II. INOSITOL METABOLISM IN THE MAMMALIAN HEART.

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THE significance of the occurrence of inositol in animal tissues is still unexplained, and it is a matter of dispute whether inositol is metabolised by animals. Rosenberger [1910] asserted that the inositol content of muscle was increased during autolysis but gave no figures. Needham [1923] compared the inositol contents of different tissues of a freshly killed rabbit and of one killed two days before: he remarks "It is certainly interesting that the muscle from the freshly killed rabbit contained 50 % less inositol than that from the rabbit which had been dead two days. It recalls the view of Rosenberger that tissues contain an 'Inositogen,' though his conclusions were drawn from the comparison of fresh rabbit muscle and commercial beef; tissues perhaps hardly comparable."

Boyland [1928], on the other hand, records three experiments with pig's heart and as a result suggests that inositol decreases during survival changes in muscle and is a precursor of lactic acid. For a review of the literature see Needham [1926].

EXPERIMENTAL.

Work on inositol has been hampered by lack of a convenient method for its estimation. Needham [1923] made use of acetone for extraction of the tissues, and precipitated the inositol with basic lead acetate: the inositol was then thrown down with strong alcohol and the carbon in the precipitate was determined. Boyland [1928] used a modification of Needham's method, the modification consisting in a direct weighing of the precipitate in place of a micro-estimation of carbon. Boyland remarks that "The method is probably not very specific, but a more reliable method could not be found." Complete extraction of even minced tissue is difficult unless large quantities of solvent are used, and then it is not easy to make the process quantitative: in addition, in the present work it was desirable to use a method by which cell enzymes could be destroyed or inactivated as soon as possible after removal of the tissue from the body. It was decided to take advantage of the stability of inositol to hot alkali. Rosenberger [1910] made use of boiling KOH solution for the estimation of inositol, but his drastic treatment has been criticised by Momose [1916]. The principle of the method employed in the present work is as follows. The tissue (30–40 g.) is disintegrated by heating with an equal volume of 10 % aqueous KOH: after neutralisation and cooling, a large amount of impurity is removed with normal lead acetate: the inositol is precipitated by basic lead acetate, and after being freed from the lead compound is precipitated by baryta in alcoholic solution. After decomposition of the barium precipitate the inositol is dried, recrystallised and weighed. The fresh heart-muscle of dogs was used throughout. The details are best described in the form of a typical experiment.

The animal is killed by severing the carotid arteries; the chest is immediately opened and the heart removed¹. Excess of fat is removed with a few strokes of the knife, the heart is divided roughly into two, one-half is immediately weighed and then cut up over a flask containing 10 % KOH at 100°; when all the pieces have been dropped into the potash a condenser is fitted and boiling is continued for the required period. The amount of KOH solution should be about 1 ml. for each gram of tissue. Meanwhile the other half of the heart is allowed to undergo survival changes, then the muscle is cut up into potash and the procedure is the same as for the first half. After the boiling with alkali is completed the fluid is made acid to litmus with glacial acetic acid, cooled to about 50°, and a rapid current of hydrogen is passed through for an hour to remove hydrogen sulphide liberated by the action of the potash on sulphur compounds in the muscle. Half the volume of water is added, and after standing for a day in the cold chamber the fluid will filter clear. To the filtrate is added a saturated solution of normal lead acetate, 0.2 ml. per g. of muscle, and a day is allowed for complete precipitation.

