ENZYMATIC SYNTHESIS OF GLUTATHIONE*

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The formation of glutathione in liver slices, in liver homogenates, and in extracts of acetone-dried pigeon liver and the effect of adenosinetriphosphate on this reaction have previously been demonstrated in this laboratory (1–3). In the present report, the synthesis of glutathione which is catalyzed by soluble liver enzymes is described in greater detail. The reaction occurs anaerobically when the constituent amino acids of the tripeptide, phosphate, magnesium, and an adenine nucleotide are present. A net synthesis of the tripeptide can be demonstrated under these conditions. The evidence suggests that the energy required for the synthetic process can be furnished by the cleavage of the pyrophosphate moiety of adenine nucleotides, in agreement with the concepts developed by Lipmann (4). Naturally occurring peptides therefore appear to be synthesized by a mechanism similar to that for the “peptidic” model compounds which have been studied previously by various investigators (5–9).

As in earlier experiments, glutathione synthesis has been measured by following the incorporation of C14-glycine and in some cases of labeled glutamic acid into the tripeptide. In a few experiments, glutathione was assayed enzymatically by the glyoxalase method.

EXPERIMENTAL

Preparations

Fresh Homogenates—The source of the enzyme was pigeon liver throughout. A survey of the enzymatic activities in liver extracts from other species (chicken, rat, rabbit, guinea pig, beef, pork) indicated that these sources were much inferior to pigeon liver. The same was true for yeast. Extracts of acetone-dried rabbit kidney or rabbit intestine showed no detectable activity.

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The experiments described in this investigation have been carried out with several enzyme preparations. The first of these was obtained by centrifugation of fresh pigeon liver homogenates in the following manner: 10 gm. of liver from freshly killed pigeons were suspended in 50 ml. of an ice-cold solution containing 100 parts of 0.1 M phosphate buffer, pH 7.4, 40 parts of 0.16 M KCl, and 4 parts of 0.16 M MgSO₄. The suspension was homogenized in a Waring blendor for 1 minute and then centrifuged in the cold room (2°) for 10 minutes at 2000 × g. The supernatant fluid obtained by decantation was used in the incubation experiments. When the enzymatic activity of the sedimented material was tested, it was resuspended in 50 ml. of the dispersion medium.

To 8 ml. of supernatant fluid or of resuspended sediment from fresh liver homogenates there were added 2.5 ml. of 0.1 M phosphate buffer, pH 7.4, 1 ml. of 0.16 M MgSO₄, 1.2 ml. of 0.16 M glutamate, 0.4 ml. of 0.16 M cysteine, and 1.2 ml. of 0.16 M C¹⁴-glycine. The final volume was 16 ml. The flasks were flushed with nitrogen or oxygen and incubated for 1 hour at 37°.

Acetone Powders—Enzyme preparations of greater stability have been obtained from acetone powders which were prepared from pigeon liver in the conventional manner. The enzymatic activity of various batches of acetone powder varied considerably, but the powders retained unchanged activity over a period of several months. For extraction, 5.0 gm. of powder were triturated with 55 ml. of an ice-cold solution containing NaCl 0.16 M, sodium bicarbonate 0.02 M, and cysteine 0.001 M. The insoluble material was removed by centrifugation at 2000 × g for 10 minutes. The extracts were used either directly (Enzyme A1) or after dialysis for varying lengths of time. Enzyme A2 was obtained from Enzyme A1 by dialysis with stirring for 4 to 7 hours against a mixture containing NaCl, NaHCO₃, and cysteine in the same concentrations as the solution used for extraction, while Enzyme A3 was a preparation which had been dialyzed for either 5 or 16 hours against distilled water, containing only 0.001 M cysteine at pH 7. Before use the dialyzed extracts were centrifuged at low speed (2000 × g) to remove any material which had precipitated during dialysis. Enzyme A4 was a preparation obtained by subjecting Enzyme A1 to high speed centrifugation (18,000 × g) for ½ hour and subsequent dialysis against distilled water containing cysteine at pH 7. This yielded a clear, reddish solution which contained the enzymatic activity, while the sediment from the high speed centrifugation was inactive. Enzyme A5 was obtained by an additional centrifugation of dialyzed Enzyme A4 at 18,000 × g. The sedimented material obtained from 1 ml. of enzyme contained 1.5 mg. of glycogen, as determined from the glucose content of the fraction which was soluble in 10 per cent trichloroacetic acid and insoluble in 50 per cent ethanol. The protein N content (in mg. of N per ml.) of the enzyme
preparations was as follows: Enzyme A2, 6.6; Enzyme A3, 5.2; Enzyme A4, 4.0; Enzyme A5, 3.3.

The standard medium for incubation contained C\textsuperscript{14}-glycine or N\textsuperscript{15}-glycine, cysteine, glutamic acid, MgSO\textsubscript{4}, KCl, and phosphate buffer, pH 7.4, in the concentrations which are given in Tables II to XI. In some experiments (Table IV) labeled glutamic acid was used. The volumes of liver extract differed in various experiments. The flasks were incubated for 1 hour in an atmosphere of nitrogen.

\textit{Materials}

\textbf{C\textsuperscript{14}-Glycine—}Glycine was prepared as described before (3). It had a specific activity of 100,000 c.p.m., counted as BaCO\textsubscript{3} under standard conditions. N\textsuperscript{15}-Glycine was prepared according to Schoenheimer and Ratner (10). It contained 33.8 atom per cent excess N\textsuperscript{15}.

\textbf{C\textsuperscript{14}-Glutamic Acid—}Glutamic acid was isolated from the proteins of algae (\textit{Scenedesmus obliquus}) which had been grown in an atmosphere of C\textsuperscript{14}O\textsubscript{2}. This was obtained from the Argonne National Laboratory through the Atomic Energy Commission. 2.2 gm. of algae containing a total of 1 mc. of C\textsuperscript{14} were extracted in succession by alcohol-ether 1:1 and cold and then hot 10 per cent trichloroacetic acid. The cell residue was hydrolyzed by 6 N HCl and glutamic acid was isolated as the hydrochloride from the hydrolysate after adsorption on Amberlite IR-4B. 49 mg. of glutamic acid hydrochloride were obtained after one recrystallization. This material, after dilution with 10 parts of non-isotopic L-glutamic acid, had an activity of 33,000 c.p.m., counted as BaCO\textsubscript{3} under standard conditions.

