METABOLISM OF GLUTATHIONE

II. DETERMINATION OF GLUTATHIONE AND PRODUCTS OF ITS HYDROLYSIS IN BLOOD*

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A method for the determination of glutathione and the sulfur-containing products of the hydrolysis of glutathione has been developed in this laboratory. This method has been applied to tissues, enzymatic digests, and blood. This report is concerned with the description of the method for blood.

The method depends upon (1) the lability of the γ-glutamyl linkage to mild hydrolysis with acid and (2) the selective response of the method of Sullivan and Hess (1) in the determination of cysteine and cysteinylglycine (2). A combination of these factors may be utilized so that cysteine, cysteinylglycine, γ-glutamylcysteine, and glutathione may be determined by a Sullivan determination on unheated and on heated portions of a filtrate of blood.

Presumptive evidence for the presence of significant amounts of cysteinylglycine and γ-glutamylcysteine in blood has been obtained. Venous blood from the normal human has been found to have a very constant content of these materials; in certain cases of disease marked variations have been observed.

EXPERIMENTAL

Hydrolysis of Glutathione and γ-Glutamylcysteine—Concentration of acid, type of acid, and time were studied as factors in the hydrolysis of glutathione and γ-glutamylcysteine. The acid of choice was found to be phosphoric acid; the concentration of acid selected was between 0.2 and 0.5 M. The optimum time of heating was found to be 90 minutes at this altitude (a water bath boils at 94°C). It is suggested that 60 minutes would be adequate at or near sea-level. Glutathione was found to be converted quantitatively to cysteinylglycine, whereas γ-glutamylcysteine was converted

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quantitatively to cysteine. Excellent recoveries of mixtures of cysteine, cysteinylglycine, and glutathione were obtained.

**Application to Blood**—The method for blood is as follows: Whole blood was collected with heparin as the anticoagulant (oxalate was found to be unsatisfactory) and a hematocrit was determined. 5 ml. of the whole blood were transferred to an Erlenmeyer flask and 10 ml. of water, saturated with digitonin, were added. After hemolysis was complete, 2 ml. of 50 per cent trichloroacetic acid were added dropwise with mixing; the flask was stoppered, shaken vigorously, and the mixture was centrifuged until a clear supernatant solution was obtained. 5 ml. portions of the supernatant solution were transferred to each of two colorimeter tubes (calibrated at 7 ml.), and 1 ml. of 2 M phosphoric acid was added to each tube. One tube was heated in a boiling water bath for 90 minutes and, after cooling, 1 ml. of 6 N sodium hydroxide was added to each tube. The volume was adjusted to 7 ml. and the method of Sullivan and Hess (1) was applied. The tubes were read (5 minutes after the addition of hydrosulfite) at 500, 540, and 580 mμ in the Coleman junior clinical spectrophotometer.

**Calculation of Results**—As has been reported (2), the reading at 540 mμ is a measure of total cysteine and cysteinylglycine. The ratio between the readings at 500 and 580 mμ is a measure of the relative concentrations of cysteine and cysteinylglycine. The 500:580 ratio for cysteine was found to be 3.4; the ratio for cysteinylglycine was 1.3. The composition of each was calculated from a graph utilizing these two values. Since all values were expressed as mg. per cent of glutathione, the standard curve was determined with solutions of glutathione. The values that were obtained were total glutathione (the 540 value of the heated tube), per cent cysteine in the heated tube (from the 500:580 ratio), cysteinylglycine plus cysteine (as glutathione) in the unheated tube, and the per cent cysteine in the unheated tube. From these values the concentration of cysteinylglycine, cysteine, glutathione, and γ-glutamylcysteine was calculated. Since no free cysteine was detected in the amount of blood used in these determinations (i.e. the ratio on the unheated tube was 1.3), only the values for cysteinylglycine, γ-glutamylcysteine, and glutathione are reported. In practice, all values are corrected to a hematocrit of 50 per cent.

The type of results obtained with the method is illustrated in Table I. Blood from five persons was analyzed over a 5 hour period to determine how much change might result from standing at room temperature. As long as heparin was used as the anticoagulant, no detectable changes were observed; therefore, each value is an average of four determinations. For purposes of comparison, reduced glutathione was estimated by an adapta-
tion of the nitroprusside method. Several hundred samples have been analyzed by our method and the values reported here are representative of the normal non-fasting group. Marked variation from these values has been observed in disease and under various types of therapy; these changes will be described elsewhere. It is interesting to note, however, that in a group of unselected diabetic patients the concentration of glutathione is low but this lowered value of glutathione is compensated for by an increase in the concentration of $\gamma$-glutamylcysteine. Since the concentration of cysteinylglycine is considerably lowered in fasting patients, it is to be expected that values for the normal human will be found to be lower in the fasting state. Our data from a large group of patients would indicate that the level of glutathione is higher in the blood of the fasting than in the blood of the non-fasting human.

**Table I**

Glutathione in Blood

Values are not corrected for differences in volume in red blood cells (hematocrit) and are expressed as mg. per cent of reduced glutathione.

<table>
<thead>
<tr>
<th>Cysteinylglycine</th>
<th>$\gamma$-Glutamylcysteine</th>
<th>Glutathione</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg. per cent</td>
<td>mg. per cent</td>
<td>mg. per cent</td>
</tr>
<tr>
<td>Normals (5), non-fasting</td>
<td>5.8 ± 2.3</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>Diabetics (10), fasting</td>
<td>1.8 ± 0.4</td>
<td>9.8 ± 3.6</td>
</tr>
</tbody>
</table>

Since the reaction with nitroprusside is positive for all sulfhydryl compounds but not for disulfide, and since by our method there is no distinction between the reduced or oxidized forms, there is no reason to expect agreement between the two methods. As is apparent from Table I, good agreement is obtained with blood of the normal human; such agreement does not apply when pathological blood is examined.

**DISCUSSION**

The method for the determination of glutathione and certain products of its hydrolysis has permitted us to make detailed studies of the metabolism of glutathione in disease. The documentation of these changes has required considerable time and effort and, in any event, we cannot hope to study more than a fraction of the available material. It is hoped, therefore, that the presentation of our methods will permit others to make a more precise study of the rôle of glutathione in physiological and pathological processes.
Methods have been developed whereby glutathione and the products of its hydrolysis (cysteine, cysteinylglycine, and \( \gamma \)-glutamylcysteine) may be determined in blood. The method is illustrated with certain values for blood of the non-fasting normal human.

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