J. Physiol. (1955) 130, 427–437

# AUTOLYTIC CHANGES AT ZERO CENTIGRADE IN GROUND MAMMALIAN TISSUES

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(*Received* 18 *April* 1955)

In a previous communication (Conway & McCormack, 1953) strong evidence was brought forward for the conclusion that the molar concentration inside mammalian tissue cells was the same as that of the plasma. The view had been entertained by Opie (1949) and Robinson (1950) that intracellular concentrations were much higher than those of the plasma (50–100% higher), and that as a consequence water entered the cells continuously *in vivo* and was pumped out again.

Briefly, the evidence on which this view depended was the swelling of tissues, or tissue slices, when these were immersed in Ringer fluid isosmotic with plasma, under conditions when metabolism was inhibited, as by cold (0° C). The water so taken up was lost again on immersion of the slices in oxygenated Ringer fluid at 37° C. Cyanide (0·002 M) also produced the same effect as low temperature and its action was shown to be reversible (Robinson, 1950). As supporting evidence the older observations of the freezing-points of tissues (Sabbatani, 1901; Gömöri & Molnár, 1932) were cited. The implications of the view that Opie and Robinson put forward are such that the total molecular concentration inside mammalian tissue cells should be about 480–640 m-mole/l. Taking mammalian skeletal muscle, for example, it has been shown (e.g. Conway, 1950*b*) that the sum of the concentrations of known substances of quantitative significance in the fibre water is about 320 m-mole/kg water, or approximately the same as the total extracellular concentration. Also, the dry weight of such substances corresponds to the total dry weight of muscle minus that of the known and analysed substances of high molecular weight. The difference between these two estimates of the total concentration of low-molecular weight substances is practically zero, while it would amount, on a conservative estimate, to 2–3 g/100 g muscle if the total osmolar concentration in the fibre water were double that of the extracellular fluid. Furthermore, it could be deduced that if such organic molecules existed they would be non-acidic, since

there is no evidence for the existence of basic groups to balance 150 to 300 extra anionic charges over and above those already considered (Conway, 1950*b*). They would also be non-nitrogenous, small in size, and because small and non-diffusible, they should be highly polar. But there is no analytical evidence for the existence of such molecules in anything like the required quantity.

At the outset these would be formidable objections to the views of Opie and of Robinson, who have ignored the existing analytical data, but also there is the positive evidence from the freezing-points taken under suitable conditions (Conway & McCormack, 1953). From these experiments it appears that the fresh tissue (immediately after removal) has the same intracellular concentration as the plasma. At the same time it was shown that with certain tissues, such as skeletal muscle and rat kidney, the molecular concentration of the frozen and ground tissue maintained at 0° C increased sharply and linearly during about 20 min. Such a rapid rise in total molecular concentration even at 0° C affords an explanation of Opie's results with the melting-points of frozen tissues (Opie, 1954) since the melting extended over 30 min. His criticism of the results of Conway & McCormack (1953) amounts apparently to the curious hypothesis that the addition of an equal amount of 0·95% NaCl to the frozen and ground tissue at 0° C may cause the disappearance from free solution of about 150–300 mM of some unknown material, calculated per kg of original cell water. It may be pointed out that maintaining the tissue without any such addition at 0° C causes a molecular increase similar to that which occurs when saline is added. Addition of saline after 30 min gives the same freezing-point depression as when added from the beginning. This shows that addition of saline does not in any case result in the disappearance from free solution of the new molecules formed on standing, and it would be surprising if it did so.

In the present circumstances we give the results of an investigation into the nature of the increased molecular concentration occurring when skeletal muscle and kidney tissue are frozen in liquid oxygen, powdered and then maintained at 0° C.