Non-isotopic glutamic acid and cysteine hydrochloride were commercial preparations. Glutathione\textsuperscript{1} was obtained from the Schwarz Laboratories. Adenosinetriphosphate dibarium salt was obtained from the Armour Laboratories or from the Sigma Chemical Company, adenosine diphosphate from the Nutritional Biochemicals Corporation, muscle adenylic acid from the Ernst Bischoff Company, yeast adenylic acid and guanylic acid from the Schwarz Laboratories.

\textit{Isolation of Glutathione Cuprous Mercaptide—}After incubation, the reaction mixture was deproteinized with 10 per cent trichloroacetic acid and 25 to 30 mg. of non-isotopic glutathione were added as carrier. Glutathione was first precipitated as the cadmium salt and then as the cuprous mercaptide, as described by Waelsch and Rittenberg (11). C\textsuperscript{14} determinations were carried out either by combustion of the samples and counting of BaCO\textsubscript{3} or by direct assay of the mercaptide. Suspensions of the mercaptide were dialyzed and a portion of one of these suspensions was acidified with HO\textsubscript{3}PO\textsubscript{4} to a total concentration of 15 mg. of mercaptide. After incubation of this solution in a water bath at 37°C for 2 hours, the reaction was stopped by the addition of 10 per cent trichloroacetic acid and the mercaptide was precipitated as described for the cadmium salt. Normal glutathione in the reaction mixture and in the cadmium salt was determined by the method of Van Slyke and Neill (12).

\textsuperscript{1} The following abbreviations are used: GSH, glutathione; ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, muscle adenylic acid; IMP, inosinic acid.
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capptide in methanol containing 0.1 per cent sodium carboxymethoxy cellulose (Hercules Powder Company) when deposited on counting cups gave samples of uniform thickness. The radioactivity of a given sample of glutathione cuprous mercaptide when counted as such was found to be 5 times greater than the radioactivity of BaCO₃ obtained by combustion of the same sample (BaCO₃ 6.0 per cent C, glutathione cuprous mercaptide 33.3 per cent C). The glyoxalase method (12) was used for enzymatic assay of glutathione. Red Star yeast was found to be a satisfactory source of glyoxalase.

Calculation of Results

The Cl⁴ concentration of glutathione cuprous mercaptide isolated after addition of a standard quantity of carrier was taken as a measure of the rate of peptide synthesis. The isotope data are expressed as counts per minute of Cl⁴ of the glycine moiety of glutathione.

The quantity of GSH synthesized may be calculated from the equation,

\[ \text{mg. of GSH synthesized} = C/C_0 \times (G_1 + G_2), \]

where \( C_0 \) = the specific activity of Cl⁴-glycine added, \( C \) = the specific activity in the glycine moiety of GSH isolated, \( G_1 \) = mg. of carrier GSH added, \( G_2 \) = mg. of GSH present in the extract before incubation.

Since the quantity of glutathione in fresh pigeon liver is known (1.5 to 2 mg. per gm. of wet tissue), it can be estimated that the quantity \( G_2 \) must in all cases be less than 5 per cent of \( G_1 \), and in the calculation \( G_2 \) may therefore be neglected. The amount of glutathione which is present initially in the extract has been determined only in one instance. By assay with the glyoxalase method 1 ml. of dialyzed Enzyme A₄ was found to contain 84 γ of GSH, or about 15 per cent of the GSH originally present in the fresh tissue. By the above equation it is calculated that under optimal conditions 0.5 to 0.9 mg. of GSH is synthesized per hour in 2.5 ml. of enzyme solution containing 10 mg. of protein nitrogen (Tables I and VII).

Results

The enzymes which catalyze the incorporation of glycine into glutathione are extractable from either fresh or acetone-dried pigeon liver. Different batches of fresh homogenates and of acetone powders were found to vary greatly in enzymatic activity. However, the range of activities found for the supernatant liquid of centrifuged homogenates and for extracts of different acetone powders was about the same as for whole homogenates (Table I). It appears therefore that most of the activity in fresh liver which is responsible for glutathione synthesis can be obtained in soluble form.

In fresh liver homogenates glycine is rapidly incorporated into glutathione.
in the presence of amino acids and salts when oxygen is the gas phase (3). When fresh liver homogenates are centrifuged (2000 × g), C\textsuperscript{14}-glycine is readily introduced into peptide linkage in the supernatant fluid (Table II). Only in one of thirteen experiments was some enzymatic activity present

<table>
<thead>
<tr>
<th>Preparation</th>
<th>No. of experiments</th>
<th>GSH synthesized* per mg. protein N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fresh homogenates</td>
<td>13</td>
<td>5–30</td>
</tr>
<tr>
<td>2. Supernatant of (1) after centrifugation at 2000 × g</td>
<td>12</td>
<td>3–20</td>
</tr>
<tr>
<td>3. Acetone powder extracts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undialyzed (Enzyme A1)</td>
<td>20</td>
<td>5–49</td>
</tr>
<tr>
<td>Dialyzed (Enzymes A2, A3)</td>
<td>10</td>
<td>6–30</td>
</tr>
<tr>
<td>&quot; and centrifuged at 18,000 × g (Enzymes A4, A5)</td>
<td>7</td>
<td>40–100</td>
</tr>
</tbody>
</table>

\* See the experimental section for calculations.

