CHARACTERIZATION OF THE PATTERN OF AMINO ACID INCORPORATION IN CELL-FREE LIVER SYSTEMS BY AUTORADIOGRAPHY OF PRECIPITIN REACTIONS

W. S. MORGAN, P. PERLMANN, and T. HULTIN. From the Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden. Dr. Morgan's permanent address is Department of Pathology, Massachusetts General Hospital, Boston

It was shown in preceding papers (13, 18) that the pattern of protein labeling in amino acid incorporation experiments can be studied in certain detail by combining immunological reactions on agar plates with autoradiography. In those experiments the labeling of different protein antigens was studied after incubation of *liver slices* with ^{14}C amino acids under varied experimental conditions. The present communication aims at a similar analysis of the labeling of proteins in *cell-free liver systems*.

METHODS

Young male rats (approx. 80 gm) were fasted overnight and killed by decapitation. The livers were perfused *in situ* with cold 0.15 M KCl. They were then removed and minced in 2 or 3 changes of ice cold 0.25 M sucrose, containing 0.01 M $MgCl_2$, 0.025 M KCl, and 0.035 M tris buffer,[1] pH 7.8. All subsequent procedures were carried out at 0–2°C. After homogenization in a motor-driven Potter-Elvehjem glass homogenizer with 2 volumes of the above medium, the homogenate was centrifuged at 12,000 *g* for 8 minutes. The incubation mixture contained, per milliliter: 0.75 ml of the mitochondrial supernatant, 1 μmole of ATP, 10 μmoles of PEP[2] (potassium salt), and 0.3 μc of each of the amino acids ^{14}C-L-valine (6.5 mc/mmole), ^{14}C-L-leucine (6.0 mc/mmole), and ^{14}C-L-isoleucine (6.1 mc/mmole).[2] The final potassium concentration was approximately 0.1 M. The time of incubation (at 37°C) varied with the individual experiment. A mixture of unlabeled amino acids was added to the incorporation system after incubation, yielding an approximately 1000-fold isotope dilution. After incubation, 4.5 ml portions of the suspension were layered, in Spinco centrifuge tubes, over 6.5 ml of the medium described above but containing 0.5 M sucrose. Microsomes were isolated from cell sap by centrifugation at 105,000 *g* for 90 minutes.

In a number of experiments, ribonucleoprotein particles were prepared from the mitochondrial supernatant by treatment with 0.5 per cent Lubrol W[2] and 1 per cent DOC in 0.6 M KCl according to the method of Rendi and Hultin (19). The particles were combined with cell sap and incubated with radioactive amino acids, ATP, and PEP, as described above. After incubation, particles and cell sap were again separated by centrifugation at 105,000 *g* for 90 minutes. For details see reference 19.

At appropriate steps during fractionation and extraction, samples were taken for the determination of protein content (12) and of protein radioactivity (19). For the immunological experiments, microsomes were extracted with 1 per cent Lubrol W or with 0.5 per cent DOC. The residues after these extractions as well as the isolated ribonucleoprotein particles were extracted with EDTA-bicarbonate at pH 8.3 (7, 1). The cell saps were freed from the fraction precipitable at pH 5.2 (6). The microsomal extracts and the pH 5 soluble fractions of the cell saps were reacted on agar plates (16) with antisera made in rabbits against rat liver cell fractions or against rat serum (1, 17). The antisera were samples from the same batches that were used in the previous study (13). For preparation of the autoradiographs and all other technical details, reference 13 should be consulted.