After centrifuging (the precipitate being discarded without washing) ammonia is added to the clear fluid until further additions give no precipitate. The precipitate is very bulky, but may be collected into two centrifuge-tubes of 25 ml. capacity if a very high speed machine such as the "Leune," made by Messrs Hearson, is used. To the clear liquid lead acetate (0.2 ml. per g. of tissue) is added, followed by ammonia. The precipitate is collected in the same cups as the first. A further precipitation, using half the quantity of lead, is performed. The combined precipitate is twice washed very carefully with 75 % ethyl alcohol, and the cups with the precipitate are then dried *in vacuo* over sulphuric acid. The precipitate is then weighed and collected in one centrifuge-cup; it is ground up with 2*N* sulphuric acid and about 5 ml. of water until the mixture is acid to litmus. About 2 ml. of acid are required for each g. of the lead compound. The mixture is centrifuged and the liquid is measured and poured into a 100 ml. flask. The precipitate is stirred up with 5 ml. of water, and sufficient acid (about 0.3 ml.) is added to make the reaction acid. The process is repeated. To the combined extracts in the flask 3 volumes of strong alcohol are added. Precipitation takes several days. The fluid is centrifuged, and the precipitate is discarded without washing. An excess of 10 % baryta in methyl alcohol is added, and after 10 minutes the liquid is centrifuged and the precipitate washed with 75 % alcohol, and suspended in 100 ml. of 75 % alcohol in a tall cylinder. Care must be taken to obtain a uniform suspension, and this is best effected by first stirring up the precipitate with very small amounts of alcohol. The barium-inositol compound is decomposed by passing in a stream of CO₂ at 40° for 2 hours. Two days are allowed for precipitation of carbonate, the fluid is centrifuged and concentrated to dryness under reduced pressure. The residue is washed with dry methyl alcohol, taken up in 5 ml. water and allowed to evaporate slowly over sulphuric acid. Usually the inositol crystallises but occasionally is left as a glass. Recrystallisation is effected by dissolving the solid in 2.5 ml. water and adding 20 ml. strong alcohol. After 2 weeks the crystals are washed with methyl alcohol, dried and weighed. The melting-points of different preparations made in this way varied between 211 and 215°. Two preparations taken at random gave the following analytical figures (micro-analyses Schoeller) C 40.36, H 6.79 %; C 40.44, H 6.75 %; C₆H₁₂O₆ requires C 40.00, H 6.66 %.

¹ Dr T. H. B. Bedford removed all the hearts, and I am grateful for the rapidity and skill which he showed in all these experiments.

To determine whether survival changes in heart muscle affected the inositol content, one half heart was immediately treated with boiling potash, while the other was kept in an atmosphere of nitrogen for 90 minutes at 37°. Each was boiled with potash for 30 minutes. The following four experiments show that the inositol content is increased on incubation of the tissue.

Muscle killed immediately mg. inositol per 100 g.	Muscle after survival changes mg. inositol per 100 g.
22.3	29.5
10.0	40.0
30.2	61.6
10.0	23.7

In the following series the time of boiling was increased, to determine whether inositol was being set free from some combination by the action of the alkali.

	Muscle killed immediately mg. inositol per 100 g.	Muscle after survival changes mg. inositol per 100 g.
Boiled for 1 hour	7.8	8.3
	54.1	54.0
	9.2	79.3
	40.5	45.0
Boiled for 2 hours	43.9	68.3
	64.9	74.5
	120.0	128.0
	122.0	166.0
Boiled for 5 hours	79.0	114.0
	112.3	122.0
	147.0	185.0

Two facts emerge from this series: the amount of inositol increases with the time of action of the alkali, and the survival changes still cause more inositol to be formed than in the corresponding half heart killed immediately. To decide whether 5 hours' boiling with 10 % KOH is sufficient to liberate the whole of the "combined" inositol, hearts were divided into two, and one half was boiled for 5 hours, the other for 12; each half was thrown into potash immediately after weighing.

5 hours' boiling mg. inositol per 100 g.	12 hours' boiling mg. inositol per 100 g.
108.3	94.0
40.2	34.4
61.7	61.0
45.7	59.5

It is evident that the maximum liberation of inositol by the potash is effected in 5 hours, within the limits of experimental error of the method, necessarily large. Four experiments were performed to estimate this error, the heart being divided into two equal pieces and the inositol estimated after boiling for 5 hours.

mg. inositol per 100 g.		Percentage error	Average error
108.8	101.2	7.5	± 13 %
119.0	106.2	12.1	
68.7	53.0	29.6	
78.9	75.3	4.8	

That the maximum yield of inositol from heart muscle is found after survival changes have taken place is confirmed by the following series of experiments, in which the hearts were divided into three approximately equal parts, A—killed

at once and boiled 30 minutes, B—killed at once and boiled 5 hours, C—incubated 90 minutes in nitrogen at 37° and boiled 5 hours.