**Table II**

*Distribution of Enzymatic Activity in Fractions of Fresh Pigeon Liver Homogenates*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Gas phase</th>
<th>Addition</th>
<th>GSH, c.p.m. C\textsuperscript{14}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O\textsubscript{2}</td>
<td>&quot;</td>
<td>Whole homogenate: 540, 470, 14 ± 7</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Supernatant fluid: 840, 620, 12 ± 7</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>ATP, 2 × 10\textsuperscript{-4} M, 8 × 10\textsuperscript{-4} &quot;</td>
<td>3400, 240, 20 ± 8</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>Nitrogen</td>
<td>135†, 1270, 770</td>
</tr>
<tr>
<td>&quot;</td>
<td>ATP, 2 × 10\textsuperscript{-4} M, 8 × 10\textsuperscript{-4} &quot;</td>
<td>1500, 1140, 1670</td>
<td></td>
</tr>
</tbody>
</table>

\* For composition of the incubation medium, see the experimental section.
† After standing at 0°C for ½ hour.

in the "particulate" material, and in general more than 50 per cent of the activity of the whole homogenates could be recovered in the soluble fraction. The enzyme preparations obtained by centrifugation of fresh liver homogenates rapidly deteriorated on standing at 0°C and were therefore not investigated extensively. A summary of the results obtained with this preparation is given in Table II. The conditions attending the formation
of labeled glutathione in these extracts differed in several aspects from those observed with whole homogenates. Thus, in crude liver dispersions the rate of synthesis was about 5 times faster under aerobic than anaerobic conditions and ATP stimulated the process markedly (3). On the other hand, in the supernatant fluid of centrifuged homogenates the formation of labeled glutathione was at least as fast in nitrogen as in oxygen and ATP failed to increase significantly the rate of the reaction (Experiment 4, Table II).

Attempts to activate the sediment remaining after centrifugation of the homogenates by addition of boiled pigeon liver extract, or of GSH, succinate, cytochrome c, or ATP, were unsuccessful. It appears therefore that in pigeon liver only soluble enzymes are concerned with the incorporation of glycine into glutathione.

**Acetone Powder Extracts**—Saline-bicarbonate extracts of acetone-dried pigeon liver catalyze peptide bond formation as actively as do whole liver homogenates and as the soluble portion of the fresh tissue (Table I). However, these extracts, in contrast to fresh tissue preparations, require for activity the presence of an adenine nucleotide. The enzymatic activity does not decline appreciably when the extracts are kept at 0° or are dialyzed for periods up to 16 hours. Enzyme solutions which have been centrifuged at 18,000 × g (Enzymes A4, A5) will retain full activity for several weeks when kept frozen at −20°. Since in such preparations several limiting conditions for glutathione synthesis can be established, acetone powder extracts were used to examine the properties of the enzyme system in some detail.

It is evident from the data in Table III that the introduction of glycine into the peptide linkage requires the presence of magnesium, phosphate, and ATP. In the absence of any of these components the C\(^{14}\) content of glutathione is reduced to less than 10 per cent of the values observed in the complete system. Failure to add glutamic acid decreases but does not entirely suppress enzymatic activity. It appears that either glutamic acid is not completely removed by dialysis or that it is liberated during incubation. The effect of omitting cysteine or glycine has not been determined, since the progress of the reaction was followed with the aid of labeled glycine, while cysteine was always present both in the extraction medium and during dialysis. The effects produced by varying concentrations of salts, amino acids, and adenine nucleotides have been studied over a limited range (Figs. 1 to 3). The absolute radioactivities observed in tests with any one of these components should not be compared with those for another component, since they were obtained from separate experiments which did not always show the same level of enzymatic activity. For example, the concentration curves for ATP and AMP (Fig. 3) should
Table III

Requirements for Glutathione Synthesis in Extracts of Acetone-Dried Pigeon Liver

<table>
<thead>
<tr>
<th></th>
<th>GSH, c.p.m. C₁₄</th>
<th>Optimal molarity of addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete*</td>
<td>900</td>
<td>1-1.5 × 10⁻⁴</td>
</tr>
<tr>
<td>Omit ATP</td>
<td>13</td>
<td>5 × 10⁻²</td>
</tr>
<tr>
<td>&quot; phosphate</td>
<td>65</td>
<td>1 × 10⁻²</td>
</tr>
<tr>
<td>&quot; MgSO₄</td>
<td>32</td>
<td>8 × 10⁻³</td>
</tr>
<tr>
<td>&quot; KCl</td>
<td>490</td>
<td>5-10 × 10⁻³</td>
</tr>
<tr>
<td>&quot; glutamic acid</td>
<td>220</td>
<td>1.5 × 10⁻²</td>
</tr>
</tbody>
</table>

* In the complete system each flask contained phosphate buffer, pH 7.4, 0.03 M, KCl 0.01 M, MgSO₄ 0.005 M, C¹⁴-glycine 0.025 M, glutamate 0.01 M, cysteine 0.01 M, ATP 0.0015 M, and 2.5 ml of Enzyme A2, dialyzed for 4 hours. Total volume 3.8 ml. Incubated in nitrogen at 37°C for 1 hour. Somewhat less C¹⁴ is incorporated into GSH under aerobic conditions, presumably because of oxidation of sulfhydryl groups.

Fig. 1. Effect of Mg++ and PO₄ concentration on GSH synthesis. When the concentration of Mg++ was varied, the samples contained KCl 0.02 M, phosphate buffer, pH 7.4, 0.03 M, C¹⁴-glycine 0.02 M, cysteine 0.007 M, glutamate 0.012 M, ATP 0.0017 M, and 2.5 ml of Enzyme A2. Total volume 4.5 ml per flask. In the experiments with varying phosphate concentrations the composition of the incubation medium was the same except for the phosphate. When phosphate was omitted, the samples contained sodium bicarbonate 0.01 M.

merely be taken to indicate that in the preparation employed (Enzyme A1) the optimal molarities of the two nucleotides are the same and that both compounds are inhibitory at higher concentrations. However, these data should not be used as a measure of the relative effectiveness of ATP and
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Fig. 2. Effect of varying concentrations of amino acids on GSH synthesis. The samples contained phosphate buffer, pH 7.4, 0.02 M, KCl 0.013 M, MgSO₄ 0.006 M, ATP 0.001 M, and 5 ml. of undialyzed extract (Enzyme A1). The total volume was 13 ml. per flask. The molarities of those amino acids whose concentrations were kept constant were as follows: C⁴⁺-glycine 0.015 M, glutamate 0.015 M, cysteine 0.005 M.