## METHODS

### *Treatment of tissue*

The tissues examined were guinea-pig abdominal muscle and diaphragm, rabbit diaphragm and rat kidney. The general method was as follows. The tissue, after its rapid removal from the anaesthetized animal, was hooked on to a piece of wire and plunged into liquid oxygen for 2 or 3 min, being kept under the surface to prevent condensation. It was then ground to a fine powder in a mortar, using occasional small amounts of liquid oxygen to maintain a low temperature. This operation was performed as quickly as possible to prevent condensation of moisture. A portion of the powder was analysed immediately for its various chemical constituents ($t_0$ values). The rest of the frozen powder was placed in an enclosed vessel, allowed to thaw to about 0° C, and then kept on crushed ice at 0° C for 1 hr before analysing. By analysing this sample ($t_{60}$) indications

of the nature of the molecular increase as found by the increase in freezing-point depressions ($F_t$) mentioned in the previous paper (Conway & McCormack, 1953) could be expected.

Apart from direct analysis of the powdered tissue as just described, the following further treatments were carried out before analyses.

*Preparation of trichloroacetic acid (TCA) extracts.* About 1 g of the frozen powder ($t_0$ sample) was introduced through a chilled funnel into a weighed centrifuge tube containing 2 ml. of cold 10% TCA. More of the 10% TCA was then added, so that the total was 5 ml. of 10% TCA per g powder. After centrifuging, the supernatant fluid was filtered through a small Buchner funnel and the filtrate analysed. The $t_0$ sample was treated similarly, about 1 g of the powder being introduced into a weighed centrifuge tube and kept at 0° C for 1 hr, the 10% TCA being subsequently added.

*Dialysis.* This was carried out on tissue-saline mixtures prepared as follows. The $t_0$ sample of powder was ground with an equal volume of 0·95% NaCl containing 0·1% $HgCl_2$. The $t_{60}$ sample was treated similarly after maintaining at 0° C for 60 min.

About 0·5 g of tissue-saline mixture was introduced into a collodion sac of about 1 ml. capacity. The sacs were then placed in glass tubes (capacity 2 ml.) containing 0·5 ml. distilled water. The tubes were stoppered and placed in the refrigerator for 100 min, the mixtures being stirred at intervals with a glass rod.

*Extraction of organic acids.* Extractions with ether were carried out on TCA extracts of $t_0$ and $t_{60}$ samples, using the Kutscher–Steudel apparatus at 45° C. The ether extract was reduced to dryness *in vacuo* and the organic matter dissolved in a small amount of water for electrometric titrations.

### *Chemical analyses*

*Glycogen* was determined by direct analysis of the frozen powder. This was boiled with alcoholic potash and the glycogen residue hydrolysed (Kerr, 1936). The resulting glucose was estimated by the method of Folin & Malmros (1929).

*Lactic acid* was estimated by hydrolysis with N-HCl (Lohmann & Meyerhof, 1934), and the resulting pyruvic acid by the method of Friedemann & Haugen (1943).

*Reducing sugars* were determined by the method of Folin & Malmros (1929).

*Organic acids* were determined by electrometric titration of the ether extracts as described below.

*Phosphate esters* of various kinds were determined after barium fractionation of the TCA extracts (Conway & Hingerty, 1946). The barium-insoluble fraction was analysed for the following substances:

(i) adenosine triphosphate by the method of Lohmann (1929);
(ii) fructose diphosphate by Roe's method (1934);
(iii) 3-phosphoglyceric acid by the method of Rapoport (1937).

The barium-soluble fraction was in turn analysed for the following:

(i) total hexose mono-esters by measuring the total phosphate remaining after precipitation of the residual unhydrolysed creatinephosphate by the method of Sachs (1944);
(ii) triose-phosphate according to Lohmann & Meyerhof (1934);
(iii) creatinephosphate by measuring the increase in inorganic phosphorus due to hydrolysis in strong acid at room temperature;
(iv) inorganic phosphorus was determined by the method of Fiske & SubbaRow (1925).

*Total nitrogen* was estimated by micro-Kjeldahl incineration and microdiffusion (Conway, 1950*a*). Total non-protein nitrogen was determined with TCA extracts.

