CXXXII. THE IODINE METHOD FOR THE DETERMINATION OF GLUTATHIONE.

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THE usual and more practical method for the estimation of glutathione in the tissue extracts is considered to be Tunnicliffe's iodine method [1925]. The end-point of the reaction is determined by the use of either starch or sodium nitroprusside as indicator. In both cases there are some difficulties in determining the true end-point. With the starch as internal indicator the end-point is very uncertain; the initial blue colour, once attained,* persists for an interval of time varying from 5 to 30 seconds with the different tissue extracts, and this time of persistence increases with the amount of iodine added after reaching the initial blue colour. With very pure samples of glutathione the end-point is much more easy to determine with precision. It is probable that this trouble is introduced by the presence in the extracts of foreign substances which react with the iodine with different speeds so that the final figures will always be larger and will not represent the true amount of the glutathione present in the tissue extracts.

The sodium nitroprusside end-point is more accurate and the difficulty in using it consists only in appreciating the point of disappearance of the red colour.

All authors have remarked on the discrepancy between the figures obtained, using starch or nitroprusside as indicator.

Voegtlin and Thompson [1926] believe that the starch is not specific enough as indicator. Kennaway and Hieger [1927], in their experiments, found large differences (from 40 to 50 %) between starch and nitroprusside end-points. Bierich and Kalle [1928] came to the same conclusions. Furthermore, Mason [1930] assumes that the method is not only subject to the error introduced by these modes of determining the end-point, but even the iodine estimation itself is not trustworthy for some tissue extracts, for example: liver, kidney and yeast. He compared the iodine method with a colorimetric one, based on the oxidation of the .SH group in glutathione with potassium ferricyanide. Mason gives a table (IV) showing results which are very suggestive, but it is difficult to understand the wide variation in the individual figures in his estimation of glutathione from yeast, using nitroprusside as external indicator. In the present experiments, using the same sample of yeast extract, the numerous estimations performed with nitroprusside as indicator, have always

given very consistent results, provided that the nitroprusside test is performed under very accurate and standardised conditions, as suggested for instance by Kennaway and Hieger [1927]

Considering the difficulties in obtaining concordance in the results of glutathione estimation from tissue extracts with the starch-iodine method, Perlzweig and Delrue [1927] proposed a modification of Tunnicliffe's method consisting in addition of an excess of 25 % potassium iodide (2 cc. for each sample ready for estimation). According to their view the potassium iodide stabilises the end-point; this is also the experience of the present author. However, the potassium iodide introduces another error if we do not take into account the acidity of the sample.

The present experiments are therefore a comparative study of the estimation of glutathione in pure solutions of this substance and in tissue extracts using one of the following indicators.

- (1) Starch in ordinary acid extracts.
- (2) Starch, acid solution and 2 cc. of 25 % potassium iodide.
- (3) Starch *plus* potassium iodide in sample neutral to litmus.
- (4) Nitroprusside.

The results are shown in the Tables.

Table I. *Glutathione estimations in various organs.*

For each estimation 10 cc. of extract.

	cc. N/100 iodine			
	2 cc. of 25 % KI			Nitro-prusside end-point
	No KI; acid	Acid	Neutral to litmus*	
<i>Sheep brain</i> †; approx. 50 g. tissue for 150 cc. extract	1.50	0.56	0.86	0.80
	1.56	0.54	0.84	0.80
	1.54	0.54	0.84	0.80
	1.54	0.54	—	0.80
<i>Beef</i> ; approx. 50 g. tissue for 150 cc. extract	0.7	0.36	0.54	0.50
	0.7	0.32	0.56	0.50
	0.7	0.36	0.50	0.50
	0.7	0.36	0.54	—
<i>Yeast</i> ; approx. 50 g. for 250 cc. extract	1.44	1.16	1.40	1.44
	1.44	1.18	1.42	1.46
	1.44	1.14	1.44	1.44
	1.44	1.14	1.42	1.46
	1.44	1.16	—	—
<i>Liver</i> ; approx. 50 g. tissue for 250 cc. extract	2.46	0.86	1.1	1.2
	2.42	0.82	1.1	1.2
	2.46	0.82	1.2	1.2
	2.40	0.82	1.1	1.2
<i>Glutathione</i> 0.225 %. Very pure sample. For each estimation 1 cc. in 10 cc. of 10 % trichloroacetic acid	0.92	0.76	0.88	0.9
	0.92	0.76	0.9	0.9
	0.90	0.78	0.86	0.9
	0.90	0.78	0.88	—

* The neutralisation was effected by addition of 2N NaOH.

† These figures do not represent the real amount of the glutathione present in tissues because the latter have been washed only two or three times.

To avoid any misunderstanding it is necessary to consider some of the major points of the method.

The tissue extracts have been made with pure (Merck) trichloroacetic acid (10 %). The filtrate was clear. The potassium iodide solution was prepared by dissolving a pure sample in freshly boiled and cooled water. Fresh solutions have always been used and were placed in the dark immediately after preparation. The nitroprusside test was performed according to the suggestions given by Kennaway and Hieger [1927].

