reported by several workers, for instance Morawetz & Hughes (1952).

The effect of pH and electrolyte concentration on the system is most marked (Fig. 3). In the absence of salt, precipitation does not occur below pH 7.0 but the addition of Cl^- or SCN^- ions shifts the pH range over which precipitation occurs towards lower pH values and decreases the amount of precipitate. The observation that thiocyanate is more effective than chloride in lowering the pH range of precipitation is in agreement with the results of Morawetz & Hughes (1952) for the precipitation of bovine-serum albumin by polyvinylamine.

The proportion of polycation to that of albumin was found to be an important factor in the precipitation studies (Fig. 4). Maximum precipitation was generally found to occur at a critical ratio of albumin to basic dextran. Above and below this ratio solubilization of the precipitate was produced—an effect similar to that obtained in an antigen-antibody-precipitin reaction.

The basic dextrans showed little effect on either the one-stage prothrombin time or the calcium clotting time. They formed insoluble salts with heparin and dextran sulphate as protamine does, so that neutralization of anticoagulant activity may be expected. It was noticed that basic dextran agglutinated suspensions of cells, e.g. *Escherichia coli*, *Staphylococcus aureus*, erythrocytes. Low concentrations of the high-molecular-weight preparations, e.g. 5–50 $\mu\text{g./ml.}$ of preparation D, were most effective. The agglutinating effect is presumably due to the fact that these cell suspensions are stabilized by electronegative charges in the cell surface which are neutralized by combination with basic dextran molecules.

SUMMARY

1. The preparation of dextran derivatives having an electropositive charge as opposed to the electronegative charge of dextran sulphates is described.

2. Some preliminary experiments on their interaction with proteins and cells are reported. The effect of pH, electrolyte concentration and the relative proportions of reactants on the formation of insoluble complexes with human-serum albumin were investigated. The optimum proportion of albumin to basic dextran lies within the range 0.256–0.41 by weight. At pH 8.6 without added electrolyte 85 % of the albumin is precipitated.

3. Suspensions of erythrocytes and bacteria were agglutinated by basic dextrans.

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Studies on Synovial Tissue

7. CONCENTRATIONS OF CREATINE AND PHOSPHOCREATINE IN SYNOVIAL TISSUE*

By E. R. COOK, D. P. PAGE THOMAS AND J. T. DINGLE

Medical Research Council, Rheumatism Research Unit, Manor Hospital, Bath, Somerset

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Dingle & Thomas (1956) showed that the synovial proliferation which takes place in joints affected by rheumatoid arthritis is accompanied by a marked rise in metabolic activity of the affected tissue. In recent years, investigations into the biochemical mechanisms which control the rates of

metabolic processes have emphasized the part played by phosphate and phosphate acceptors (Lardy & Wellman, 1952; Krebs, 1957; Kornberg & Krebs, 1957), and it was therefore considered that a study of the levels of energy-supplying phosphates and phosphate acceptors in normal and proliferating synovial tissue would further clarify the metabolic changes induced by the disease

* Part 6: Thomas, Dingle & Cook (1960).

process. However, no information relating to the concentrations of these compounds in synovial tissue could be found in the literature, and, as a preliminary step, a study of the estimation of creatine and phosphocreatine in this tissue was initiated.

The widely used method of determining phosphocreatine in tissue extracts by the estimation of the acid-labile phosphate group has been criticized on the grounds of non-specificity (review by Ennor & Morrison, 1958). We have therefore preferred to use the method of Ennor & Rosenberg (1952), which employs an alkaline solution of diacetyl and 1-naphthol to measure creatine as such, before and after hydrolysis, the difference representing phosphocreatine. Preliminary work demonstrated that the technique, as described, was not sufficiently sensitive to measure the low concentrations of creatine in the small samples of synovial membrane available, and further that this tissue was too tough and fibrous to break up by conventional homogenization.

It was, however, found possible to simplify the various stages in the Ennor & Rosenberg method, and also to increase the sensitivity of the colorimetric estimation by decreasing the volumes of sample and reagents. This modified technique was then applied to extracts obtained by treating the finely sliced tissue with a detergent solution which also contained ethylenediaminetetra-acetate, *p*-chloromercuribenzoic acid and alkali (Rosenberg & Ennor, 1955) in order to inhibit enzyme activity and stabilize the liberated phosphocreatine.

