XXVII. THE USE OF SODIUM SULPHATE FOR THE PREPARATION OF CONCENTRATED PROTEIN-FREE TISSUE EXTRACTS

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(*Received 15 November 1937*)

SODIUM sulphate has been used as an alternative to ammonium sulphate for the precipitation of proteins since the last century, and its special advantages were exploited by Pinkus [1901], who drew attention to the importance of the facts that this salt is relatively insoluble at 0°, and that it crystallizes as a decahydrate. But these properties, which Pinkus utilized for the purification of proteins, lend themselves more directly to the isolation of non-protein substances contained in protein solutions or in tissues [M. G. & P. Eggleton, 1932].

Briefly, if 50 g. of anhydrous Na_2SO_4 are added to 100 g. of water at 32°, a saturated solution is formed, and if protein is present it is completely precipitated and may be filtered off. If this saturated solution is now cooled to 0° it separates into 110 g. of $Na_2SO_4.10H_2O$ and 40 g. of 4% Na_2SO_4 solution. The water-soluble constituents originally present in the 100 g. of water are now contained in 40 g. and free from protein. The principle is applicable without modification to minced muscle tissue, and since 100 g. water are contained in 125 g. muscle, this quantity should yield 40 g. of a protein-free extract, three times as concentrated as in the original tissue.

The following table, taken from a typical experiment shows that this is the case.

Table I

	Concentration in muscle mg. per 100 g.	Concentration in Na_2SO_4 extract mg. per 100 g.	Concentration ratio
Carnosine (by Pauly reaction)	35	112	3·2
Non-protein nitrogen	247	690	2·8
Lactate	250	700	2·8
Total phosphorus	118	303	2·6

The use of trichloroacetic acid, or similar protein precipitant would give about 500 g. of extract, about twelve times more dilute.

We have applied this technique to tissues other than muscle and obtained protein-free extracts from liver, brain, defibrinated blood and milk.

Actually manipulative losses are greater with the Na_2SO_4 technique, but in certain cases this disadvantage is outweighed by the smallness of the bulk to be handled, the rapidity of the process, and the mildness of the reagent employed. 1000 g. finely minced tissue are thoroughly mixed with 400 g. of powdered anhydrous sodium sulphate. The mixture is warmed to 32°, pressed out with the aid of a small fruit press previously warmed to the same temperature and, if necessary, the resulting extract is filtered (under suction). Care must be taken

to prevent much sodium sulphate from crystallizing out in the press or on the filter, as then some of the protein may dissolve and go into the filtrate.

The clear colourless oily solution (about 600 g.) is chilled to 0°, and the mother liquor separated by filtration, or better in a centrifugal crystal dryer. It is an economy to separate first at room temperature, chill the separated fluid to 0°, and filter off the further crop of crystals on a Büchner funnel. Yield 160 g. of extract (50 %).

We obtained the same average yield of 50–60 % of extract from muscle-, liver- and brain-tissues, and defibrinated blood. No doubt this yield can be increased if a more efficient press is available.

One pint of milk (300 g.) mixed with 135 g. anhydrous Na_2SO_4, warmed to 32°, filtered warm on a Büchner funnel, cooled down to 0° and filtered again, yielded 99 g. of extract, corresponding to a yield of over 90 %.

This method of extraction has also been used with success to obtain quickly extracts from blood for pharmacological testing. 100 ml. of blood (37°) is well mixed with 40 g. anhydrous Na_2SO_4 and immediately filtered under suction into a flask cooled in ice. The cooled filtrate is poured away from the Na_2SO_4, $10H_2O$ crystals, and can be injected at once in cases where the presence of 4 % of Na_2SO_4 does not matter. If necessary the extract can be treated with a $CaCl_2$ solution, the $CaSO_4$ filtered off, and the volume made up to six times the original volume of extract. The product is then isotonic and any active principles are present in a concentration about half that in the original blood.