Table II. *The effect of varying concentrations of KI in acid and neutral solutions.*

	cc. of 25 % KI	cc. N/100 iodine	
		Acid	Neutral
<i>Liver</i>	0.0	1.84 and 1.84	1.1 and 1.1
	0.1	1.3 " 1.34	1.06 " 1.1
	0.2	1.08 " 1.08	—
	0.5	0.92 " 0.94	—
	1.0	0.82 " 0.82	1.1 " 1.08
	2.0	0.68 " 0.68	—
	3.0	—	1.06 " 1.08
<i>Glutathione</i> 0.225 %	0.0	0.90	0.88
	0.1	0.82	0.86
	0.2	0.82	0.88
	0.5	0.82	0.88
	1.0	0.80	0.86
	2.0	0.76	0.88
	3.0	0.70	0.84 and 0.86

Table III. *The effect of p_H on the glutathione estimation in the presence of excess of potassium iodide.*

Glutathione 0.25 % (for each estimation 1 cc. glutathione solution plus 10 cc. phosphate mixture plus 2 cc. 25 % KI).

p_H	cc. iodine	5.9	6.46	6.8	6.9	7.16	Strongly acid (control)	
							KI	No KI
		0.80	0.84	0.88	0.94	1.00	0.8	1.0
	N/100	0.82	0.84	0.88	0.94	1.00	0.8	1.0
		0.80	0.84	—	0.92	—	0.8	—

We notice that the depressant action of the potassium iodide is present even with samples of very pure glutathione (Table I).

Table IV. *Sheep brain.*

70 g. for 250 cc. extract; comparative estimation of the glutathione in acid or neutral solution, with or without KI.

	Acid			Neutral	
	No KI	2 cc. of 25 % KI		No KI	2 cc. of 25 % KI
1.34		0.50	Nitroprusside end-point	1.22	0.76
1.38		0.52		1.16	0.8
1.42		0.50		1.2	0.78
1.38		—		1.2	—
—		—		1.2	—

DISCUSSION.

This error introduced by the presence of potassium iodide was noticed by Okuda [1925] in his experiments on the estimation of cysteine. He believed that the discrepancies might be explained by assuming different chemical reactions between cysteine and iodide under the different conditions of estimation, but he did not prove this point experimentally. However, he correlated the difference in the reaction with the temperature, acidity and presence of potassium iodide. To avoid the effect of these different factors which interfere with the results, he proposed for the cysteine estimation specially standardised conditions.

If we consider now the figures from the present paper, we notice the following very suggestive facts.

In the estimation of pure samples of glutathione there is no difference between starch-iodide (no KI) and nitroprusside end-points.

The same statement holds for the yeast samples. The same comparison for beef, brain and liver extracts gives respectively the average differences 29 %, 47 %, and 50 %. Do these differences between starch and nitroprusside end-points represent some foreign substances interfering with the estimation of the glutathione? Probably, but if so, why are there no differences between these figures when the estimation with starch as indicator is made in neutral solution?

Indeed we notice from our Tables that, if the nitroprusside end-point in the estimation of .SH groups is the most specific one, the iodine-starch-KI method may be very accurate, provided we take into account some special condition with regard to the acidity of the sample.

In acid solution the use of excess KI produces lower figures than the nitroprusside. Near, or just at the neutral point of the solution, the figures obtained with excess potassium iodide are concordant with the nitroprusside figures (Tables I and II).

If the estimation is made at the neutral point but without adding potassium iodide in excess, the figures obtained are higher than those indicated by nitroprusside. Table IV shows this comparative experiment. Therefore the KI seems to be necessary for a good estimation.

Table III is a good demonstration of the relations between the p_H and the estimation of pure glutathione when the KI is present in excess.

The depressant action of KI in acid solution depends also upon its concentration (see Table II), but by neutralising the sample this effect of concentration is eliminated. What is the explanation of this fact?

First we must remember that in the ordinary estimation of sodium thiosulphate with iodine, the addition of KI in acid solution in no way affects the end-point, using starch as indicator. A fundamental difference between the thiosulphate and the glutathione estimations seems to be that in the former there is no liberation of HI, whereas HI is liberated in the latter. The trouble may thus be introduced by the formation of the HI which in the presence of

KI reaches some equilibrium with the liberation of free iodine. That the amount of free iodine is correlated with the p_H of the sample is suggested by our experimental results (see Table II).

We can consider a second explanation. In acid solution, in the presence of certain compounds contained in the tissue extracts, the KI could liberate iodine, and the free iodine would then react with the .SH groups to some extent before the titration was commenced. This view is not supported by the following experimental fact. If we leave some glutathione or extract in the presence of 2 cc. of 25 % KI, estimating then the glutathione at different time intervals, we find no difference between the figures.

The most probable explanation is the physico-chemical explanation of equilibrium between KI-HI-I, this equilibrium being correlated with the p_H of the sample. Further investigation will perhaps indicate how the equilibrium is to be formulated.

CONCLUSIONS.

A comparative study has been made of glutathione estimation in tissue extracts, either with starch or nitroprusside as indicators.

In comparison with the nitroprusside end-point the starch in acid samples and without KI gives very high figures. In the presence of excess of KI on the contrary, lower results are obtained.

The figures obtained in the estimation of glutathione in pure solution and in tissue extracts, using excess of potassium iodide and making the sample neutral to litmus, are concordant with the figures obtained, using the nitroprusside end-point.

The author proposes the above modification of Tunncliffe's method which is very accurate when compared with the methods employing nitroprusside as an indicator.

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