A STABILIZED ENZYME SYSTEM FOR AMINO ACID INCORPORATION*

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(Received for publication, February 18, 1957)

Previous studies (1) of glutamic acid metabolism suggested an investigation of the role of this amino acid and its thio esters (2, 3) in amino acid incorporation. The studies by Zamecnik et al. (4, 5) on amino acid incorporation into freshly prepared rat liver microsomes in the presence of the soluble cell fraction and an ATP-regenerating system appeared to provide a suitable test system for our purposes. However, considerable thioesterase activity was found in the microsomal preparations and still more in the soluble cell fraction (6), a fact which made a meaningful study of the utilization of glutamic acid thio esters in amino acid incorporation not feasible. It was then attempted to investigate the rate of incorporation of glutamic acid into microsomal preparations. In the absence of cysteine the incorporation of glutamic acid was of the order of magnitude of that of C\(^{14}\)-leucine, whereas in the presence of cysteine considerably more radioactivity was found in the proteins. This latter activity could be removed by treating the proteins with either SH compounds, such as excess cysteine and thioglycolic acid, or with performic acid. These observations suggested that glutamylcysteine (or GSH) formed during incubation was attached to the proteins by S—S linkages and was removed by the subsequent treatment.\(^{2}\) These findings are reminiscent of the observations that C\(^{14}\)-glycine incorporated into proteins of partially fractionated liver

* This work was supported in part by grants from the National Institute of Neurological Diseases and Blindness (grant No. B-226) of the National Institutes of Health, Public Health Service, and by a contract between the Office of Naval Research and the Psychiatric Institute.

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1 The following abbreviations have been used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; S-protein, 105,000 × g supernatant protein; GSH, glutathione; GDP, guanosine diphosphate; GMP, guanosine monophosphate; TCA, trichloroacetic acid.

2 Unpublished results (also cf. Sachs and Neidle (7)); in order to conserve space the detailed data on these experiments are not reported but will be made available upon request.
homogenates could be partly removed by treatment with SH compounds, a fact which was interpreted as indicating the attachment of newly formed GSH (containing labeled glycine) to the proteins by S—S linkages (8).

In order to define more carefully the properties of the incorporating system, the requirements for amino acid incorporation were therefore studied with the aid of C14-leucine and C14-phenylalanine. The lability and variability of each microsome preparation have hitherto necessitated the preparation of the enzyme system from the livers of freshly killed animals before each experiment. This difficulty was to a great extent overcome by the development of a microsome preparation (7) which could be prepared in relatively large amounts with an activity which remained constant for several months. Some of the studies on the requirements, kinetics, and properties of the amino acid-incorporating system of the stable microsome preparation are reported below.

EXPERIMENTAL

Materials and Methods—L-Leucine-C14, L-phenylalanine-C14, and L-lysine-C14 (all uniformly labeled) were obtained from the Nuclear Instrument and Chemical Corporation. Random samples of these amino acids were found to be isotopically pure when tested by the use of paper chromatography (phenol-water, butanol-acetic acid-water) and paper ionophoresis (acetate buffer, pH 4.5).

The following commercial preparations were used: L-leucine, L-lysine, L-phenylalanine (California Foundation for Biochemical Research); GSH (Schwarz); ATP (Pabst); sodium phosphocreatine, GDP, and GMP (Sigma); ADP, barium salt (Pabst), was converted to the sodium salt by the use of Dowex 50 (sodium form) at 4°. The concentration of the various nucleotides was usually measured in the Beckman spectrophotometer.

Purified ATP-creatine transphosphorylase was prepared according to Kuby et al. (9). The Tris salt of phosphocreatine was obtained by passing a solution of the sodium salt through a column of Dowex 50 (X4) in the Tris form at 4°.

Phosphocreatine was determined as described by LePage (10) or by the difference between the inorganic phosphate measured in the Lowry-Lopez (11) and Fiske-Subbarow (12) procedures. Soluble protein was measured on aliquots by the biuret procedure (13) with crystalline bovine serum albumin as a standard. Microsomal protein could not be estimated in this manner (without pretreatment with TCA, lipide solvents, etc.) because the amounts required for accurate determinations led to turbid solutions. Routinely, microsomal material was washed three times with cold 5 per cent TCA, suspended in 5 per cent TCA at 90° for 15 minutes, and washed once more with cold TCA. The wet pellet was heated for about 10 min-
utes at 100° to drive off residual TCA and then extracted with lipide solvents. There was no significant difference in the values obtained with bovine serum albumin as a standard or microsomal protein washed as described above and dried to constant weight. Microsomal protein was also more conveniently determined directly by the procedure of Lowry et al. (15), in which case very small aliquots could be used without leading to any noticeable turbidity. These two determinations agreed within 10 per cent.