This paper presents details of the method, together with results from its application to various tissues, especially human and rabbit synovial membranes.

EXPERIMENTAL

Reagents

Detergent solution. This was an aqueous solution containing 0.4% of Tween 80 (L. Light and Co. Ltd., Colnbrook, Bucks.), 1% of NaOH, 0.2% of *p*-chloromercuribenzoic acid (sodium salt) (L. Light and Co. Ltd., or Sigma Chemical Co., St Louis, Mo.) and 0.4% of ethylenediaminetetra-acetic acid (disodium salt). This solution is kept at 4° and the gelatinous precipitate which slowly forms may be redissolved easily by gentle warming.

Alkali solution. This was an aqueous solution containing 68.4 g. of NaOH and 181 g. of anhydrous Na_2CO_3 /l. Although stored at 4°, this reagent does not keep, probably because of absorption of atmospheric CO_2 , and should be prepared weekly.

Aqueous diacetyl. This was a 1% solution of diacetyl; no purification is necessary and the solution appears to be stable for several weeks in the deep freeze. The solution was diluted 1:20 with water just before use.

***p*-Chloromercuribenzoic acid.** The sodium salt (1 g.) was dissolved in 55 ml. of water; the solution precipitates on standing and should be shaken vigorously before use.

Stock creatine solution. An aqueous solution containing 400 $\mu\text{g.}/\text{ml.}$ was kept at 4°. The working standard was prepared by diluting 1 ml. of stock solution to 20 ml. with detergent solution just before use.

Methods

Rats and guinea pigs were killed by a blow on the head. Rabbits were generally killed by air injection, but a few received Pentothal before the injection. In all cases the tissues were dissected out as quickly as possible and dropped into ethanol containing solid CO_2 . Most tissues were placed in the freezing mixture within 30 sec. of the death of the animal, but even with practice it was not found possible to remove the synovium in less than 1½–1¾ min. After a few minutes' immersion the tissues were blotted with filter paper and placed in dry tubes standing in a bath of ethanol-solid CO_2 .

Preparation of sample. The method described is appropriate for 200–400 mg. of synovial tissue; adjustment in volume of detergent and weight of sample must be made for other tissues. Where practicable the sample taken for colorimetric estimation should preferably contain 5–10 $\mu\text{g.}$ of total creatine, but this is not feasible for synovial tissue, for which the sample usually contains 2–4 $\mu\text{g.}$

Frozen synovial tissue (200–400 mg.) was rapidly weighed and placed in a Pyrex basin, 6.25 cm. diam., supported on a coarse mush of powdered solid CO_2 and ethanol in a small porcelain mortar. The tissue was held in a pair of Spencer-Wells forceps and sliced as finely as possible with a scalpel blade (Gillette shape A for standard Swann-Morton no. 4 holders has proved very convenient); the shavings were then crushed into a fine powder with a glass rod. The instruments were chilled on solid CO_2 before use. The technique requires care and cannot be hurried.

The finely divided tissue was then transferred to a homogenizing tube previously chilled in ethanol- CO_2 ; 1.5 ml. of detergent solution was added and the tube placed in cold water until the partly frozen contents had liquefied. This process is accelerated by manual stirring with the homogenizer plunger. The contents of the tube were then placed in the deep-freeze until frozen solid and then thawed in running tap water, the plunger being used to break up the fat and tissue agglomerates. This process was repeated once more. A further 0.5 ml. of detergent was added, and the solution placed in an ice bath. Gentle homogenizing was carried out for brief periods totalling 2 min., with adequate intervals for chilling.

Homogenization is unnecessary for the disruption of softer tissues such as lung, kidney, spleen and muscle, which can be completely broken up by freezing and thawing the mixture of detergent and powdered tissue four times, followed by the addition of 0.5 ml. of detergent and freezing and thawing twice more. The values reported in Tables 1 and 2 for such tissues were obtained by this technique.