*Amino acids* were estimated by treating with ninhydrin (Van Slyke, Dillon, MacFadyen & Hamilton, 1941) and the ammonia measured by microdiffusion (Conway, 1950*a*).

*Peptides* were estimated as amino acids after hydrolysis for 10 hr in 20% HCl.

*Amide nitrogen* was estimated as ammonia after hydrolysis of TCA extracts with 3N-$H_2SO_4$ (Conway, 1950*a*).

*Creatine* was estimated after conversion to creatinine (Böhm & Grüner, 1936).

*Purine nucleotides* and *nucleosides* were determined after precipitation (Kerr & Blish, 1932), Kjeldahl incineration and microdiffusion.

*Bicarbonate, chloride* and *ammonia* were estimated by microdiffusion (Conway, 1950*a*).

*Sodium, potassium* and *magnesium* were estimated by flame photometry (magnesium after precipitation with 8-hydroxy-quinoline).

*Calcium* was precipitated with oxalate and titrated with permanganate (Clark & Collip, 1925)

*Electrometric titrations* were carried out on TCA extracts and saline mixtures prepared as for dialysis but using five times the volume of saline. One ml. of fluid was titrated with N/2·5 acid or alkali from a micro-syringe. The temperature of the sample was kept as near 0° C as possible, and in the case of the saline the surface of the liquid was covered with a layer of paraffin to prevent appreciable changes in $CO_2$.

*Freezing-point determinations* were carried out as described by Conway & McCormack (1953).

## RESULTS

### *Electrometric titrations*

*Trichloroacetic acid extracts of muscle (abdominal; guinea-pig).* Titrations were carried out as described in Methods on TCA extracts at $t_0$ and $t_{60}$ of guinea-pig abdominal muscle and diaphragm. Figs. 1 and 2 refer to diaphragm, but since the curves for abdominal muscle were very similar they will not be described here.

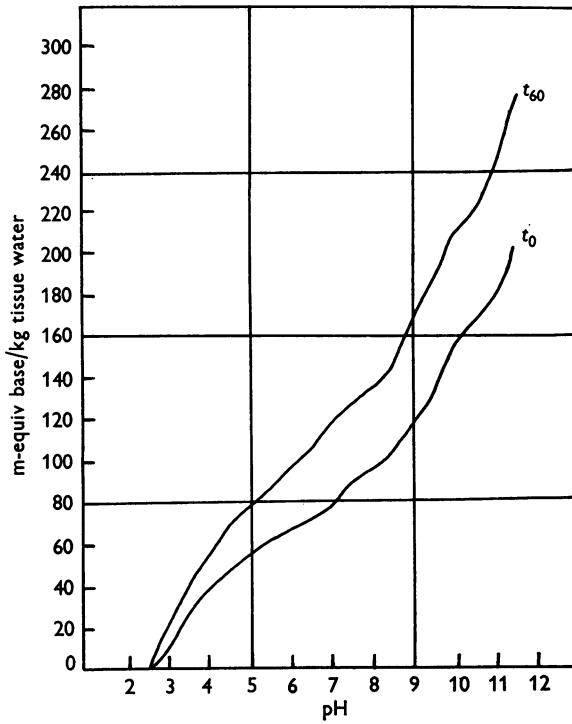


Fig. 1. Titration curves for guinea-pig diaphragm extracted at $t_0$ and $t_{60}$ with trichloroacetic acid.

Fig. 1 shows the titration curves for TCA extracts of diaphragm at $t_0$ and $t_{60}$. The buffering distribution at $t_0$ is shown in Fig. 2, together with the difference between the distributions for $t_0$ and $t_{60}$. From these results the following may be stated: (1) There occurs a marked increase in titration values on maintaining the previously frozen and powdered tissue at 0° C for 1 hr. The titration difference between pH 2·5 and 11·0 may be expressed as about 75 m-equiv/kg of original tissue water. (2) The zones of increased buffering appear around pH levels of 3·0, 4·25, 6·65, 9·0 and 11·0.