Stable Microsome Preparations—Adult rats were decapitated and bled, and the livers quickly excised and placed in ice-cold 0.25 M sucrose. The livers were blotted, trimmed, minced with scissors, and homogenized in 2 volumes of 0.25 M sucrose in an all-glass Potter-Elvehjem homogenizer for 1 to 2 minutes. The homogenate was centrifuged at 15,000 × g for 10 minutes in the Spinco preparative centrifuge (rotor No. 30). The supernatant fluid (15,000 × g supernatant) was decanted, shell-frozen, and lyophilized. 100 ml. of supernatant fluid yielded approximately 12 gm. of dry powder which could be stored at -20° over a period of several months without any appreciable loss of activity.

Two preparations of microsomes were used in the incorporation experiments. Suspensions of 1 gm. of powder in cold 0.25 M sucrose solution to give a total volume of approximately 60 ml. (dilute suspension) or 12 ml. (concentrated suspension), respectively, were centrifuged at 40,000 r.p.m. for 35 minutes at 0°. The pellet material of the dilute suspension was suspended in 1.6 ml. of cold 0.25 M sucrose solution and, unless otherwise noted, 0.4 ml. of this preparation (equivalent to approximately 10 to 12 mg. of microsomal protein) was used in a final volume of 1.0 ml. The microsomes of the concentrated suspension were resuspended in 12 ml. of 0.25 M sucrose solution and recentrifuged as above. The pellet was suspended in 1.6 ml. of 0.25 M sucrose and 0.4 ml. of this suspension (washed microsomes) was used. All additions were adjusted to approximately pH 7.5 before use.

Measurement of Radioactivity—After incubation of the enzyme system in the presence of labeled amino acids (for specific conditions, see the legends to Figs. 1 to 7) the proteins were precipitated with 10 per cent TCA and washed essentially as described by Siekevitz (16). The protein was suspended in ether and plated with suction on Whatman No. 1 filter paper (1 cm. in diameter) placed on perforated Teflon planchets. The planchets

3 If the heating step (100°) was omitted, then 10 to 30 per cent of the protein was extracted by the lipide solvents. Drying the protein before lipide extraction was suggested by Dr. H. Meltzer (unpublished experiments). The success of the procedure used is understandable in the light of the recent observations of Korner and Debro (14) on the solubility of albumin in alcohol after precipitation with TCA.

4 We are indebted to Dr. P. R. Srinivasan for familiarizing us with this technique.
were weighed before and after plating. Counting of radioactive materials was performed with a thin window Geiger-Müller tube and scaler (Nuclear Instrument and Chemical Corporation). In many instances the protein after counting was scraped from the filter paper, treated with thioglycolate (16) or performic acid (8), and recounted. All the samples were corrected to counts per minute at infinite thickness, empirical self-absorption curves of the specific material under investigation being used (e.g. radioactive protein or amino acids6). The data are expressed in terms of either relative specific activity (counts per minute at infinite thickness), total counts (counts per minute per mg. at infinite thickness multiplied by the total mg. of protein), or per cent of maximal incorporation obtained for each series of experiments carried out simultaneously.

Fractionation of Protein of Soluble Cell Fraction (ε-Protein) with Protamine-Ethanol—In order to obtain the protein of the soluble cell fraction essentially free from salts, other low molecular weight compounds, and nucleo-proteins, the fractionation procedure was carried out thus: The crude soluble cell fraction (4) (140 ml., prepared in 0.25 M sucrose solution and obtained essentially free from particulate components by centrifugation at 40,000 r.p.m. for 1 hour at 0°) containing 29 mg. of protein per ml., at pH 7.0, was cooled to 0°. Approximately 200 mg. of protamine sulfate in 13 ml. of H2O were added dropwise over a period of 30 minutes with stirring, which was continued for 15 minutes in the cold, and the suspension was centrifuged in the Servall angle centrifuge at 4° for 20 minutes. The precipitate was discarded and the supernatant fluid (approximately 18 to 20 mg. of protein per ml.) was fractionated with ethanol at -15°. The fraction precipitating between 11 and 23 per cent ethanol (final temperature, -10°) was centrifuged at 15,000 $\times g$ for 10 minutes (International refrigerated centrifuge, high speed attachment). The precipitate was dissolved in 0.01 M phosphate buffer, pH 7.5, to give a protein concentration of 42 mg. per ml. This solution was subsequently dialyzed against 10-4 M GSH, 10-4 M EDTA, pH 7.5, and then treated with Norit A (25 mg. of Norit per ml.) with stirring for 1 hour at 4°. The Norit was separated by centrifugation.