The extract was then acidified with an equal volume of chilled 14% trichloroacetic acid solution, this volume being taken as the sum of the detergent added, plus the weight of the tissue taken. Experience has shown that this is a sufficiently accurate estimate of the volume required. The tube was stoppered, well shaken and centrifuged briefly in chilled centrifuge tubes, followed by filtration through a small Whatman no. 541 filter paper into test tubes standing in ice water. The filtrate was occasionally cloudy, but this

precipitate dissolved in alkaline solution and did not therefore give erroneous values in the colorimetric reaction.

Colour development. To 1 ml. of filtrate, 2 ml. of a mixture of diacetyl, 1-naphthol, *p*-chloromercuribenzoate and alkali was added. This reagent solution is prepared by adding 10 ml. of water and 2.5 ml. of *p*-chloromercuribenzoate suspension to 0.08 g. of 1-naphthol, and then, immediately before use, adding 5 ml. of alkali solution followed by 2.5 ml. of a 1:20 dilution with water of the stock 1% diacetyl solution. It is sometimes necessary to remove a slight turbidity by centrifuging. The tube containing the reaction mixture was well shaken and incubated for 35 min. in the dark at 20°. The resulting pink colour was measured at 525 m μ in cells of 1 cm. light path. This estimates the free creatine present. It is essential that the tubes should be shielded from sunlight and fluorescent lighting, both of which cause a reaction between trichloroacetate and 1-naphthol with the formation of a blue colour.

A further 1 ml. of the trichloroacetic acid filtrate was heated at 60° for 10 min. and then cooled. This treatment hydrolyses the phosphocreatine present, and the total creatine present in the sample was measured by the colorimetric technique described, with a fresh batch of reagent.

The difference between free and total creatine represents phosphocreatine, and since this value is generally a small fraction of the total, it is important that the estimations are carried out as accurately as possible. We have also adopted the precaution of running appropriate standards with every estimation to allow for slight variation in colour development.

The stock creatine solution (400 μ g./ml.) was diluted with detergent solution to concentrations of 20, 10 and 5 μ g. of creatine/ml., treated with an equal volume of chilled 14% trichloroacetic acid and filtered through Whatman no. 541 paper. The filtrate was often turbid but a clear solution was produced on the addition of the alkaline reagent mixture.

Samples (1 ml.) were taken for the estimation of free and total creatine, the determinations being carried out in parallel with the tissue extracts.

Deoxyribonucleic acid. Burton's (1956) modification of the diphenylamine reaction was applied to the protein and fat precipitate produced by addition of 14% trichloroacetic acid.

RESULTS

Tissue disintegration. Hodes, Palmer & Williams (1958) have commented that the non-ionic detergents Tween 80 and Triton X-102 will produce cytolysis of tissues but do not liberate the nuclei.

We have examined histologically the centrifuged debris from sliced liver, spleen, kidney, heart, skeletal muscle and synovial tissue, attacked by alkaline Tween 80 solution in the manner described, and find complete disruption of cells and nuclei.

In another investigation, the completeness of cellular breakdown with detergent was tested by chemical means. The total creatine in a number of tissues was estimated, and then the residue from the 14% trichloroacetic acid precipitation, which would contain any fragments of intact tissue, was homogenized vigorously with 5% trichloroacetic acid, and the creatine content of the filtrate was estimated. As Table 1 shows, the softer, more friable tissues were completely disintegrated by detergent, the mean extraction being 97%. However, the tougher synovial tissue is more resistant, with a mean extraction of 87%.

Recovery of creatine and phosphocreatine. Phosphocreatine appears to be quite stable in the alkaline-detergent solution for at least 1 hr. at room temperature, but under these conditions creatine solutions lose about 5%.

However, in the analytical technique described, the mixture of tissue and detergent never attains room temperature for this length of time, and, as Table 1 shows, the recovery of creatine added to various tissue is 102–104% and phosphocreatine about 97%.

These recovery experiments were made by extracting tissues with detergent which contained known amounts of creatine or phosphocreatine. Parallel controls without added substance were also carried out.

Hydrolysis of phosphocreatine and estimation of creatine. Ennor & Rosenberg (1952) hydrolysed the phosphocreatine present in neutral trichloroacetic acid extracts of tissue by treatment with 0.1N-HCl and heating at 65°. The chilled solution must be neutralized before the colorimetric technique can be applied.