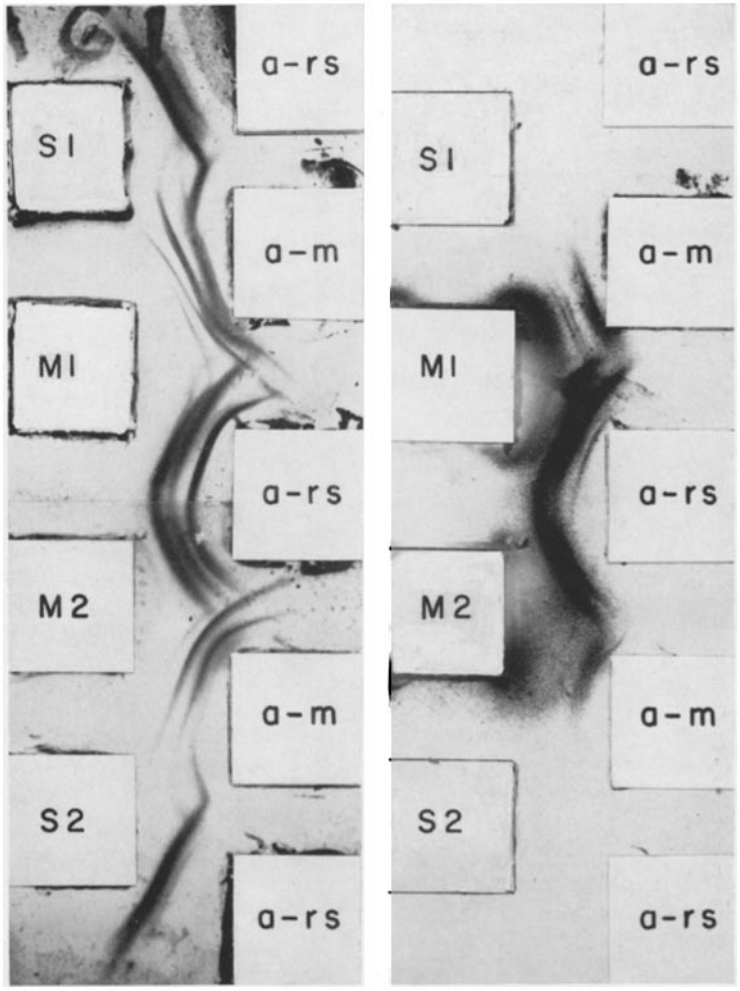
RESULTS AND DISCUSSION

In previously reported experiments (13, 18) autoradiographs of Ouchterlony plates were made with extracts of liver slices, incubated with ^{14}C amino acids. The results indicated that most of the label first appeared in those antigens which could be extracted from the microsomal fraction by means of detergents. Among the many microsomal antigens, those with serum protein–like specificities were most rapidly and intensely

[1] Abbreviations: tris, tris(hydroxymethyl)aminomethane; ATP, adenosine triphosphate; PEP, phosphoenolpyruvate; EDTA, ethylenediamine tetraacetate; DOC, sodium deoxycholate.

[2] The labeled amino acids were supplied by the Radiochemical Centre, Amersham, England; phosphoenolpyruvate was purchased from C. F. Boehringer und Soehne, Mannheim, Germany, and was recrystallized (2); Lubrol W (cetyl alcohol polyoxyethylene condensate) was obtained from Imperial Chemical Industries, Ltd., Manchester, England.

labeled. In the present experiments a similar pattern of labeling was obtained with microsomal extracts of previously incubated mitochondrial supernatants. Figs. 1 *a* and *b* show the results obtained with the microsomal extracts and the pH 5 soluble fractions of the cell saps of two independent experiments, reacted on the same agar plate with two different antisera. The photograph of the Ouchterlony reaction (Fig. 1 *a*) shows the usual spectrum of precipitates formed by fraction-specific and common "liver" antigens, or by serum proteins (17, 1). The autoradiograph (Fig. 1 *b*) reveals that even in this cell-free system, the labeling occurs primarily in the serum protein–like antigens which react with the antiserum against rat serum. Only a few of the precipitates



a *b*

FIGURE 1

Fig. 1 *a*, photograph, Fig. 1 *b*, autoradiograph of Ouchterlony plate, made with Lubrol extracts of microsomes (*M1*, *M2*) and pH 5 soluble fractions of the cell saps (*S1*, *S2*) of mitochondrial supernatants from the homogenates 1 and 2, respectively. Prior to fractionation, the two mitochondrial supernatants were separately incubated for 15 minutes with ^{14}C amino acids. Radioactivity (counts/min/mg protein): *mitochondrial supernatant 1*, 305; *S1*, 81; *mitochondrial supernatant 2*, 625; *S2*, 89.

a-rs, antiserum against rat serum; *a-m*, antiserum against rat liver microsomes. Before use, the anti-microsomal serum was partially absorbed with lyophilized rat serum. Exposure time of autoradiograph, 45 days.

formed by the antigens reacting with antibodies in the anti-microsomal sera were visibly labeled. The nature of the latter antigens is unknown.