Results

Activity of Lyophilized Microsomes—The lyophilized preparations most active in amino acid incorporation were obtained when the liver was ho-

* Although up to 10 per cent of the activity was sometimes removed from C14-leucine-labeled protein by these time-consuming manipulations, they were not adopted as a routine procedure except to check small differences, low incorporation values, etc.

* The curve obtained with C14-leucine was almost identical with the curve for C14-leucine-labeled protein. Under these conditions infinite thickness was obtained by approximately 11 mg. of material.
mogenized in 0.25 M sucrose solution instead of the salt medium of Zamec-
nik and Keller (4) and when the 15,000 × g supernatant fluid containing
microsomes and S-protein was used for lyophilization. When this powder
was resuspended in 0.25 M sucrose and centrifuged at 105,000 × g, the
microsomes and S-protein upon recombination were fully as active in
amino acid incorporation as was a fresh preparation. If the microsomes
were sedimented first and then lyophilized, 50 to 75 per cent of the activity
was lost. Serum albumin did not appear to be capable of preventing this
loss of activity. Also, microsomes prepared in the salt medium, especially
in dilute suspensions and washed with salt medium in the absence of S-pro-
tein, were inferior to microsomes prepared in sucrose (see “Effect of salts”).

Requirements for Incorporation—The requirements for maximal incor-
poration into microsomes obtained from the dried powder were essentially
the same as those for a fresh preparation (4, 17, 18). In this as yet crude
system (Fig. 1), the pH optimum was approximately 7.5. Under the con-
ditions described, the requirements and optimal concentrations for the
incorporation of C¹⁴-leucine are given in Figs. 1 to 7. The extent of in-
corporation varied with each powder, as it did with the freshly prepared
controls, and ranged from approximately 0.1 to 0.8 μmole of leucine per
gm. of protein during a 30 minute incubation period. The highest in-
corporation of isotope was found to occur into ribonucleoprotein fractions
of microsomes (19, 20).

Increased amounts of C¹⁴-leucine, GDP (below 1 × 10⁻³ M; cf. Keller
and Zamecnik (18)), or ATP-creatine transphosphorylase did not affect
the incorporation. However, the other components (ATP, GSH (Fig. 1),
phosphocreatine, MgCl₂ (Fig. 2), KCl (Figs. 3 to 5), and S-protein (Fig. 6))
all exhibited optimal concentrations above which incorporation was in-
hhibited.

Effect of —SH—The stimulation by GSH (Fig. 1) was most marked when
the S-protein was either dialyzed free from endogenous, low molecular
weight compounds or precipitated (acid, salt, or alcohol) and subsequently
treated with Norit. These results are essentially in accordance with
those of Zamecnik and Keller (4). If S-protein was dialyzed overnight
against 5 × 10⁻³ M phosphate buffer, pH 7.5, in the absence of SH, almost
complete inactivation occurred. GSH was the most efficient of the com-
pounds tested (cysteine, ascorbate, thioglycolate) in stimulating incorpora-
tion in such a system. S-protein could also be dialyzed against dilute
buffer or sucrose containing 10⁻⁴ M GSH and 10⁻⁴ M EDTA, pH 7.5, without
loss of activity. Dialysis against any of a variety of metal-chelating or
complex-forming agents at a concentration of 10⁻³ M (cyanide, EDTA,
8-hydroxyquinoline, etc.) did not appear to affect the activity of S-protein.

Effect of Mg⁺⁺—Under the conditions described (Fig. 2), the optimal con-
centration of MgCl₂ was about 5 × 10⁻⁵ M. On either side of this value
incorporation fell off rather sharply. However, the system could tolerate higher concentrations of magnesium ions in the presence of increased

![Diagram](http://www.jbc.org/)

**Fig. 1.** The effect of ATP (○) and GSH (×) on the incorporation of C\(^{14}\)-leucine. Each tube contained microsomes from a lyophilized preparation (see “Methods;” dilute suspension), 11 to 12 mg. of protein, S-protein (protamine, ethanol fraction, see “Methods”), 2 mg., 0.03 M Tris buffer, pH 7.5, phosphocreatine sodium salt, 0.02 M, ATP-creatine transphosphorylase, 14 units, GDP, 10\(^{-4}\) M, C\(^{14}\)-L-leucine, 10\(^{-4}\) M (2 × 10\(^6\) c.p.m. per mg. at infinite thickness), potassium phosphate buffer, pH 7.5, 0.02 M, MgCl\(_2\), 5 × 10\(^{-3}\) M, ATP, sodium salt (GSH, 2.5 × 10\(^{-3}\) M), and GSH (ATP, 1 × 10\(^{-3}\) M) additions as indicated, all in a final volume of 1.0 ml. Incubated at 37° for 30 minutes. Maximal incorporation equivalent to 0.1 μmole of C\(^{14}\)-leucine per gm. of protein (273 c.p.m. at infinite thickness; infinite thickness at 11 mg. of protein).

amounts of S-protein. Furthermore, the optimal concentration of MgCl\(_2\) was a function of the ATP concentration as described below.