Muscle killed before survival changes		After survival changes
A. Boiled $\frac{1}{2}$ hour	B. Boiled 5 hours	C. Boiled 5 hours
mg. inositol per 100 g. tissue.		
59.7	83.0	102.0
12.8	18.6	36.8
49.5	71.0	102.0
83.0	112.0	141.0

In individual hearts, the value of A is that of the "free" with a small amount of the "combined" inositol, for it must be assumed that boiling for even half an hour with potash liberates some inositol from combination: the difference between A and B gives the value for the "combined" inositol (too low, for the reason already mentioned), and the difference between B and C is the amount of inositol produced during survival changes in the muscle; this will be referred to as the "new-formed" inositol. All the above experiments were carried out with a minimum of cell destruction. To obtain further light on the mechanism of production of the "new-formed" inositol, it was desirable to determine whether moderate cell disintegration, as in mincing, interferes appreciably with the production of the "new-formed" inositol. The hearts were minced and divided into two nearly equal parts. One, after weighing, was placed at once in twice the usual volume of boiling potash (10 % in 0.9 % sodium chloride) and the other was incubated in 0.9 % saline (1 ml. for each g. tissue) for 90 minutes at 37° in an atmosphere of nitrogen. At the end of this time 20 % potash in amount equal to the original volume of saline was added. Boiling was for 5 hours in each case.

Muscle killed immediately	After survival changes
mg. inositol per 100 g. tissue.	
60.0	69.5
78.6	96.6
87.6	95.0
78.4	97.0

It is evident that new formation of inositol has taken place. The possible effect of phosphate buffers of different p_H was then tried. The hearts were minced and divided as nearly as possible into three equal parts. Column A refers to the tissue which was placed at once in 10 % potash. In column B are the figures for the tissue incubated for 1½ hours in buffer p_H 5, in column C those for p_H 8.0. All were boiled for 5 hours with the same volume and concentration of alkali.

A	B	C
mg. inositol per 100 g. tissue.		
72.0	66.0	66.0
44.0	73.0	65.7
58.7	61.6	71.5
62.9	92.0	87.0

In three of the experiments the yield of inositol was increased on incubation of the muscle, but there was no significant difference between the acid and alkaline media.

In the following series of experiments a known amount of inositol was added to one half heart, and the whole extraction process was carried through to estimate how much of the substance is lost in the method of extraction and to obtain approximate figures for the free, combined, and "new-formed" inositol in the dog's heart. The average recovery was 73.2 %.

mg. inositol added	mg. inositol in each half heart		Difference	Percentage recovered
	28.4	80.0		
71.9	13.6	65.5	51.6	71.8
76.0	16.2	78.6	51.9	68.1
85.0	15.5	78.8	62.4	73.5
79.2			63.2	79.3

DISCUSSION.

Though the experimental error of the method is considerable, it has the advantage of certainty that inositol is being estimated, and it seems safe to conclude that in the heart muscle of the dog the inositol is of three kinds. Firstly, that lying free in the muscle: it is difficult to say what proportion this bears to the whole, for survival changes were occurring between the death of the animal and the killing of the muscle cells in the boiling potash, and there is also the hydrolytic action of the alkali to be considered during the 30 minutes' boiling. It may be that there is no free inositol in muscle, and if steps were taken to reduce survival changes to a minimum, a very small value might be obtained. Secondly, the combined inositol, that which is liberated completely after 5 hours' boiling with potash: the source of this is unknown. Thirdly, the "new-formed" inositol, that which appears during survival changes in the muscle on incubation in an atmosphere of nitrogen: the possibilities are that it is synthesised, or that it is liberated by enzymic action from some combination which is not split by boiling alkali. These problems must be left for further consideration.

SUMMARY.

1. A method is described for the estimation of inositol in muscle.
2. Survival changes in the heart muscle of the dog are accompanied by an increase in the inositol content.
3. Evidence is brought forward for the existence of free, combined and "new-formed" inositol in the heart muscle.

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