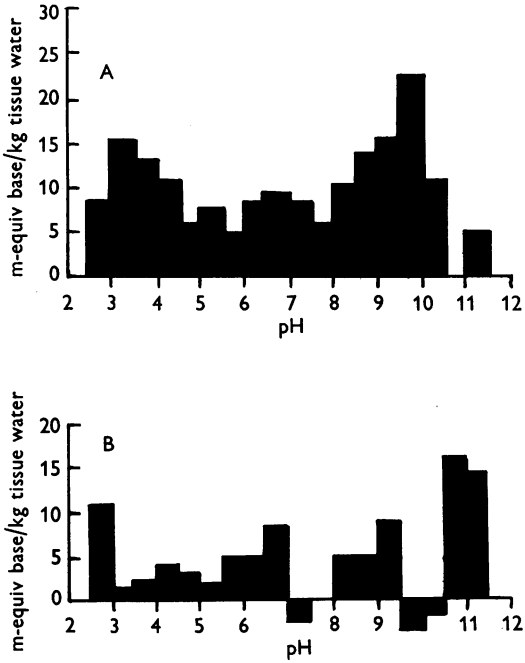


Fig. 2. Buffering distribution of trichloroacetic acid extracts of guinea-pig diaphragm. The buffering is expressed for each 0·5 pH range as m-equiv base/kg tissue water/unit pH. A, Buffering of $t_0$ sample; B, increase in buffering due to maintaining at 0° C for 60 min.

*Trichloroacetic acid extracts of rat kidney.* The experiments were carried out in a similar way to those with muscle. Fig. 3 shows the difference between the $t_0$ and $t_{60}$ titrations expressed as buffering power. This kidney buffer graph shows a much smaller increase in the region pH 6–7 than the corresponding muscle curve. The increases above pH 10 and below pH 3 are somewhat similar to muscle.

## *Chemical analyses*

*Abdominal muscle (guinea-pig).*

Table 1 shows the results of analyses of the TCA extracts of the $t_0$ preparation of guinea-pig abdominal muscle, and the increase in molecular

concentration after 60 min at 0° C. Only the analyses showing a significant change are given. All concentrations are given as m-mole/kg of original tissue water.

It will be seen that a number of labile substances break down rapidly, even at 0° C, and between ATP, hexose esters, creatinephosphate and glycogen, one can account for a 28% increase over an external 0·30 osmolar fluid and a possible 44% increase. These increases are referred to tissue water, but if referred to the original intracellular fluid of the abdominal muscle the increase amounts to 40–60%. Similar figures were obtained for diaphragm.

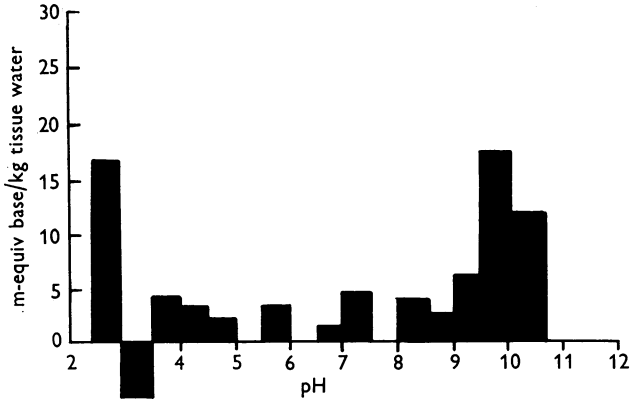


Fig. 3. Increase in buffering of trichloroacetic acid extracts of rat kidney after 60 min at 0° C, calculated from the difference between the titrations of the $t_0$ and $t_{60}$ samples. Buffering levels expressed as in Fig. 2.