Fig. 3. Effect of ATP and AMP in crude liver extracts. Curve A, ATP; the samples contained phosphate buffer, pH 7.4, 0.02 M, KCl 0.012 M, MgSO₄ 0.006 M, C⁴⁺-glycine 0.015 M, cysteine 0.005 M, and glutamate 0.03 M. 5 ml. of Enzyme A1 per flask; total volume 12.5 ml. Curve B, AMP; the samples contained phosphate buffer, pH 7.4, 0.03 M, KCl 0.02 M, MgSO₄ 0.01 M, C⁴⁺-glycine 0.03 M, cysteine, and glutamic acid 0.01 M. 2.5 ml. Enzyme A1 per flask. Total volume 3.9 ml.

Fig. 4. Effect of pH on the incorporation of C⁴⁺-glycine into GSH. The samples contained KCl 0.013 M, MgSO₄ 0.007 M, glutamate 0.024 M, C⁴⁺-glycine 0.012 M, cysteine 0.008 M, and 5 ml. of Enzyme A1. Total volume per flask 12.1 ml. Acetate buffer was employed at pH 5 and below, phosphate buffer between pH 5 and 8, and borate buffer at pH 8 and above.

AMP. It may also be pointed out that the concentration effects observed in crude extracts do not necessarily reflect the true requirements for the
formation of the cysteine-glycine bond in glutathione. More probably they are the resultant of a variety of subsidiary or competing reactions and they may change with progressing purification of the enzyme.

pH—The effect of pH on the incorporation of C\textsuperscript{14}-glycine into glutathione has been studied in undialyzed extracts (Enzyme A1) (Fig. 4). Activity is confined to a narrow range between pH 6.5 and 7.5.

Formation of Glutaminylcysteine Moiety of Glutathione—If the incorporation of labeled glycine represents a total synthesis of GSH, then the formation of the glutaminylcysteine bond in the tripeptide should be demonstrable under the same conditions. This was found to be the case in earlier experiments with N\textsuperscript{15}-DL-glutamic acid when fresh pigeon liver homogenates were the source of the enzyme (3). Extracts of acetone-dried liver likewise catalyze the formation of both peptide bonds of GSH. Table IV gives the results from experiments in which two isotopic substrates were used, glycine containing N\textsuperscript{15} and L-glutamate labeled by C\textsuperscript{14}. From the relative concentrations of C\textsuperscript{14} and N\textsuperscript{15} in the tripeptide the relative number of glycine and glutamate molecules which had entered into peptide linkage can be calculated. Under the conditions investigated the molar ratio of the two labeled amino acids in the peptide was close to unity. This result is most readily explained by assuming that the rate of formation of the glutaminylcysteine bond is the same as that of the cysteinylglycine bond. Whether this is the case also when the relative concentrations of the amino

<table>
<thead>
<tr>
<th>Addition</th>
<th>Glutamate</th>
<th>Glycine</th>
<th>C.p.m.</th>
<th>RIC\textsuperscript{†}</th>
<th>Atom per cent excess</th>
<th>RIC\textsuperscript{‡}</th>
<th>Molar ratio glycine glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.013</td>
<td>0.026</td>
<td>4</td>
<td>0.01</td>
<td>0.010</td>
<td>0.284</td>
<td>0.92</td>
</tr>
<tr>
<td>AMP, 1.5 × 10\textsuperscript{-3}</td>
<td>0.026</td>
<td>0.026</td>
<td>102</td>
<td>0.31</td>
<td>0.096</td>
<td>0.222</td>
<td>1.01</td>
</tr>
<tr>
<td>ATP, 1.5 × 10\textsuperscript{-3}</td>
<td>0.013</td>
<td>0.026</td>
<td>72</td>
<td>0.22</td>
<td>0.075</td>
<td>0.222</td>
<td></td>
</tr>
</tbody>
</table>

Each flask contained phosphate buffer, pH 7.4, 0.05 M, KCl 0.02 M, MgSO\textsubscript{4} 0.01 M, cysteine 0.008 M, C\textsuperscript{14}-glycine, and N\textsuperscript{15}-glutamate as indicated in the table and 2.5 ml. of Enzyme A3.

\* Calculated for the glutamic acid carbon atoms of GSH.
\† Calculated for the glycine nitrogen atoms of GSH.
\‡ Relative isotope concentration =

\[
\frac{\text{Isotope concentration of amino acid in GSH}}{\text{Isotope concentration of amino acid in medium}}
\]
acids in solution are greatly different remains to be determined. The data in Table IV also demonstrate that the uptake of labeled glutamate is negligible in the absence of added AMP or ATP. It thus appears that adenine nucleotides participate in the synthesis of both peptide linkages of glutathione.