If the yield is important, it is profitable to carry out a second extraction, by taking the tissue residue and crystallized Na_2SO_4, $10H_2O$ left over from the first extraction, adding enough water to make a saturated solution of sodium sulphate at 32°, or preferably the same amount of 4 % Na_2SO_4 solution as the first extract, mixing thoroughly, warming to 32°, pressing and filtering at this temperature, cooling down to 0° and filtering again.

1000 g. muscle on second extraction (750–800 g. tissue residue + 420–440 g. Na_2SO_4, $10H_2O$ + 160 g. 4 % Na_2SO_4 solution) yield again about 160 g. of extract. This second extract is only half as concentrated as the first one, thus corresponding to a yield of 25 % and increasing the total yield from both extractions to 75 %.

Our original intention in developing this method of extraction was to isolate from fresh muscle several substances, notably creatinephosphoric acid, carnosine and anserine, in order to discover whether they were present in the living tissue in the form of derivatives unstable towards the usual protein precipitants. No evidence of this was obtained in the cases examined.

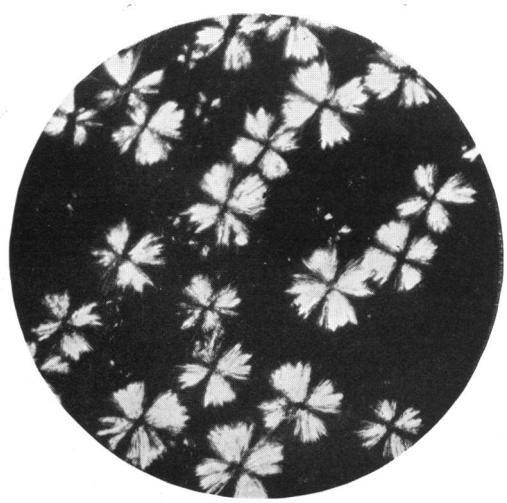
We have applied the Na_2SO_4 technique also to the isolation of creatine and lactic acid from butcher's meat, and to the isolation of various other substances. Creatine can be precipitated immediately from the extract by addition of acetone, and lactic acid was isolated as the zinc salt by continuous extraction of the acidified extract with ether, removal of the ether and treatment with zinc carbonate [Ghaffar, 1935].

In these cases it is the rapidity of the process and the small bulk to be handled, which present an advantage over other methods of extraction.

Barium creatinephosphate.[1] Twenty Hungarian frogs are kept for a day at 0–4°. They are then pithed, skinned and the hind limbs cut off and minced with Na_2SO_4 and a small addition (totalling 1–2 ml.) of conc. ammonia, to keep the reaction alkaline. More Na_2SO_4 is added at the end, to bring the ratio of Na_2SO_4 to muscle + bone up to 2 : 5.

[1] The experiments on the isolation of this substance were performed in association with Dr S. M. Ling of Peiping.

Barium creatinephosphate × 80.



Barium creatinephosphate under crossed nicols × 60.

The final extract (60 g. from 375 g. muscle + bone) is treated with an excess of saturated barium acetate solution and the volume is doubled by addition of acetone. After thorough mixing, the precipitate is filtered off on a Büchner funnel or centrifuged, washed successively with 50% acetone and acetone, and finally dried in a desiccator.

The precipitate is extracted thoroughly with water and the extract precipitated with an equal volume of acetone. This is best done by successive extractions with the smallest volume it is convenient to work with. The extraction is rendered less efficient by the adsorption of barium creatinephosphate on the barium sulphate precipitate as was recorded by Fiske & Subbarow [1929], but by a sufficient number of extractions the barium creatinephosphate can be got out from the precipitate. The combined precipitate is filtered off and dried.

The crude barium creatinephosphate is taken up in five times its weight of water, the solution filtered, and methyl alcohol added until a slight but permanent turbidity shows that saturation has been reached. If the side of the vessel is scratched crystallization of the barium salt begins, sometimes immediately, sometimes after a few hours. Recrystallization is effected in the same way.

