VI. THE PREPARATION OF GLUTATHIONE FROM YEAST AND LIVER.

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VARIOUS workers have published modifications of the original Hopkins [1921] method for the preparation of glutathione without increasing markedly either the ease of the method or the purity of the final product. Hopkins [1929] obtained pure crystalline glutathione by a very much simplified technique in which the precipitation of glutathione as its crystalline cuprous salt was the most important feature. The still more simple method now to be described depends entirely for its success on the extreme specificity of this cuprous oxide precipitation in acid solution.

The yield of glutathione by the Hopkins [1929] method is very variable although always large; it appeared that the yield became smaller when large ($\frac{1}{2}$ cwt.) quantities of yeast were used than when only a few kilograms were worked up at a time. This loss seemed to be due to the difficulty of washing a large mercuric sulphide precipitate so as to remove all the adsorbed glutathione. An attempt was therefore made to dispense with the precipitation with mercuric sulphate.

The extract obtained on boiling yeast in water was found to give an unsatisfactory cuprous salt when cuprous oxide was added directly to the acidified filtrate. Treatment with quantities of alcohol varying from 2000 to 150 cc. per kg. of pressed yeast was also unsatisfactory, although a filtrate rich in glutathione, as estimated by the method of Perlzweig and Delrue [1927], was obtained. The filtrates obtained from mixtures of pressed yeast with common salt, ammonium sulphate or small quantities of 30 % sulphuric acid contained very little glutathione.

It was observed that the addition of a little ether to pressed yeast resulted in the formation of a smooth cream after a few hours' standing. This was very difficult to filter but filtration was much improved by the addition of sulphuric acid; acid has the further advantage of diminishing autoxidation in the filtrate. In the final method adopted a mixture of alcohol, ether and sulphuric acid is used, the concentration of sulphuric acid must be high both on account of the great buffering power of yeast and also to prevent frothing which occurs in mixtures of yeast, alcohol, ether and insufficient sulphuric acid. This mixture filters somewhat slowly but centrifuges easily and where the latter method of

treatment is possible it is to be recommended. By this method the preparation of glutathione is remarkably easy and, using small quantities of yeast, crystalline glutathione may easily be obtained in 3 days.

Since this work was completed Kendall, McKenzie and Mason [1929] have published a method of preparation in which benzene is used to liberate the glutathione from yeast. I had tried toluene and butyl alcohol, adding 5 cc. to 100 g. of pressed yeast, but abandoned them in favour of ether.

EXPERIMENTAL.

20 cc. of concentrated sulphuric acid are added slowly to 100 cc. of 89 % alcohol, the mixture being kept cool; when cold 80 cc. of ether are added. This mixture is poured on to 2000 g. of pressed baker's yeast, coarsely crumbled, in a large jar. The mixture is stirred for a few minutes till homogeneous and poured on to two large Büchner funnels (it is necessary to put kieselguhr on the papers to get clear filtration). Filtration is somewhat slow and after about 4 hours nearly stops, at this point it is best to pour the fluid upper layer on to another funnel and allow the cake to suck dry for a few hours more. Too high a vacuum should not be maintained in the filter flask at first since the filtrate froths as the ether boils off.

The combined filtrate amounts to about 1100 cc., a sample is titrated with sodium hydroxide using Congo red as indicator (the filtrate is generally just acid to Congo red) and the amount of 20 % sulphuric acid necessary to bring the filtrate to 0.5 *N* is added.

Precipitation with cuprous oxide is carried out as in Hopkins's method. The reaction proceeds satisfactorily at 20° but is somewhat more rapid at 40°. About 1 g. of cuprous oxide is required and this is most conveniently added in 2 cc. lots when suspended in about 30 cc. water. When about half the cuprous oxide has been added the salt should be allowed to settle and the mother-liquor poured off for further addition of oxide. At a later stage the addition of cuprous oxide will cause the re-solution of some of the cuprous-glutathione compound already formed; when this occurs the cuprous salt may be reprecipitated by blowing air through the solution for about an hour. The precipitate redissolves on very prolonged aeration. Although flocculent and amorphous this compound has the normal copper content (17.0 %) and gives crystalline glutathione on decomposition.