### Table V

**Effect of Nucleotides on Synthesis of Glutathione**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Addition</th>
<th>GSH, c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Enzyme A2, dialyzed</td>
<td>None</td>
<td>22 ± 4</td>
</tr>
<tr>
<td></td>
<td>ATP, 1.7 x 10^{-3} M</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>Adenosine, 1.7 x 10^{-3} M</td>
<td>24 ± 4</td>
</tr>
<tr>
<td></td>
<td>Adenine, 1.7 x 10^{-3} M</td>
<td>6 ± 4</td>
</tr>
<tr>
<td>2. Enzyme A1, undialyzed</td>
<td>ATP, 2.0 x 10^{-3} M</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>ADP, 2.0 x 10^{-3} M</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td>5-AMP, 2.0 x 10^{-3} M</td>
<td>370</td>
</tr>
<tr>
<td>3. Enzyme A2, dialyzed</td>
<td>ATP, 1.7 x 10^{-3} M</td>
<td>1380</td>
</tr>
<tr>
<td></td>
<td>AMP, 1.7 x 10^{-3} M</td>
<td>2740</td>
</tr>
<tr>
<td></td>
<td>3-AMP, 1.7 x 10^{-3} M</td>
<td>490</td>
</tr>
<tr>
<td>4. Enzyme A1, undialyzed</td>
<td>ATP, 2.0 x 10^{-3} M</td>
<td>2780</td>
</tr>
<tr>
<td></td>
<td>5-AMP, 2.0 x 10^{-3} M</td>
<td>2500</td>
</tr>
<tr>
<td></td>
<td>5-IMP, 2.0 x 10^{-3} M</td>
<td>900</td>
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<tr>
<td>5. Enzyme A2, dialyzed</td>
<td>None</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>5-AMP, 2.0 x 10^{-3} M</td>
<td>620</td>
</tr>
<tr>
<td></td>
<td>5-IMP, 2.0 x 10^{-3} M</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>Guanylic acid, 2.0 x 10^{-3} M</td>
<td>110</td>
</tr>
</tbody>
</table>

In Experiments 1 and 2, each flask contained phosphate 0.036 M, KCl 0.011 M, MgSO_4 0.006 M, C^{14}-glycine 0.027 M, cysteine 0.009 M, glutamate 0.013 M, and 5 ml. of enzyme. Total volume 7 ml.

In Experiment 3, each flask contained phosphate 0.028 M, KCl 0.02 M, MgSO_4 0.003 M, C^{14}-glycine 0.02 M, cysteine 0.007 M, glutamate 0.007 M, and 2.5 ml. of enzyme. Total volume 4.5 ml.

In Experiments 4 and 5, each flask contained phosphate 0.032 M, KCl 0.02 M, MgSO_4 0.01 M, C^{14}-glycine 0.08 M, cysteine 0.01 M, glutamate 0.008 M, and 2.5 ml. of enzyme. Total volume 3.8 ml. In Experiment 5, each flask also contained GSH 0.0015 M.

**Effect of Nucleotides**—The experiments listed in Tables III to V and Fig. 3 illustrate the need for a purine nucleotide in glutathione synthesis. The participation of ATP in the formation of other peptidic compounds has been well established (4, 5, 7–9). It seemed reasonable to expect, therefore, that glutathione synthesis also was associated with a utilization of phosphate bond energy. It was felt to be of interest, nevertheless, to test nucleotides other than ATP. It will be seen that in crude extracts, either before or after dialysis, muscle adenylic acid and adenosinediphosphate
are as effective as ATP in supporting glutathione formation (Experiments 2 to 4, Table V). On the other hand, no activity in excess of the blank values is observed with adenosine and adenine (Experiment 1, Table V). The effect given by yeast adenylic acid (3-AMP) is small but significant (Experiment 3, Table V). Inosinic acid could partially replace the adenine nucleotides in undialyzed extracts, but the effect was much smaller in extracts which had been dialyzed (Experiments 4 and 5, Table V). It may be concluded from these results that in crude extracts of acetone-dried pigeon liver a number of nucleotides, notably inosinic acid, ADP, and adenylic acid, can be converted to ATP or compounds which function similarly to ATP in peptide synthesis.

### Table VI

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Enzyme</th>
<th>Addition</th>
<th>GSH, c.p.m. C(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A1, undialyzed</td>
<td>ATP, (2.0 \times 10^{-3}) M</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AMP, (2.0 \times 10^{-3}) M</td>
<td>370</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>ATP, (2.0 \times 10^{-3}) M</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AMP, (2.0 \times 10^{-3}) M</td>
<td>830</td>
</tr>
<tr>
<td>3</td>
<td>A2, dialyzed 7 hrs.</td>
<td>ATP, (1.7 \times 10^{-3}) M</td>
<td>1370</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AMP, (1.7 \times 10^{-3}) M</td>
<td>2740</td>
</tr>
<tr>
<td>4</td>
<td>A3, 10</td>
<td>ATP, (1.7 \times 10^{-3}) M</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AMP, (1.7 \times 10^{-3}) M</td>
<td>1000</td>
</tr>
<tr>
<td>5</td>
<td>A3, 5</td>
<td>ATP, (1.5 \times 10^{-3}) M</td>
<td>490</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AMP, (1.5 \times 10^{-3}) M</td>
<td>880</td>
</tr>
</tbody>
</table>

In all experiments, the incubation medium contained phosphate buffer, pH 7.4, 0.03 M, KCl 0.02 M, MgSO\(_4\) 0.01 M, C\(^14\)-glycine 0.025 M, cysteine and glutamate 0.008 M, and 2.5 ml. of enzyme per flask. Total volume 3.7 ml. Time of incubation 1 hour.

Since under the conditions of our experiments peptide formation was not specifically dependent on ATP, it became necessary to make a more detailed study of the nucleotide effect. Table VI contains a record of the activities of ATP and AMP under comparable conditions. In extracts prior to dialysis, AMP and ATP, when used in the same concentrations, support glutathione synthesis equally well and the two nucleotides show optimal activity at the same molarities (Experiments 1 and 2, Table VI). On the other hand, in extracts which had been dialyzed for varying lengths of time, AMP and ATP support glutathione synthesis in combination with various amides. These findings, which indicate the enzymatic conversion of inosinic acid to an adenine nucleotide, will be the subject of a forthcoming publication.