TABLE 1. Analyses of trichloroacetic acid extracts of guinea-pig abdominal muscle. Concentrations expressed in m-mole/kg tissue water unless otherwise stated.

| Source of the molecular increase in free solution | No. of experiments | $t_0$ values | Change in concentration over 60 min | Increase in total molecular concentration over 60 min | Possible maximum molecular increase | Nature of the increase |
|---|---|---|---|---|---|---|
| ATP | 2 | 7·6 | −7·5 | 22·5 | 22·5 | Inosinic acid, ammonia and phosphate |
| Hexose esters | 2 | 36·3 | −10·9 | 10·9 | 21·8 | Phosphate and and triose or 3-carbon compounds |
| Creatinephosphate | 2 | 11·3 | −9·8 | 9·8 | 9·8 | Creatine and phosphate |
| Glycogen (m-mole hexose per kg tissue water) | 2 | 56·0 | −35·5 | 35·5 | 71·0 | See Table 2 |
| Non-protein nitrogen sources (mg-atoms N per kg tissue water) | 6 | 160·3 | 5·8 | 5·8 | 5·8 | |
| | | | Total | 84·5 | 130·9 | |

Such increases due to the breakdown of organic compounds, observed on the TCA filtrates, do not represent the total produced. Potassium ions are to some extent rendered osmotically inactive by attachment to negatively charged protein, and the same applies in greater relative amounts to magnesium. A proportion of these attached ions will be set free. This is evidenced by the dialysis experiments, which show that after 60 min at 0° C, there is more diffusible magnesium and also somewhat more diffusible potassium. The amounts of potassium and magnesium so liberated are approximately 5·0 and 5·5 m-mole/l. of tissue water.

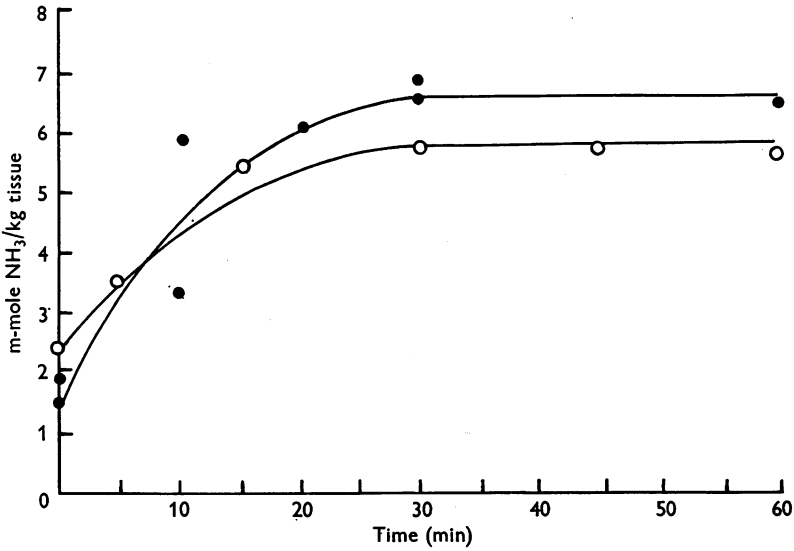


Fig. 4. Increase in free ammonia in frozen and powdered guinea-pig diaphragm, maintained at 0° C. The results for two animals are given.

*Notes on the breakdown of the labile substances*

*ATP.* This breaks down to inosinic acid, phosphate and ammonia. The course of the breakdown can be readily followed by the free ammonia figures which come from the deamination of the adenylic acid produced by dephosphorylation of ATP. Each mole of ATP disappearing gives rise to four new moles. Fig. 4 shows the rate at which ATP breaks down in the ground tissue mixture maintained at 0° C. The data represent the increase in the free ammonia content, resulting from the deamination of adenylic acid.

*Hexose esters.* These also break down quickly, and presumably into phosphate plus triose derivatives, or at least to 3-carbon compounds, since hexose esters are not appreciably found after the 60 min at 0° C. One mole of hexose ester disappearing may cause therefore the appearance of three new moles.