**Effect of ATP**—The effect of ATP above 1 × 10\(^{-3}\) M was striking. As little as 5 × 10\(^{-3}\) M ATP reduced the incorporation to 15 per cent of the maximal value (Fig. 1), but this inhibition by ATP (5 × 10\(^{-8}\) M) was completely overcome by increasing the MgCl\(_2\) concentration from 5 × 10\(^{-3}\) M
to $1 \times 10^{-2}$ M. The effect of MgCl$_2$ on the inhibition by ATP was observed in the presence of either limiting or maximal concentrations of S-protein.

Further information on the effect of ATP on the incorporating system was obtained from centrifugation studies. If, before incubation under optimal conditions for incorporation, washed microsomes (2 to 3 mg. of protein per ml.) were suspended and resedimented in a medium consisting of 0.25 M sucrose, $2 \times 10^{-3}$ M ATP, pH 7.4, these microsomes were only about 20 to 50 per cent as active as microsomes centrifuged from 0.25 M sucrose, $5 \times 10^{-3}$ M sodium phosphate buffer, pH 7.0 to 7.4 (Table I). Spectrophotometric measurements at 260 m$\mu$ of cold perchloric acid extracts of the microsomal pellets indicated that a maximum of 0.1 to 0.2 $\mu$ mole of ATP was either occluded or adsorbed per 10 mg. of protein. Therefore, the observed inhibition cannot be ascribed to the presence of

![Figure 2. The effect of Mg$^{++}$ (O) and phosphocreatine, sodium salt (□) on the incorporation of C$^{14}$-leucine. Conditions as described in legend, Fig. 1; ATP, sodium salt, 0.001 M, GSH, 0.025 M, MgCl$_2$ (phosphocreatine, $1 \times 10^{-2}$ M), and phosphocreatine, sodium salt (MgCl$_2$, $5 \times 10^{-3}$ M) additions as indicated. Maximal incorporation, 0.1 pmole of C$^{14}$-leucine per gm. of protein.](http://www.jbc.org/).
increased amounts of ATP during subsequent incubation. The effects obtained with ATP were independent of the ATP preparation used. Similar results were obtained with several preparations of either potassium ATP (Sigma) or crystalline ATP (Pabst). When washed microsomes were centrifuged in the presence of an acid hydrolysate of ATP (N HCl, 100°, 7 minutes), then the activity of the final pellet was about equal to that ob-

Table I

Incorporation of C\textsuperscript{14}-Leucine into Washed Microsomes
Centrifuged in Presence of Various Nucleotides

<table>
<thead>
<tr>
<th>Centrifugation medium</th>
<th>Per cent of maximal incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 5 × 10\textsuperscript{-3} m phosphate</td>
<td>100</td>
</tr>
<tr>
<td>2. 2 × 10\textsuperscript{-3} &quot; AMP</td>
<td>84</td>
</tr>
<tr>
<td>3. 2 × 10\textsuperscript{-3} &quot; ADP</td>
<td>96</td>
</tr>
<tr>
<td>4. 2 × 10\textsuperscript{-3} &quot; ATP</td>
<td>17</td>
</tr>
<tr>
<td>5. 1 × 10\textsuperscript{-3} &quot; GDP</td>
<td>90</td>
</tr>
<tr>
<td>6. 2 × 10\textsuperscript{-3} &quot; ATP</td>
<td>23</td>
</tr>
<tr>
<td>1 × 10\textsuperscript{-3} &quot; GDP</td>
<td>39</td>
</tr>
<tr>
<td>7. 2 × 10\textsuperscript{-3} &quot; ATP</td>
<td>120</td>
</tr>
<tr>
<td>2 times concentration of microsomes in 4</td>
<td></td>
</tr>
<tr>
<td>8. 2 × 10\textsuperscript{-3} m ATP</td>
<td></td>
</tr>
<tr>
<td>4 × 10\textsuperscript{-3} &quot; MgCl\textsubscript{2}</td>
<td></td>
</tr>
<tr>
<td>9. 2 × 10\textsuperscript{-3} &quot; ATP (hydrolyzed)*</td>
<td>79</td>
</tr>
</tbody>
</table>