This hydrolytic stage may be simplified by using warm trichloroacetic acid to remove the labile-phosphate group. The detergent extract of the tissue is acidified with an equal volume of 14%

Table 1. *Quantitative evaluation of the modified Ennor & Rosenberg (1952) technique*

The 'soft' tissues included spleen, kidney, lung, heart, liver, brain, uterus and skeletal muscle of rat or rabbit. Figures in parentheses are S.E.M. values.

Type of tissue	Completeness of tissue extraction		Added creatine		Added phosphocreatine	
	No. of expts.	Mean extraction (%)	No. of expts.	Mean recovery (%)	No. of expts.	Mean recovery (%)
'Soft'	13	97.1 (1.2)	11	101.8 (4.2)	13	96.7 (1.4)
Rabbit and human synovia	21	86.9 (1.2)	12	104.3 (0.7)	11	97.5 (2.4)

trichloroacetic acid, and the protein and fat precipitate removed by filtration. A portion (1 ml.) of the filtrate, which contains approximately 5% of free trichloroacetic acid, is heated at 60° for 10 min. This procedure brings about complete hydrolysis of the bound creatine and does not cause conversion of creatine into creatinine.

The technique of the colorimetric reaction is similar to that of Ennor & Rosenberg (1952), but uses smaller volumes and a higher concentration of alkali to neutralize the free trichloroacetic acid in the tissue filtrates. Multiple pipetting is avoided by employing a mixed reagent, but it is necessary to use the solution without delay. The development curve of the pink reaction colour rapidly reaches a gradually rising plateau which falls off very quickly after 1 hr. and we have arbitrarily chosen 35 min. as a convenient length of time for incubation. A standard curve may be drawn from 0–20 µg. of creatine, but since there is a slight day-to-day variation of approximately 5% in the slopes of the straight-line curves, we prefer to include standards of 10, 5 and 2.5 µg. of creatine with every batch of estimations.

Concentrations of creatine and creatine phosphate in tissues. Ennor & Rosenberg (1952) have commented that it is difficult to compare the concentrations of creatine phosphate, estimated by the diacetyl reaction, with values obtained by the probably less specific estimation of the labile-phosphate group. Nevertheless, the concentrations for various rat tissues, given in Table 2, agree sensibly with those reported by Ennor & Morrison (1958).

Table 3 shows the concentrations found in synovial tissues of rabbit and human origin. The 'normal' human membranes were obtained at the time of operation from patients undergoing meniscectomy. These cases had a traumatic aetiology but effusion had subsided before the operation. Four rheumatoid membranes have been included in the group of 12 human tissues, since their values were indistinguishable from those of the normal tissues. The table shows that there is a close similarity, on a wet-weight basis, between membranes from human and rabbit. However, in rheumatoid-villous tissue there is a slight elevation in the level of free creatine and a considerable decrease in bound creatine.

The synovial tissues examined always contained variable amounts of adherent fat, and consequently it was considered preferable to relate the creatine values to the tissue deoxyribonucleic acid content (Table 3), generally regarded as a more reliable index of cell number (e.g. Hotchkiss, 1955). On this basis there is a difference between the membranous tissue of rabbit and human, and a very marked reduction of both ratios for rheumatoid villi.

DISCUSSION

The various synovial tissues estimated in this investigation always contained a proportion of fat, and it is probable that the mean levels of creatine and phosphocreatine reported in Table 3 are low by several per cent. On a wet-weight basis there is a close similarity between human and rabbit membrane but when related to deoxyribonucleic acid-

Table 2. *Concentrations of phosphocreatine and creatine in normal rat tissues*

Rat tissue	No. of separate expts.	Concn. (mg./100 g. wet wt.)		Bound creatine (% of total)
		Free creatine	Phosphocreatine	
Spleen	11	8.8 (0.6)	3.1 (0.3)	18.1 (1.6)
Lung	8	9.5 (1.0)	2.5 (0.4)	14.5 (1.9)
Kidney	6	14.7 (3.0)	2.7 (0.8)	9.7 (1.6)
Brain	7	118.3 (4.5)	6.9 (1.9)	3.6 (1.0)
Muscle (cardiac)	5	145.5 (29.4)	28.7 (6.2)	12.3 (3.2)
Muscle (skeletal)	10	211.1 (28.9)	290.2 (23.5)	47.9 (4.1)

Figures in parentheses are S.E.M. values.