\(^2\) In Enzyme A3, A4, and A5 inosinic acid shows no detectable activity, but will support glutathione synthesis in combination with various amides.
of time, either against bicarbonate buffer (Enzyme A2) or against distilled water (Enzyme A3), the response given by ATP is consistently inferior to that shown by AMP (Experiments 3 to 5, Table VI). In a total of seven experiments which were carried out with dialyzed extracts, ATP was on the average only half as effective as AMP under the same conditions. These findings seemed difficult to reconcile with the view that ATP was the active agent in the process under study. The poorer response shown by ATP in dialyzed extracts might be attributed to the presence of an inhibitor. As shown by the data in Fig. 3, ATP is indeed strongly inhibitory at higher concentrations, but the same is also true for high concentrations of adenylic acid. Moreover, inhibition by a contaminant in the ATP preparation should be evident in undialyzed as well as in dialyzed extracts. This was not the case, however. The relatively low activity of ATP found under the conditions of Experiments 3 to 5, Table VI, remains therefore unexplained.

**Table VII**

**Effect of High Speed Centrifugation of Liver Extracts (18,000 x g) on GSH Synthesis**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Enzyme</th>
<th>Glutathione C.p.m.</th>
<th>C\textsuperscript{14} / 10 mg protein N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dialyzed extract, A3</td>
<td>740</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>&quot; after centrifugation (A4)</td>
<td>3200</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>Sediment from A4</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Dialyzed extract, A3</td>
<td>550</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>&quot; after centrifugation (A4)</td>
<td>3600</td>
<td>900</td>
</tr>
</tbody>
</table>

The samples contained phosphate buffer, pH 7.4, 0.046 M, KCl 0.02 M, MgSO\textsubscript{4} 0.01 M, cysteine and glutamate 0.008 M, C\textsuperscript{14}-glycine 0.0025 M, ATP 0.0015 M, and 2.5 ml. of enzyme.

**Centrifugation of Liver Extracts**—Evidence to indicate that the active factor in glutathione synthesis is indeed ATP has been obtained by an additional step in the preparation of the enzyme. Centrifugation of the liver extracts at 18,000 x g yields preparations exhibiting markedly increased activity. The material which is sedimentable at high speed fails to catalyze glutathione formation, while the supernatant liquid is several times as active as the same extract prior to centrifugation (Table VII). The sedimented material contained only about 30 per cent of the total protein of the original extract, and therefore the several fold increase in potency of the soluble fraction is probably due to the removal of enzymes which interfere with GSH synthesis.
In the more active enzyme preparation which had been subjected to high speed centrifugation prior to dialysis (Enzyme A4), ATP and AMP are indistinguishable in their effect on GSH synthesis (Curves I and II, Fig. 5) and the two nucleotides exert their effect without any time lag. If it is assumed that the action of adenylic acid is indirect, and is preceded by its conversion to a pyrophosphate ester, then the anaerobic reactions which generate energy-rich phosphate must occur more rapidly than peptide synthesis. Since the dialyzed pigeon liver extracts were found to contain substantial amounts of glycogen, glycolysis and hence generation of high energy phosphate are the most likely cause for the observed activity of adenylic acid. This glycogen can be removed by a second centrifugation at 18,000 g if this step is carried out after dialysis of the enzyme against distilled water. In the supernatant liquid obtained by this step (Enzyme A5) the rate of glutathione synthesis in the presence of ATP is unaffected, but the activity of adenylic acid is greatly diminished and becomes evident only after a considerable time lag (Curve IV, Fig. 5). It is clear therefore that adenylic acid by itself is incapable of supporting peptide bond formation. The delayed occurrence of glutathione synthesis with Enzyme A5 in the presence of adenylic acid suggests that after this second high speed centrifugation formation of pyrophosphate derivatives continues to a limited extent.

Glutathione synthesis in the presence of AMP was completely suppressed by 0.001 M fluoride (Table VIII), a result which would be obtained if the

![Fig. 5. Comparison of ATP and AMP after one (Enzyme A4) and two (Enzyme A5) centrifugations at 18,000 × g. All flasks contained salts and amino acids in the same concentrations as in the experiments of Table VII. The adenine nucleotides were present at 0.0015 M concentration. Curve I, Enzyme A4 and AMP; Curve II, Enzyme A4 and ATP; Curve III, Enzyme A5 and ATP; Curve IV, Enzyme A5 and AMP.](http://www.jbc.org/)

by guest on October 19, 2017 http://www.jbc.org/ Downloaded from
activation of the monophosphate was due to glycolytic reactions. On the other hand, fluoride was equally inhibitory in experiments performed with ATP. The action of fluoride may therefore be concerned with the utilization as well as with the generation of high energy phosphate. A similar, though less marked inhibition of enzymatic activity by fluoride has been observed by Speck in his study of the enzymatic synthesis of glutamine from glutamate, ammonia, and ATP (13). For these reasons the nature of the reactions which bring about the conversion of adenylic acid into what would appear to be a pyrophosphate derivative remains to be determined.

Adenosinediphosphate—In all enzyme preparations investigated so far, ADP has been found to be as active as ATP in supporting GSH synthesis. It will be noted that this is the case also with Enzyme A5, a preparation in which the activation of adenylic acid occurs only after a considerable time lag. While it is conceivable that the utilization of ADP is preceded

<table>
<thead>
<tr>
<th>Addition</th>
<th>GSH, c.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>620</td>
</tr>
<tr>
<td>&quot; + NaF, 5 $\times 10^{-3}$ M</td>
<td>4</td>
</tr>
<tr>
<td>&quot; + &quot; 1 $\times 10^{-3}$ &quot;</td>
<td>0</td>
</tr>
<tr>
<td>ATP + &quot; 5 $\times 10^{-3}$ &quot;</td>
<td>13</td>
</tr>
<tr>
<td>&quot; + &quot; 1 $\times 10^{-3}$ &quot;</td>
<td>10</td>
</tr>
</tbody>
</table>

The concentrations of salts and amino acids were the same as those in the experiments of Table VII. The adenine nucleotides were present at 1.5 $\times 10^{-3}$ M concentration. 2.5 ml. of Enzyme A4 per flask.

by its dismutation to the mono- and triphosphate, no convincing evidence exists that pigeon liver extracts contain an enzyme similar in function to myokinase (14). On the other hand, the data presented in Fig. 6, particularly the absence of a lag period with ADP, are consistent with the assumption that ADP participates directly in glutathione synthesis and that the splitting of the terminal phosphate of ADP as well as the splitting of the third phosphate grouping of ATP can supply energy for peptide formation.