*Creatinephosphate.* This disappears rapidly, giving rise to free phosphate and creatine.

*Glycogen.* A considerable portion disappears after 60 min but only a relatively small proportion appears as lactic acid as seen in Table 2. Glyceric acid also results, and the total of ether-extractable acids found amounts to about one half the total breakdown of glycogen (reckoned as m-mole of hexose). Fig. 5 shows the increased buffering due to these acids extracted from abdominal muscle.

TABLE 2. Details of the breakdown of glycogen in guinea-pig abdominal muscle. All concentrations given as m-mole/kg of tissue water

| Nature of the molecular increase in free solution | No of expts. | $t_0$ values | Change in molecular concentration over 60 min |
|---|---|---|---|
| Lactic acid | 2 | 4·4 | +17·6 |
| Glyceric acid (phosphate) | 1 | 2·2 | +14·0 |
| Keto-acids | 1 | 0·7 | + 0·03 |
| Total of ether extractable organic acids | 2 | 51·0 | +32·0 |

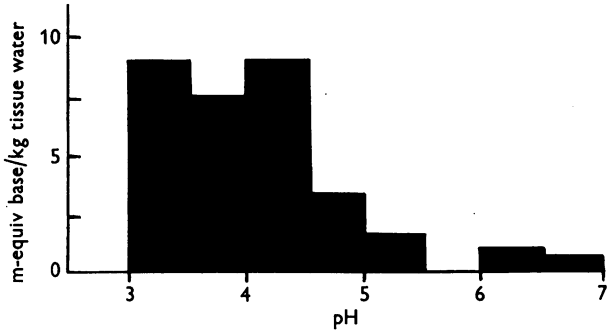


Fig. 5. Increase in buffering due to organic acids, in guinea-pig abdominal muscle, after 60 min at 0° C. Buffering expressed as in Fig. 2.

*Kidney (rat)*

It can be seen from Table 3 that in the case of rat kidney there is also a rapid breakdown of labile substances on maintaining the ground tissue at 0° C for 60 min. The principal substances contributing to the molecular increase are ATP, hexose esters and organic acids. There is also a marked increase in non-protein nitrogen which is shown in further detail in Table 4. The increase in buffering due to the ether-extractable organic acids is shown in Fig. 6.

TABLE 3. Results of analyses of the trichloroacetic acid extracts of rat kidneys. Only the analyses showing a significant change are given. Concentrations expressed in m-mole/kg tissue water unless otherwise stated

| Source of the molecular increase in free solution | No. of expts. | $t_0$ values | Change in concentration over 60 min at 0° C | Increase in total molecular concentration over 60 min | Possible maximum molecular increase | Nature of increased products |
|---|---|---|---|---|---|---|
| ATP | 2 | 1·5 | −1·5 | 4·5 | 4·5 | Inosinic acid, ammonia, phosphate |
| Hexose esters | 2 | 20·2 | −9·3 | 9·3 | 18·6 | Phosphate and triose or 3-carbon compounds |
| Organic acid sources (ether-extractable) | 1 | 51·0 | +35·0 | 35·0 | 35·0 | |
| Non-protein nitrogen sources (mg-atoms N per kg tissue water) | 5 | 194·0 | +40·4 | 40·4 | 40·4 | |
| | | | Total | 89·2 | 98·5 | |

TABLE 4. Details of the increase of non-protein nitrogen.