The labeling of the antigens was energy-dependent; without ATP and PEP in the incubation mixture no labeling of the precipitin lines was observed. Controls, in which unrelated immune systems were added in different combinations, demonstrated that the labeling of the precipitates was not due to an unspecific adsorption of radioactive material (13). Evidence for the serum protein nature of the most heavily labeled antigens was also obtained by a study of the reactions of

Experiments of this kind provide evidence that even in the mitochondrial supernatant an energy-dependent incorporation of isotope takes place into several immunologically well defined proteins (*cf.* also 3, 15). With regard to the microsomal extracts, the pattern of incorporation appears, at least roughly, similar to that found after incubation of liver slices (13). With regard to the pH 5 soluble fraction of the cell sap the situation is different. In experiments with liver *slices*, weakly labeled precipitates of radioactive serum proteins were formed by the cell sap after 15 minutes of incubation with ^{14}C amino acids. After 60 or 120

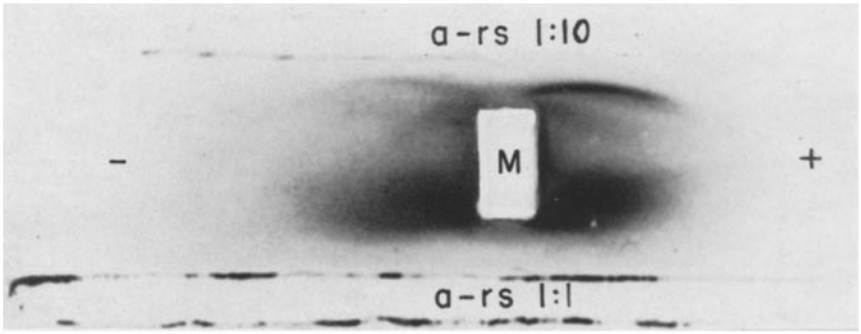


FIGURE 2

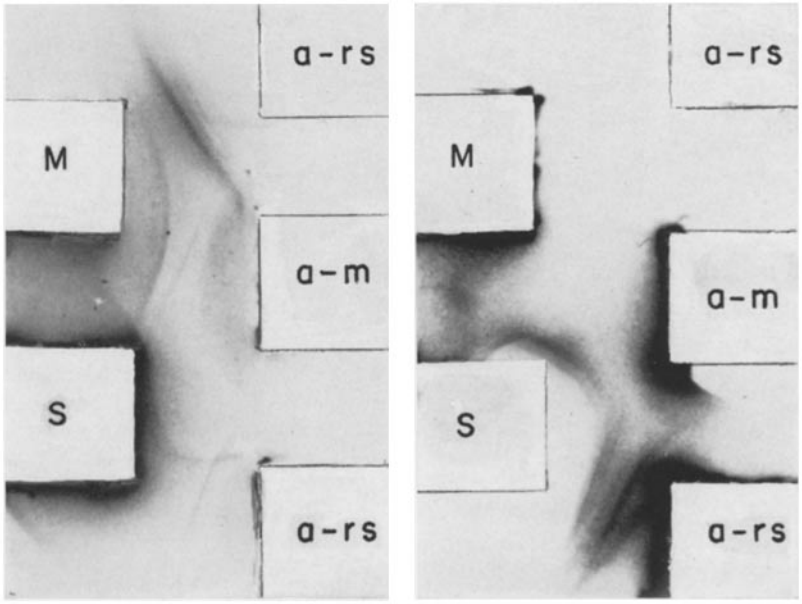
Autoradiograph of immuno-electrophoretic plate, made with Lubrol extracts of microsomes isolated from liver homogenate. Prior to fractionation, the mitochondrial supernatant was incubated for 30 minutes with ^{14}C amino acids. Radioactivity of the *mitochondrial supernatant*, 369 counts/min/mg protein. For electrophoresis, 0.2 ml of the Lubrol extract was added to the agar plate at *M*. After completion of electrophoresis (10 hours, barbiturate buffer pH 8.2, μ = 0.05, 0.4 ma/cm cross-section, agar layer 2 mm high), diluted antiserum against rat serum (*a-rs*) was added from the upper, and undiluted antiserum from the lower, longitudinal basin. Exposure time of autoradiograph, 245 days. (The diffuse blackening seen in the lower part of the autoradiograph is due to the gradual displacement of precipitates in antibody excess.)