The yield of once recrystallized salt from a preparation on this scale is about 0·4–0·5 g. By carrying out a second extraction, the yield can be increased to 0·6–0·7 g. For analysis the substance is dried in high vacuum over P_2O_5 at 20°. (Found: C, 13·7; H, 2·8; N, 11·7; P, 8·6; Ba, 37·3%. $C_4H_8O_5N_3PBa$, H_2O requires: C, 13·2; H, 3·0; N, 11·5; P, 8·5; Ba, 37·7%.)

The crystal form is illustrated in Plate IV.

Carnosine and anserine. Both these substances have been isolated from vertebrate skeletal muscle [*v.* Wolff & Wright Wilson, 1935, for further references]. For the isolation of these substances we adopted a simplified technique, working under the mildest possible conditions in order to ascertain whether carnosine and anserine are present in muscle as such or in form of unstable derivatives. As already mentioned, no evidence of the latter could be obtained.

Copper carbonate (freshly prepared from copper sulphate and sodium bicarbonate) is stirred into the Na_2SO_4 extract from muscle, until there is an excess. No heat is necessary. The solution is filtered into a distilling flask and concentrated under reduced pressure (at 30–35°) until the bulk is reduced to one-quarter, or until the copper carnosine (or copper anserine) crystallizes out. It is then set aside overnight, and the crystals filtered off. The yield can be increased slightly by further concentrating the mother liquor or adding acetone.

As an alternative to the concentration under reduced pressure, 2 vols. of acetone may be added to the filtered copper salt solution (or more if necessary) and the copper carnosine (or copper anserine) precipitates as a thick syrup. This on extraction with acetone may crystallize, but usually gives an amorphous solid.

The product from either preparation is dissolved in a minimal amount of conc. ammonia solution, filtered from a colourless crystalline residue, and the ammonia is aspirated off. Crystallization usually begins before all the ammonia is gone, but occasionally it is necessary to leave the ammonia-free solution overnight to crystallize. Recrystallization is effected in the same way.

The copper carnosine and copper anserine were recrystallized three times and dried *in vacuo* over sulphuric acid at 20°.

Horse muscle yielded 0·8–1 g. pure copper carnosine per 1000 g. muscle (extracted twice). (Found: C, 33·6; H, 5·0; N, 16·8%. $C_9H_{14}O_3N_4CuO$, H_2O requires: C, 33·4; H, 4·95; N, 17·3%.)

The pure copper salt was freed from copper by H_2S and the free base converted into the nitrate, following the directions given by Ackermann *et al.* [1929],

for anserine. The nitrate was recrystallized three times from hot dilute alcohol and dried *in vacuo* over P_2O_5 at 100°. M.P. 227° (decomp.), $[\alpha]_D^{20°} + 23{\cdot}6°$ in water ($c = 3{\cdot}5$, 1 dm. tube). (Found: C, 37·3; H, 5·3; N, 24·0; $(N)CH_3$, 0·0%. $C_9H_{14}O_3N_4.HNO_3$ requires: C, 37·35; H, 5·2; N, 24·2; $(N)CH_3$, 0·0%.)

We have also isolated carnosine from the Na_2SO_4 extract of frog muscle. (Found: C, 33·4; H, 4·9; N, 17·0%. $C_9H_{14}O_3N_4CuO$, H_2O requires: C, 33·4; H, 4·95; N, 17·3%.)

Rabbit muscle yielded 1·5–2 g. pure copper anserine per 1000 g. muscle (extracted twice). (Found: C, 37·5; H, 5·1; N, 17·0%. $C_{10}H_{16}O_3N_4CuO$ requires: C, 37·6; H, 5·0; N, 17·5%.)