Washed microsomes were suspended in the medium indicated (all containing 0.25 m sucrose), adjusted to pH 7.0 to 7.4 with NaOH, to give a final concentration of 2 to 3 mg. of protein per ml., and centrifuged at 40,000 r.p.m. for 45 minutes at 0°. The pellet material in each tube was suspended in 0.25 m sucrose and an aliquot of each suspension (7 to 10 mg. of protein) was added to separate tubes containing 3 to 5 mg. of S-protein, 0.03 m Tris buffer, pH 7.5, sodium phosphocreatine, 0.02 m, ATP, sodium salt, 0.001 m, ATP-creatine phosphokinase, 14 units, GSH, 0.025 m, GDP, 0.0001 m, C\textsuperscript{14}-L-leucine, 0.0001 m (2 × 10\textsuperscript{6} c.p.m. per mg. at infinite thickness), KCl, 0.06 m, MgCl\textsubscript{2}, 0.005 m, all in a final volume of 1.0 ml. Incubated at 37° for 30 minutes. Maximal incorporation, 100 per cent (370 c.p.m. at infinite thickness) 0.13 μmole of C\textsuperscript{14}-leucine per gm. of protein.

* N HCl, 100°, 7 minutes.

served with microsomes sedimented from a centrifugation medium containing AMP.

Under the conditions described (Table I), the extent of the inhibition observed with ATP was a function of the concentration of microsomes as well as the MgCl\textsubscript{2} in the centrifugation medium. At constant ATP (2 × 7 The crystalline ATP used in these experiments were chromatographed according to Krebs and Hems (21) and showed only small amounts of ADP as well as ATP. This solvent system failed to reveal the presence of any new, acid-soluble ultraviolet-absorbing material released from microsomes centrifuged in the presence of ATP.
doubling the concentration of microsomes decreased the inhibition by about one-half. Addition of MgCl₂ (4 × 10⁻³ M) to the centrifugation medium prevented completely the inhibition obtained with 2 × 10⁻³ M ATP. Also, if the MgCl₂ concentration was increased from the usual 5 × 10⁻³ M to 1 × 10⁻² M in an incubation mixture containing microsomes

**Fig. 3**

The effect of sodium (○) and potassium chloride (●) on the incorporation of C¹⁴-leucine. Each tube contained microsomes obtained from a lyophilized preparation (see "Methods;" washed microsomes), 8 to 10 mg. of protein, S-protein (dialyzed 6 hours against 0.3 M sucrose containing 10⁻⁴ M GSH, 10⁻⁴ M EDTA, pH 7.5), 1.5 mg., 0.03 M Tris buffer, pH 7.5, Tris-phosphocreatine, 0.02 M, ATP, sodium salt, 0.001 M, ATP-creatine transphosphorylase, 14 units, GSH, Tris salt, 0.025 M, MgCl₂, 5 × 10⁻³ M, GDP, 10⁻⁴ M, C¹⁴-L-leucine, 10⁻⁴ M (2 × 10⁶ c.p.m. per mg. at infinite thickness), NaCl and KCl additions as indicated, all in a final volume of 1.0 ml. Incubated at 37° for 30 minutes. Maximal incorporation (293 c.p.m. at infinite thickness) 0.1 μmole of C¹⁴-leucine per gm. of protein.

**Fig. 4**

The effect of varying the ratio of potassium and sodium on the incorporation of C¹⁴-leucine. Conditions as described in legend, Fig. 3; total concentration of chlorides, 0.08 M. Maximal incorporation (245 c.p.m. at infinite thickness) 0.09 μmole of C¹⁴-leucine per gm. of protein.

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10⁻³ M), doubling the concentration of microsomes decreased the inhibition by about one-half. Addition of MgCl₂ (4 × 10⁻³ M) to the centrifugation
which had been centrifuged from sucrose-ATP, then the incorporation was stimulated by about 50 to 100 per cent.

In Table I comparative data are given on the activity of microsomes centrifuged in the presence of AMP, ADP, or GDP rather than ATP.

**Effect of Salts**—Amino acid incorporation into microsomes was stimulated about 2-fold by sodium and very markedly (approximately 10-fold) by potassium ions (cf. Sachs and Neidle (7)). The effect of increasing the sodium or potassium concentration on the incorporation is shown in Fig. 5.