All results are given as milligram atoms of nitrogen/kg tissue water

| | $t_0$ | $t_{60}$ | Change |
|---|---|---|---|
| Total non-protein nitrogen | 194 | 234 | +40 |
| Amide nitrogen | 14 | 16 | +2 |
| Amino nitrogen | 27 | 32 | +5 |
| Peptide nitrogen | 74 | 84 | +10 |
| Nucleotide nitrogen | 18 | 30·5 | +12·5 |
| Nucleoside and purine nitrogen | 29·7 | 33·5 | +3·8 |

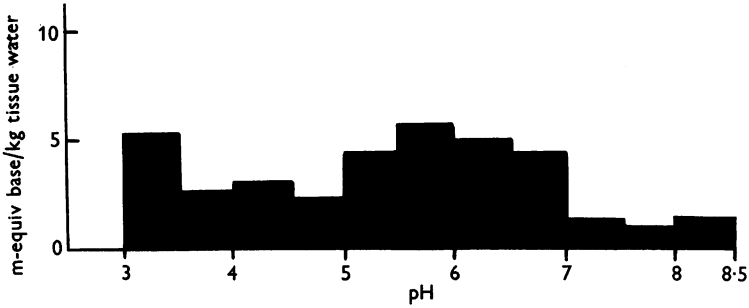


Fig. 6. Increase in buffering due to organic acids, in rat kidney, after 60 min at 0° C. Buffering expressed as in Fig. 2.

## DISCUSSION

It is obvious from these studies that in ground tissue, even at 0° C, there is a surprising rapidity of breakdown of substances such as ATP, hexose esters and glycogen (although only a small fraction of the breakdown of glycogen passes to lactic acid over 60 min). The increase in molecular concentration as found analytically approaches that calculated from the freezing-point

depression, and would probably account for the total $F_i$ change if all the substances were recognized and determined.

One half of the ATP is broken down to inosinic acid, ammonia and phosphate in less than 10 min. At 37° C such breakdown would no doubt proceed at a greatly increased rate. It is very likely that in frozen and thawed tissue, maintained at about 0° C there is a similar rapid increase and this would account for such results as those of Opie (1954). The relatively large increase of amino acid and peptide nitrogen, as well as the increase in ether-extractable organic acids in the ground kidney, are of special interest. They suggest that when, in rat kidney slices immersed in Ringer fluid, swelling occurs as the result of metabolic inhibition by low temperature (0–3° C) or cyanide (Robinson, 1950), the breakdown of such labile substances is a major cause of the swelling. This question is dealt with in a subsequent paper. Here account may be taken of the short note published by Brodsky, Rehm & McIntosh (1953) on osmotic equilibria in tissues, using a technique in some respects similar to that employed by Conway & McCormack (1953). The fresh tissue of dogs was frozen in liquid nitrogen, then crushed in a Carver press, the freezing-point depression being measured by a cryoscopic method. The results suggest to them the conclusion that internal isotonicity is maintained in organs which do not produce a secretion, such as skeletal muscle, while internal hypertonicity is restricted to those organs concerned with the production of a secretory fluid (kidney, stomach, liver, etc.). With regard to Robinson's comment on this note (Robinson, 1954) we do not agree that similar precautions were taken in the determinations. No mention, for instance, is made of the effect of time after the preparation of the cell-free homogenate on the freezing-point depressions and it is clear from the results obtained in this laboratory that the molecular increase is so rapid in certain tissues after disintegration that it could quite easily account for the observed facts.

## SUMMARY

1. The nature of the increased molecular concentration occurring in frozen and powdered mammalian tissues maintained at 0° C has been investigated.

2. With skeletal muscle so treated there occurs a rapid breakdown of ATP, hexose esters, creatinephosphate and glycogen during the 60 min period. The total of extra molecules produced accounts for a rise in the molecular concentration in the tissue water of about 28–44% over an external 0·30 osmolar solution. This will tend to be a little further increased by changes in diffusible potassium and magnesium.

3. There is a similar breakdown of labile substances in the rat kidney during the 60 min at 0° C. Such substances are ATP, hexose esters and precursors of organic acids, and of increased free non-protein nitrogen. The increase

in the molecular concentration is calculated as 30–33% over an external 0·30 osmolar solution.

4. It is suggested that a similar molecular breakdown is an important cause of the swelling of tissue slices in Ringer fluid during anoxia, as found by other workers. Other causes of this swelling are discussed in a subsequent paper.

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