Table 3. *Concentrations of creatine and phosphocreatine in synovial tissue from human and rabbit*

Figures in parentheses are S.E.M. values.

Source and type of tissue	No. of tissues	Concn. (mg./100 g. wet wt.)			Bound creatine (% of total)
		Deoxyribonucleic acid-phosphorus	Free creatine	Phosphocreatine	
Human membrane, 'normal' and rheumatoid	12	5.39 (0.6)	5.0 (0.5)	2.4 (0.4)	21.1 (1.9)
Rheumatoid-villous tissue	9	23.4 (1.8)	7.4 (0.8)	0.9 (0.2)	7.4 (1.6)
Rabbit normal membrane	4 experiments (32 synovia)	3.74 (0.3)	5.8 (0.6)	2.6 (0.2)	22.2 (1.6)

phosphorus content the latter tissue contains 50 % more creatine and phosphocreatine than the comparable human material.

Table 3 shows quite clearly that in proliferative rheumatoid-villous tissue there is not only a considerable reduction in the proportion of bound creatine but also a marked drop in the levels of total creatine relative to the tissue deoxyribonucleic acid-phosphorus content. However, the massive synovial proliferation which takes place in the active-disease state is accompanied by a considerable change in subsynovial-cell types (Thomas & Dingle, 1959), and the changes observed in the ratios of creatine and phosphocreatine to deoxyribonucleic acid-phosphorus may well reflect these alterations in cell populations.

SUMMARY

1. The method of Ennor & Rosenberg (1952) for the estimation of creatine and phosphocreatine has been simplified and applied to various tissues including synovial tissue from human and rabbit.

2. Cellular disruption was brought about by a solution containing Tween 80, ethylenediamine-tetra-acetic acid, *p*-chloromercuribenzoic acid and alkali. The extraction of creatine from the sliced softer tissues was 97 %, but synovial tissue proved more resistant, with an average extraction of 87 %.

3. Quantitative recoveries of added creatine and phosphocreatine were obtained, and results for rat tissues agree with those found by other authors.

4. Human synovial membrane contained 5.0 mg. of creatine and 2.4 mg. of phosphocreatine/100 g.

of wet tissue, and normal rabbit synovial tissue contained 5.8 and 2.6 mg. respectively. The proportion of creatine bound as phosphate was roughly 22 % for each type of tissue. Rheumatoid-villous tissue contained 7.4 mg. of creatine and only 0.9 mg. of phosphocreatine/100 g. of wet tissue.

5. There was a considerable decrease of creatine (in relation to the amount of deoxyribonucleic acid) in rheumatoid-villous tissue, but this may be due to the considerable redistribution of cell types which accompanies the active disease state.

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A Structural Function of Mucopolysaccharide in Connective Tissue

By J. H. FESSLER*

Department of Biochemistry, University of Oxford, and Department of Medicine, Harvard Medical School, and the Massachusetts General Hospital, Boston, U.S.A.

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The function of hyaluronic acid in connective tissue is unknown. Studies described here show that one possible function of hyaluronic acid is a mechanical one: a combined structure of hyaluronic acid, water and collagen fibres can be made which has a definite resistance to compression.

A general investigation of the Wharton's jelly of umbilical cord was made and led to comparisons

with hyaluronic acid complex (Ogston & Stanier, 1950) of synovial fluid. Model experiments were then made in which mixtures of synovial fluid and fibres of soluble collagen were ultracentrifuged, and they showed properties consistent with the mechanical functions proposed. It is suggested that any suitable combination of water, a high polymer of open structure and fibres, can form a system resistant to temporary mechanical compression. This view, together with a summary of some of the work, has been described (Fessler, 1957).

* Present address: M.R.C. Group for Research on Body Temperature Regulation, Department of the Regius Professor of Medicine, The Radcliffe Infirmary, Oxford.