![Graph showing the effect of potassium chloride concentration on amino acid incorporation](image)

**Fig. 5.** The effect of low (▲), optimal (○), and high (●) concentrations of KCl on the time-course of the incorporation of C\(^{14}\)-leucine. Low KCl (▲), 0.02 M, optimal (○), 0.08 M, high (●), 0.17 M. Conditions as described in legend, Fig. 3.

3. The optimal potassium concentration extended over the range 0.06 to 0.1 M, incorporation falling off on either side of the plateau. However, in the presence of larger amounts of S-protein this plateau was extended into even higher salt regions. If the total salt concentration was kept constant at 0.08 M and the ratio of sodium and potassium was varied, the effect of these two ions on the incorporation appeared to be additive over a limited range. This is apparent from a comparison of the curves in Figs. 3 and 4. In experiments designed to test the role of various anions, chloride salts were replaced by sodium or potassium salts of either di- or polyvalent anions. Whereas, under the conditions described, KCl above 0.1 M became increasingly inhibitory, potassium salts of di- or polyvalent anions (under
equivalent conditions of ionic strength, potassium and S-protein concentration, etc.) were inhibitory at lower concentrations. The inhibition by

![Graph showing the effect of varying the concentration of S-protein and microsomal protein on the incorporation of C14-leucine into microsomes.](image)

**Fig. 6.** The effect of varying the concentration of S-protein (○) and microsomal protein (●) (●) on the incorporation of C14-leucine into microsomes. Conditions as described in legends, Figs. 1 and 2; S-protein (dialyzed 6 hours against 0.3 M sucrose containing 10⁻⁴ M GSH, 10⁻⁴ M EDTA, pH 7.5) additions as indicated, (○); microsome concentration, 9 to 10 mg. of protein, the volume of S-protein added was kept constant by adding the required amount of dialysate; after incubation at 37° for 30 minutes the microsomes were centrifuged at 40,000 r.p.m. for 1 hour, washed, and counted. Maximal incorporation (650 c.p.m. at infinite thickness) 0.2 μmole of C14-leucine per gm. of protein. Microsomal protein additions as indicated; (●) in the presence of 1.7 mg. of S-protein; (△) 5.7 mg. of S-protein; processed as described above; for small amounts of microsomal protein, three or four tubes were run simultaneously and pooled after incubation. Incorporation expressed as per cent of total counts (c.p.m. per mg. at infinite thickness X total mg. of microsomal protein); maximal total counts, 1014 c.p.m.

higher levels of sodium phosphocreatine (Fig. 2) was probably due to such an effect. Aspects of the mechanism of action of polyvalent anions on the incorporation system have been reported briefly (20) and will be the subject of a subsequent communication.
In a system depleted of sodium or potassium ions (washed microsomes, dialyzed S-protein, Na, and K replaced by Tris), increasing the osmotic strength with sucrose stimulated the incorporation only slightly (20 per cent).

Fig. 7. The effect of varying the concentration of S-protein on the time-course of the incorporation of C\textsuperscript{14}-leucine. (○) no added S-protein, (●) S-protein, 1.4 mg. per ml., (□) 2.8 mg. per ml. Each vessel contained microsomes (8 to 10 mg. per ml.) from a lyophilized preparation (dilute suspension), standard additions (see legend, Table I), and S-protein as indicated; \( t = 37^\circ \). Additions and protein equilibrated separately and mixed at zero time. The zero time value was used as background. Data expressed as relative specific activity = counts per minute at infinite thickness.

The kinetics of leucine incorporation was studied in the presence of low, high, and optimal concentrations of KCl (Fig. 5). Under these conditions the initial rates of incorporation differ in the experiments containing either optimal or low concentration of KCl. These initial rates did not decline until about 6 and 9 minutes, respectively. However, when potassium chloride was present in high concentration, the initial rate appeared to be maintained for only the first few minutes.

Effect of Microsomes and S-Protein Concentration—The extent of incor-
poration of C\(^{14}\)-leucine also depends upon the concentration of microsomes and S-protein (Fig. 6). Under standard conditions, microsomes (expressed in mg. of protein) at low concentrations appeared to be limiting. At higher concentrations of microsomes, the amount of S-protein was limiting, although strict proportionality was not observed. Under such conditions, increasing amounts of S-protein had little effect on the initial rates but appeared instead to maintain for a longer time the ability of the system to incorporate C\(^{14}\)-leucine (Fig. 7). However, once the incorporation had leveled off (after about 30 minutes at 37\(^{\circ}\)), subsequent additions of an ATP-generating system, S-protein, GDP, or these components in combination with another C\(^{14}\)-amino acid did not lead to significant further bursts of incorporation. Also, no further incorporation was observed with microsomes separated from the original incubation medium by centrifugation. When the time-course of incorporation was studied at 18\(^{\circ}\) instead of 37\(^{\circ}\), under otherwise identical conditions as in Fig. 7, increasing the S-protein concentration had little effect on the initial rate or the extent of incorporation. Strikingly, the requirement for S-protein was greatly increased with acetone-dried microsomes.\(^8\)