Net Synthesis of Glutathione The incorporation of labeled amino acids into glutathione demonstrates peptide bond formation but does not establish that a net synthesis of peptide takes place. It is also possible that the amino acid residues of glutathione are being replaced by a process which involves little expenditure of energy. Such enzyme-catalyzed exchange reactions have been observed with the aid of isotopic tracers; e.g., the exchange between inorganic phosphate and glucose-1-phosphate (15) and between ammonia and hippurylamide (16). The occurrence of a re-
action involving an exchange between non-isotopic glycyl residues of the peptide and C\textsuperscript{14}-glycine in the medium was a possibility, since our extracts contained, even after dialysis, considerable amounts of glutathione. An assay for glutathione by the glyoxalase method\textsuperscript{3} showed the presence of 200 $\gamma$ of GSH in 2.5 ml. of dialyzed extract (Enzyme A3 and A4). In the same volume of extract before high speed centrifugation (Enzyme A3) the quantity of C\textsuperscript{14} incorporated was, on the average, equivalent to the synthesis of 180 $\gamma$ of GSH; i.e., a quantity similar to that of the endogenous tripeptide. In view of the close correspondence of these two quantities, it seemed possible that the uptake of C\textsuperscript{14}-glycine under these conditions was due to an exchange reaction.

Apparent support for this interpretation may be seen in the finding that the addition of small quantities of GSH to the reaction medium before incubation raised the C\textsuperscript{14} concentration in the reaction product (Curve I, Fig. 7). When Enzyme A3 was used, the isotope concentration of the isolated GSH was, in a narrow range, proportional to the quantity of GSH which had been added initially, suggesting that the formation of the cyst-

\textsuperscript{3} We are indebted to Mr. S. Yanari for some of the GSH assays by the glyoxalase method.
teinylglycine linkage depended, at least in a limited range, on the quantity of tripeptide already present. However, in the more active preparations which are obtained by high speed centrifugation (Enzymes A4, A5) the incorporation of labeled glycine can no longer be accounted for by an exchange mechanism. Thus, in a volume of enzyme solution containing initially 200 $\gamma$ of GSH, the quantity of C$^{14}$ found in the GSH isolated after incubation corresponded to 900 $\gamma$ of tripeptide (Table VII). Hence, the GSH content of the system must have increased by at least 700 $\gamma$. Enzymatic assay of the reaction mixtures by the glyoxalase method$^3$ demonstrates that the incorporation of C$^{14}$-glycine is indeed a measure of the net synthesis of GSH. Glutathione determinations both by C$^{14}$ analysis and by the glyoxalase method in aliquots of the same experimental solution gave results which agree satisfactorily (Table IX).

### Table IX

**Net Synthesis of Glutathione**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Addition</th>
<th>GSH synthesized</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>By C$^{14}$ analysis</td>
<td>By glyoxalase assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\gamma$</td>
<td>$\gamma$</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>1100</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ATP, 1.5 $\times$ 10$^{-3}$ M</td>
<td>890</td>
<td></td>
</tr>
</tbody>
</table>

The experimental conditions were the same as those given in Table VII, 2.5 ml. of Enzyme A4 per flask. C$^{14}$ analyses and glyoxalase assays were carried out on aliquots of the same experimental sample.

A utilization of ATP in stoichiometric amounts in the synthesis of the amide bond of glutamine has been demonstrated by Speck (7, 13). The net synthesis of glutathione from its constituent amino acids would accordingly be expected to require the splitting of one energy-rich phosphate link for each peptide bond, or a total of two per molecule of glutathione. Since under our experimental conditions relatively high concentrations of inorganic phosphate were necessary for enzymatic activity, it was not feasible to determine the release of inorganic phosphate from the adenine nucleotides in the course of the reaction. Moreover, the presence of ATPase activity and of enzyme systems capable of generating high energy phosphate linkages, as shown by the results obtained with adenylic acid, have so far precluded an accurate assessment of the quantitative relation between the energy source and the reaction product. However, the data given in Table X suggest that energy-rich phosphate is consumed in peptide synthesis in stoichiometric amounts. These data are taken from experi-
ments performed with Enzyme A5 at time intervals at which adenylic acid showed no effect. Thus, under these conditions the endogenous formation of high energy phosphate was presumably small. For each micromole of ATP added, approximately 1 \( \mu \text{M} \) of tripeptide was formed. If the assumption is correct that the splitting of 1 mole of high energy phosphate is necessary for the synthesis of each of the two bonds of GSH, it would follow that the bond energy derived from more than one pyrophosphate linkage of ATP was utilized in the synthetic process.

The data obtained with ADP show that 1 \( \mu \text{M} \) of the diphosphate afforded roughly 0.5 \( \mu \text{M} \) of GSH. Regardless of whether or not ADP is utilized after dismutation to ATP and AMP, these results also appear to indicate that both pyrophosphate linkages in the adenine nucleotides can supply energy for peptide synthesis. However, this conclusion must be regarded as tentative until a direct utilization of ADP can be demonstrated in enzyme preparations of greater purity.

**Synthesis and Hydrolysis of GSH**—The marked increase of synthetic activity which is observed after centrifugation of crude extracts at 18,000 \( \times \) g and which permits the demonstration of GSH synthesis by a balance method may be attributed either to the removal of inhibitors or of lytic enzymes which act upon the reactants, the reaction product, or both. A comparison of the various enzyme preparations shows that before high speed centrifugation ATP at \( 5 \times 10^{-4} \) \( \text{M} \) concentration is without effect on GSH synthesis (Fig. 3), but that after removal of sedimentable material this same concentration of the adenine nucleotide affords substantial activity (Table X). This indicates that the concentration of ATP-splitting enzymes had been greatly diminished by this step.