The pure copper salt was converted into the nitrate in the same way as in the case of carnosine. The nitrate was recrystallized three times from hot dilute alcohol and dried *in vacuo* over P_2O_5 at 100°. M.P. 226° (decomp.), $[\alpha]_D^{20°} + 14{\cdot}0°$ and $+12{\cdot}8°$ in water ($c = 3{\cdot}74$ and 6·5, 1 dm. tube). (Found: C, 39·7; H, 5·7; N, 23·4; $(N)CH_3$, 5·0%. $C_{10}H_{16}O_3N_4.HNO_3$ requires: C, 39·6; H, 5·65; N, 23·1; $(N)CH_3$, 4·95%.) The carnosine content of the preparation, as measured by the Pauly reaction, was less than 0·5%.

In addition we have isolated anserine from the muscles of sheep and goat. (Found: C, 34·4; H, 5·4; N, 15·4% (sheep) and C, 34·4; H, 5·5; N, 15·4% (goat). $C_{10}H_{16}O_3N_4CuO$, $2H_2O$ requires: C, 33·8; H, 5·6; N, 15·75%.) The carnosine content of these preparations, as measured by the Pauly reaction, was 1·7% (sheep) and 3·7% (goat).

From sheep muscle Smorodinzew [1914] reported the isolation of carnosine. The analyses of minced goat muscle by means of the Pauly reaction indicated a high carnosine content (350 mg. per 100 g., if the Pauly reaction were attributable entirely to carnosine). But with the simplified technique we used one never obtains both carnosine and anserine from the same animal. There is always an appreciable residue of uncrystallizable copper salts, which may be a mixture of anserine, carnosine and other copper salts.

It has been known for some years [Clifford, 1921] that the muscles of invertebrates give no Pauly reaction, and therefore contain no carnosine [*v.* also Flössner, 1932; Broude, 1933]. In order to ascertain whether anserine could be isolated, the isolation technique just described was applied to the flesh of lobster (*Homarus vulgaris*), scallop (*Pecten maximus*), limpet (species not identified) and sea anemone (*Tealia crassicornis*). Only in the case of pecten was a crystalline copper salt obtained. This proved to be copper glycine, and the large yield suggested that there must be about 2% of free glycine in this animal. Both the "quick" and "slow" muscles were used in the preparation, so it is not possible to say if either muscle contains none.

The copper glycine obtained from pecten was recrystallized, and dried *in vacuo* over sulphuric acid at 20°. (Found: N, 12·1%. $(C_2H_5O_2N)_2CuO$ requires: N, 12·2%.)

Glycine was detected by Chittenden [1875] in *Pecten irradians* and later by Kelly [1904] in *P. opercularis*. [For further references see Kutscher & Ackermann, 1936.]

An attempt to isolate carnosine or anserine from the brain of a horse confirmed the negative result of Tschernow, reported by Hefter [1925] in that the extract proved to contain very little of any compound capable of giving a copper salt in neutral solution from $CuCO_3$ and no crystalline substance could be isolated.

SUMMARY

1. A method is described for obtaining concentrated protein-free extracts of animal tissues under very mild conditions, by the use of sodium sulphate.

2. Examples of the application of this technique to the preparation of concentrated extracts from muscle, liver, brain, blood and milk are given.

3. The method has been used to ascertain whether creatinephosphate, carnosine and anserine are present in muscle in free condition or in unstable combination with other tissue constituents. No evidence of such unstable combinations was obtained.

4. Barium creatinephosphate obtained by this method from fresh muscle crystallizes as soon as it is separated from insoluble barium salts. The yield is sufficiently good for the method to be recommended for the isolation of small quantities of pure creatinephosphate.

5. In confirmation of earlier workers carnosine has been isolated from the muscles of the horse and anserine from the muscles of the rabbit. Carnosine has also been isolated from frog muscle, and anserine from the muscles of sheep and goat.

6. Certain other applications of the technique are described.

The expenses of this work were borne by grants from the Moray Fund (to A. D.) and from the Medical Research Council (to P. E.).

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