The protective action of S-protein on microsomes was further demonstrated by preincubation experiments. Microsomes were preincubated at 37\(^{\circ}\) for 10 to 15 minutes in Tris buffer, buffer and an ATP-generating system, or buffer and ribonuclease, and then tested under standard conditions for incorporation. In all cases in which either crude or dialyzed S-protein was present during the preincubation, a several-fold higher rate of incorporation was observed compared to controls preincubated in the absence of S-protein (Table II).

S-proteins not only from rat liver but from a variety of sources have been found to be effective in stimulating amino acid incorporation into rat liver microsomes. These include guinea pig, cat, and monkey liver, guinea pig, rat, and monkey brain cortex. Preliminary experiments have indicated that S-proteins may be subjected to the usual fractionation procedures (e.g. acid, salt, organic solvents, gels, etc.) (cf. Hoagland et al. (17)).

The stabilizing effect of S-protein made it possible to dialyze microsomes

\(^8\) Acetone drying of microsomes has up to now yielded preparations with variable degrees of inactivation. However, all active preparations required relatively large amounts of S-protein for maximal activity (compared to freshly prepared or lyophilized microsomes). The best preparations were obtained in the following manner: fresh, concentrated suspensions of microsomes were added to 50 to 75 volumes of acetone at 0\(^{\circ}\), stirred for about 10 minutes, filtered, and washed with acetone. The powder was resuspended in acetone and treated as above to yield a white, fluffy powder. In view of these results the possibility cannot be excluded that some lipid-soluble cofactor is involved.
in the presence of a 10-fold excess of S-protein for 3 to 4 hours at 4° against a 0.25 M sucrose solution containing $10^{-4}$ M GSH, $10^{-4}$ M EDTA, pH 7.5. These microsomes still retained considerable incorporating activity, i.e. up to 0.2 µmole of leucine per gm. of protein. Microsomes treated in this manner failed to show any requirement for a complete mixture of amino acids.

**Table II**

*Effect of Preincubating Microsomes under Various Conditions in Presence and Absence of Supernatant Protein*

<table>
<thead>
<tr>
<th>Additions during preincubation</th>
<th>Per cent of maximal incorporation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>S-protein during preincubation</td>
</tr>
<tr>
<td></td>
<td>−</td>
</tr>
<tr>
<td>1. None</td>
<td>0</td>
</tr>
<tr>
<td>2. “</td>
<td>37</td>
</tr>
<tr>
<td>3. ATP-generating system*</td>
<td>37</td>
</tr>
<tr>
<td>4. 0.3 γ ribonuclease</td>
<td>37</td>
</tr>
</tbody>
</table>

Each tube contained microsomes (10 to 12 mg. of protein) from a lyophilized preparation (dilute suspension), 0.04 M Tris buffer, pH 7.5, and additions as indicated in a volume of 0.5 ml.; preincubated with or without dialyzed S-protein (6 mg.) for 10 minutes at 37°; the standard additions (Table I) including 2 mg. of dialyzed S-protein (kept at 0°) were then added to each tube and the incubation was continued for 30 minutes at 37°. Those tubes preincubated without S-protein also received along with the standard additions 6 mg. of S-protein which had been incubated for 10 minutes at 37°. Similar results were obtained if, after preincubation, the microsomes from each vessel were sedimented (all in the presence of identical amounts of S-protein) at 40,000 r.p.m. for 35 minutes at 0° and then reincubated under standard conditions. For these latter experiments more concentrated suspensions of microsomes and S-protein were employed. Maximal incorporation (180 c.p.m. at infinite thickness), 0.06 µmole of C¹⁴-leucine per gm. of protein.

* ATP, $10^{-4}$ M, Tris-phosphocreatine, $10^{-2}$ M, ATP-creatine transphosphorylase, 14 units.

