BEFORE AND AFTER HYDROLYSIS IN 0.1 *N* HCl AT 65° FOR 9 min IN PRESENCE AND ABSENCE OF AMMONIUM MOLYBDATE ($0.5 \cdot 10^{-3}$ *M*)

Treatment of Extract	Cr. μg	Crine μg	Cr. ÷ Crine μg
Unhydrolyzed	29.3	0	0
Hydrolyzed — no addition	48.0	1.7	2.0
Hydrolyzed — + molybdate	30.6	16.2	18.8

This experiment was carried out taking the trichloracetic acid extract of a guinea pig liver which had been freed of Ba-insoluble salts by treatment with $BaCl_2$ at p_H 9.0. Ba was removed by precipitation as $BaSO_4$ and the p_H adjusted to 7.0. Creatine and creatinine were determined before and after hydrolysis in 0.1 *N* HCl for 9 min at 65° with and without the addition of molybdate. The True Bound creatine (Table III) is arrived at by the difference between the creatine determined before and after simple acid hydrolysis plus the creatine equivalent of the creatinine measured after hydrolysis,

References p. 277.

i.e., (48.0 — 29.3) + 2.0 = 20.7 μg. The difference between the True Bound creatine arrived at by the two methods is within experimental error. Authentic phosphocreatine subjected to acid hydrolysis in the presence of molybdate is so changed[1] that only 7% of the creatine equivalent to the phosphocreatine appears as such. In the case of the TCA extract from liver the corresponding figure is $\frac{(30.6 - 29.3)}{19.9} = 6.5\%$. This is within good agreement with the previous work[1] and may be regarded as additional proof of the presence of phosphocreatine in liver.

Paper Chromatography

Solutions

1. A solution of the Na salts prepared from the Ba-soluble fraction of a TCA extract of 3 guinea pig livers prepared as described above; 2. KH_2PO_4 (200 μg P/ml); 3. NaPC (2 mg/ml).

Runs of 18 hours duration were carried out at 20° using No. 1 Whatman filter paper which had been well washed with *N*/10 HCl and then with distilled water until acid free. In all cases the paper was spotted with 3 μl drops of the appropriate solution and the chromatogram developed in an alkaline medium made up of 60 ml *n*-propanol/30 ml conc. ammonia/10 ml water as described by HANES AND ISHERWOOD[10]. Following completion of the run, the paper was removed from the jar and the solvent dried off in a current of warm air. The paper was then sprayed with a mixture of 50 ml of *N* HCl, 20 ml of 5% (w/v) ammonium molybdate and 30 ml of water and dried in an air oven at 80°. The phospho-molybdate formed by this procedure was reduced with H_2S as described by HANES AND ISHERWOOD[10]. Under these conditions as little as 6 μg of phosphocreatine can be readily detected about 10 cm from the starting line after an 18 hour run. Inorganic phosphate if present appears about 2 cm behind phosphocreatine. In the case of the extract a well-defined spot appeared precisely opposite that of authentic phosphocreatine.

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SUMMARY

1. The nature of the acid-molybdate-labile P compound occurring in trichloracetic acid extracts of livers from guinea pigs treated with carbon tetrachloride has been investigated.

2. On the basis of the P:creatine ratio, the nature and determination of the compounds liberated in the presence of acid-molybdate, and the behaviour as judged by paper chromatography it is concluded that the compound is phosphocreatine.

References p. 277.

RÉSUMÉ

1. Nous avons étudié la nature du composé phosphorique, labile en présence de molybdate en solution acide, qui se trouve dans les extraits trichloracétiques de foies de cobayes traités au tetrachlorure de carbone.

2. En nous basant sur le rapport P:créatine, la nature et la détermination des composés libérés en présence de molybdate en solution acide, ainsi que sur le comportement lors de la chromatographie sur papier, nous concluons que le composé étudié est de la phosphocréatine.

ZUSAMMENFASSUNG

1. Die Natur der Säure-Molybdat-labilen Phosphorverbindung, die in Trichloressigsäure-Extrakten von Lebern von Meerschweinchen vorkommt, welche mit Tetrachlorkohlenstoff vorbehandelt waren, wurde untersucht.

2. Auf Grund des Verhältnisses P:Kreatin, der Natur und Bestimmung der in Gegenwart von Säure-Molybdat in Freiheit gesetzten Verbindungen, sowie der Ergebnisse der Papierchromatographie wird geschlossen, dass die untersuchte Verbindung Phosphokreatin ist.

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