A further quantity of glutathione may be extracted from the yeast by 1000 cc. of 1 % (by vol.) sulphuric acid warmed to about 40°. Mixing is most easily effected by hand and the filtrate is treated precisely as before. A third extract prepared in the same way will also give some cuprous salt. The combined extracts from which the cuprous salt has been separated give a copious precipitate with the mercuric sulphate reagent used by Hopkins. This, after removal of the mercury with H₂S, gives a further yield of cuprous salt.

After a preliminary washing on the centrifuge with 0.5 *N* sulphuric acid the cuprous salt must be washed with water till the washings are free from

sulphate. This is very laborious if a centrifuge is used since the washing must be repeated about ten times. Washing may be carried out by filtration if the surface at which filtration is occurring is situated above instead of below the precipitate to be washed. A convenient device may be made with two Jena sintered glass filters (as some filters of grade 3 allow the precipitate to pass, it is safer to use grade 4) of a suitable size. The cuprous salt is put in one and the other placed upside down on top, a watertight joint being made with a piece of wide rubber tubing. The lower funnel is connected to a reservoir of distilled water about a metre above it and the upper one is attached to a flask and filter pump. Washing is continued till the filtrate is free from sulphate and remains so on shaking the filtration apparatus.

After decomposition with H_2S the filtrate from cuprous sulphide is evaporated to small bulk in a vacuum desiccator at room temperature. If the volume of filtrate is too large for this and it is essential to distil *in vacuo* to concentrate the solution, a good pump should be used and the distillation carried out at about 25° . Hopkins (unpublished work) has shown that a solution of wholly crystalline glutathione is converted in part to a gummy material by a few hours' exposure to a temperature of 40° . Crystallisation is generally spontaneous but should the solution reach a syrupy consistency without the appearance of foci rubbing with a glass rod will start crystallisation.

By this method 2 g. of crystalline glutathione are obtained from 2 kg. yeast. From 25 kg. yeast 31 g. glutathione, of which 29 were perfectly crystalline, were obtained. In this case only 7 g. came from the decomposed mercury precipitate. This probably represents glutathione which was autoxidised during the initial filtration and, when small quantities are used, or if the mixture is centrifuged, the extra yield does not repay the labour involved by mercury precipitation.

The same method has been applied satisfactorily to liver. In the case of rabbit liver, 20 cc. of the alcohol-ether-sulphuric acid mixture are added to 100 g. fresh tissue and the mixture is ground to a smooth paste with a little sand. The filtrate generally requires refiltration through kieselguhr. Pig or ox liver is put through a mincing machine before the addition of the alcohol mixture. To avoid the autolytic formation of free cysteine in liver these preliminary operations are carried out as rapidly as possible and in every case the p_{H} of the tissue has been lowered, by the sulphuric acid, to about 2, *i.e.* out of the range of peptidase action, within 40 minutes of the death of the animal. None of the liver preparations has given any colour with the Sullivan reagent under conditions such that 0.1 mg. cysteine hydrochloride could be detected clearly in the presence of 20 mg. glutathione. It would appear therefore that, as Bierich and Kalle [1928] have suggested, only traces of free cysteine are present in fresh liver, since it has been shown in this laboratory that cysteine forms a cuprous salt similar to that of glutathione¹.

¹ Glycylcysteine and glycylcysteine diketopiperazine also form cuprous derivatives. I hope to describe these compounds in a paper at present in preparation.

On addition of the alcohol mixture, minced liver becomes dry and granular. It is therefore squeezed in a press and the extract, amounting to 1080 cc. from 2300 g. of ox liver and 300 cc. of the alcohol mixture, filtered through kieselguhr. The filtrate is now treated exactly as in the case of yeast. Glutathione prepared from liver by this method is not wholly crystalline and the analytical figures have been somewhat low, *e.g.* N = 12.5 %, S = 9.93 %, while the ash is sometimes as high as 2 % (the analyses were made by Mr A. R. Colwell in this laboratory). The product seems however, as mentioned above, to be free from cysteine.

Attempts to isolate glutathione by this method from muscle and blood have not so far given satisfactory results.

SUMMARY.

A simple method for the isolation of glutathione from yeast is given. The yield is greater than by any of the methods described hitherto.

The preparation of glutathione from liver is described.

I am grateful to Sir F. G. Hopkins for his interest in this work.

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