**DISCUSSION**

By the lyophilization procedure described in this report, relatively large amounts of powder could be prepared from rat livers containing microsomes whose amino acid-incorporating activity remained constant over a period of at least several months. In addition, the requirements for amino acid incorporation did not appear to be altered in any way by the lyophilization step. In this respect, some of the findings reported here corroborate many of the results of Zamecnik *et al.* (4, 17, 18) on their studies with freshly prepared rat liver microsomes. However, there are a number of additional observations which are pertinent and are discussed briefly below.
The inhibition of amino acid incorporation by relatively low levels of ATP presents a paradoxical situation since ATP is required at some point during incorporation. It would appear that the inhibition by ATP arises from its ability to disrupt or distort some essential microsome structure. Presumably ATP binds some metal ion (probably magnesium) essential for the integrity of microsomes. Recent observations by Petermann and Hamilton\(^9\) (22, 23) on the stabilizing effect of magnesium ions on rat liver ribonucleoprotein particles support such an interpretation of the ATP inhibition.

The effects of electrolytes on a large number of enzymatic processes have been well documented and especially the role of potassium in phosphorylating systems (24). It would appear that we are dealing with two effects of electrolytes on the incorporating system: one related unspecifically to the ionic strength and the other specifically to the potassium concentration. The inhibition observed with high concentrations of salt where the incorporation ceases after a few minutes is probably due to the dissociation of the ribonucleoprotein granules on rat liver microsomes (25). The complexity of this crude system precluded a more detailed analysis of these salt effects.

Allfrey and Mirsky (26) have reported on the effect of sodium ions on the incorporation of amino acids into isolated thymus nuclei. In their system, replacement of sodium by potassium decreased the incorporation to 15 per cent of the optimal value. The requirement for potassium in the cytoplasmic and of sodium in the nuclear amino acid-incorporating system may conceivably represent a mechanism for the control of protein synthesis in these two cell compartments during various stages of growth. The role (if any) of the adrenocortical steroids in such a process must obviously await further experimentation.

Zamecnik et al. (4) have clearly demonstrated the requirement for soluble protein for amino acid incorporation into rat liver microsomes. Hoagland et al. (17, 27) have found enzymes in this soluble fraction of liver capable of activating the carboxyl group of a number of amino acids and they have postulated that this reaction represents the first step in amino acid incorporation. From the data presented here, it would appear that one of the functions of S-protein (which may be unrelated to any enzymatic properties) is the preservation of some essential ribonucleoprotein structure of microsomes during incorporation. This conclusion is consistent with the ability of S-protein to protect microsomes against the deleterious effects of ribonuclease, lyophilization, heat, and high concentrations of salt, ATP, and polyvalent anions (20). The cessation of amino acid incorporation after relatively short time intervals is probably due to an eventual irreversi-

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\(^9\) Dr. M. L. Petermann, personal communication.
ble dissociation of these highly organized granules (cf. Petermann et al. (23, 28)). According to these studies the most reasonable mechanism whereby S-protein protects microsomes would involve an intimate association of these two components. The nature and specificity of this combination and the extent to which S-protein forms an integral, active part of the microsome-incorporating system are presently under investigation.

The author wishes to express his gratitude to Dr. Heinrich Waelsch for his continued interest and counsel and to Mr. Amos Neidle for his active collaboration during the early phases of this work.

**SUMMARY**

The microsome fraction from rat liver, active in amino acid incorporation, was obtained in the form of a stable lyophilized powder. Full activity was retained only when the microsomes were lyophilized in the presence of the soluble protein fraction. The requirements for amino acid incorporation into microsomes of such a preparation were studied. Although adenosine triphosphate (ATP) and an ATP-generating system were required for incorporation, concentrations of ATP above $10^{-3} \text{ M}$ were inhibitory. This inhibition did not appear to function via a phosphorylating mechanism and could be overcome by increased amounts of magnesium ions. Washed microsomes centrifuged in the presence of $10^{-3} \text{ M}$ ATP at $0^\circ$ were also inhibited. Magnesium ions added to the centrifugation medium were effective in preventing this inactivation. Amino acid incorporation was stimulated about 10-fold by KCl. This effect appeared to be specific for potassium ions. In the presence of concentrations of KCl above $0.1 \text{ M}$ the initial rate of incorporation of $^{14}$C-leucine declined rapidly after the first few minutes. The rate and extent of incorporation of $^{14}$C-leucine into microsomes depended also on the concentration of microsomes and supernatant protein. Microsomal protein at low concentrations appeared to be limiting. At higher concentrations of microsomes, the incorporation was a function of the concentration of supernatant protein. However, under these conditions, increasing amounts of supernatant protein had little effect on the initial rates but appeared instead to increase the ability of the system to incorporate $^{14}$C-leucine for a longer time. Supernatant protein was also shown to be capable of stabilizing microsomes in the presence of high concentrations of salt, heat, dialysis, and ribonuclease.

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