### Table X

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Concentration ( \mu \text{M} )</th>
<th>ATP Concentration ( \mu \text{M} )</th>
<th>GSH synthesized ( \mu \text{M} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>3.0</td>
<td>( 1.6 \times 10^{-3} )</td>
<td>1.27</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.5</td>
<td>( 0.8 \times 10^{-3} )</td>
<td>0.76</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.0</td>
<td>( 0.53 \times 10^{-3} )</td>
<td>0.68</td>
</tr>
<tr>
<td>ATP</td>
<td>2.0</td>
<td>( 1.06 \times 10^{-3} )</td>
<td>1.90</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.0</td>
<td>( 0.53 \times 10^{-3} )</td>
<td>0.92</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.5</td>
<td>( 0.026 \times 10^{-3} )</td>
<td>0.37</td>
</tr>
</tbody>
</table>

The composition of the incubation medium was the same as in the experiments of Table VII, 1.25 ml. of Enzyme A5 per flask, incubated for 40 minutes.

* By glyoxalase assay.

† Based on determination of 7 minute-hydrolyzable phosphate in the nucleotides.
That the same treatment eliminates a hydrolytic factor which acts on glutathione itself is indicated by an experiment designed to test simultaneously the formation and the splitting of the cysteinylglycine bond of GSH. Glutathione labeled in the glycine moiety was prepared by incubation of liver homogenates with $\text{C}^{14}$-glycine. 1 mg. of the labeled tripeptide was added to the standard incubation medium which contained glycine, labeled in this case by $\text{N}^{15}$ instead of $\text{C}^{14}$. One aliquot of the mixture was incubated with Enzyme A3 and another with the enzyme obtained by high speed centrifugation (Enzyme A4). After incubation, 30 mg. of non-isotopic glutathione were added as carrier to both flasks and the isolated GSH cuprous mercaptide was analyzed for $\text{C}^{14}$ and for $\text{N}^{15}$ (Table XI). Flasks

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Glutathione</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C.p.m. C$^{14}$</td>
</tr>
<tr>
<td>A3</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>&quot; control†</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>A4</td>
<td>111 ± 6</td>
</tr>
<tr>
<td>&quot; control†</td>
<td>105 ± 5</td>
</tr>
</tbody>
</table>

All flasks contained salts and amino acids in the same concentrations as in the experiments of Table VII. 5 ml. of enzyme and 1 mg. of $\text{C}^{14}$-glutathione were added to each flask before and 30 mg. of carrier glutathione after incubation.

* Calculated for the glycine N of GSH.
† Trichloroacetic acid added immediately after mixing.

Glutathione isolated from the experiment with Enzyme A3 contained about one-fourth as much $\text{C}^{14}$ as the GSH isolated from the control flask (Table XI). From these data it is calculated that of the 1 mg. of $\text{C}^{14}$-glutathione added initially only 0.22 mg. remained after incubation; i.e., 0.78 mg. of the labeled tripeptide had been split with the loss of $\text{C}^{14}$-glycine. The $\text{N}^{15}$ content of the same sample of GSH corresponded to a synthesis of 0.4 mg. of peptide. Thus with Enzyme A3 the rate of hy-
drolysis was greater than the rate of synthesis. In this case a balance method would have failed to detect glutathione synthesis. On the other hand, in the experiment with Enzyme A4, the reisolated GSH had the same C\textsuperscript{14} concentration as in the control, showing that the cysteinylglycine linkages of the labeled GSH which had been added initially had remained intact. The enzymatic activity which is responsible for the splitting of GSH is therefore no longer present after centrifugation of the extracts at high speed.\textsuperscript{4} The incorporation of N\textsuperscript{15} into GSH isolated from the same experiment with Enzyme A4 was equivalent to the synthesis of 1.69 mg. of peptide and must have occurred in the absence of any splitting of cysteinylglycine bonds. These findings provide an explanation of the relatively low and variable activity of preparations of Enzyme A1 to A3. Here, enzymes capable of splitting glutathione as well as synthetic enzymes appear to be present, and, if the two opposing reactions occur at similar rates, the newly synthesized peptide will not accumulate. This interpretation also explains why glutathione itself has a stimulatory effect on the formation of C\textsuperscript{14}-glutathione in crude dialyzed extracts (Enzyme A3, Fig. 7) but not in the fractions which have been subjected to centrifugation at 18,000 \( \times \) \( g \). When the system contains a large quantity of glutathione initially, the proportion of labeled tripeptide which is split by the hydrolytic enzyme will be smaller than in the absence of any added glutathione, and hence the isotope concentration in the glutathione isolated at the end will be greater. After the hydrolytic enzyme has been removed, the newly synthesized tripeptide accumulates and net synthesis will be demonstrable. Under these latter conditions the addition of glutathione prior to incubation should be without stimulatory effect. This was found to be the case (Fig. 7, Curve II).

The separation of enzymatic activity capable of splitting glutathione from the synthetic system is of interest with regard to the much debated identity of proteolytic and protein-synthesizing enzymes. Our results appear to indicate that, at least in the case of glutathione, the enzymes which catalyze the synthesis and the hydrolysis of peptide bonds are distinct entities.

\textbf{SUMMARY}

1. Extracts of acetone-dried pigeon liver catalyze reactions leading to the synthesis of glutathione from glutamic acid, cysteine, and glycine. Mg\textsuperscript{++} ion, phosphate, and an adenine nucleotide are necessary for the synthesis of the tripeptide. The preparation and some properties of the enzyme system are described.

\textsuperscript{4} The sedimented material obtained by this step has recently been found to split GSH.
2. Adenosinediphosphate and adenosinetriphosphate are equally effective in supporting glutathione synthesis. Adenylic acid can replace ATP in crude extracts, presumably because the monophosphate is converted to ADP or ATP.

3. Crude liver extracts catalyze both the splitting and the synthesis of glutathione. From these preparations the hydrolytic activity can be separated by centrifugation at high speed.

The authors are indebted to Mr. W. Kramer for assistance in the course of this work.

BIBLIOGRAPHY