identity (16) appearing when rat serum was added as reference in adjacent containers of an agar plate (13). Moreover, that these antigens are serum proteins is also demonstrated in Fig. 2. This shows the autoradiograph of an immuno-electrophoretic experiment, made with the Lubrol extract of a microsomal preparation and reacted with antiserum against rat serum. The most heavily labeled antigens have the mobilities of serum albumin and β-1-globulin. In addition, a number of weakly labeled α- and β-globulins were present. It may be mentioned that in this experiment no unlabeled rat serum was added as carrier. The precipitates were actually too weak to be visualized on the original agar plate.

minutes of incubation, the specific radioactivity of the serum proteins in the cell sap was considerable, and labeling could also be found in additional "cell sap specific" antigens (13). In contrast, in the *mitochondrial supernatants* labeled serum proteins were never found in the cell saps after incubation times of 15 minutes (Fig. 1). If the mitochondrial supernatants were incubated for long periods (60 to 120 minutes), labeled serum protein-like antigens were also recovered in the cell sap (Fig. 3 *a* and *b*), in spite of the fact that in these systems no incorporation of isotope into protein takes place after about 15 minutes of incubation (4). However, at the same time, no more visible precipitates were formed by serum protein-like antigens in

the microsomal extracts and antiserum against rat serum, and only a few by microsomal antigens and anti-microsomal serum. Labeled "cell sap specific" antigens could never be detected in such supernatants. It may therefore be assumed that the appearance of labeled antigens in the cell sap of the mitochondrial supernatants after prolonged incubation is due to an artificial release of soluble components from the microsomal structures.

extract from particles incubated for 60 minutes as described above. For comparison, a corresponding extract of a microsomal residue from liver slices (60 minutes of incubation) was reacted with antiserum on the same plate. In contrast to what is typical for EDTA-bicarbonate extracts of particles prepared from incubated slices (13), the extracts of the isolated and directly incubated particles consistently yielded a number of hardly visible but



a *b*

FIGURE 3

Autoradiographs of Ouchterlony plates, made with Lubrol extracts of microsomes (*M*) and pH 5 soluble fraction of the cell sap (*S*) of a liver mitochondrial supernatant, divided into two parts before incubation and fractionation. *a-rs*, antiserum against rat serum; *a-m*, antiserum against rat liver microsomes, partially absorbed with lyophilized rat serum. Exposure time of autoradiographs, 30 days.

Fig. 3 *a*, mitochondrial supernatant incubated for 60 minutes with ^{14}C amino acids. Radioactivity (counts/min/mg protein): *mitochondrial supernatant*, 341; *S*, 76.

Fig. 3 *b*, mitochondrial supernatant incubated for 120 minutes with ^{14}C amino acids. Radioactivity (counts/min/mg protein): *mitochondrial supernatant*, 310; *S*, 186.

It has been shown that the period of active incorporation of isotope is considerably longer in systems containing *isolated ribonucleoprotein particles* than in *mitochondrial supernatants* (4, 9). It has recently been reported that appropriate incubation of such particles leads to the incorporation of radioactive amino acids into immunologically well defined proteins (5, 10, 11). This was also found in the present study. Fig. 4 presents the results obtained with the EDTA-bicarbonate

strongly labeled precipitates, formed by serum protein–like antigens. The serum protein nature of the labeled antigens could again be verified in immuno-electrophoretic experiments. The results suggest that antigenic proteins may be at least partially synthesized by isolated ribonucleoprotein particles. (See also reference 5.) In addition, in these systems, labeled antigens of the same kind were also found in the pH 5 soluble fraction of the cell sap when the time of incubation was 30

minutes or more. No radioactivity has been detected so far in the precipitates formed by the various "liver" antigens reacting with antibodies in the anti-microsomal and anti–cell sap sera. We assume that the solubilization of labeled serum protein–like antigens is not due to an artificial disintegration of particles, since it has been shown that the release of labeled proteins from isolated

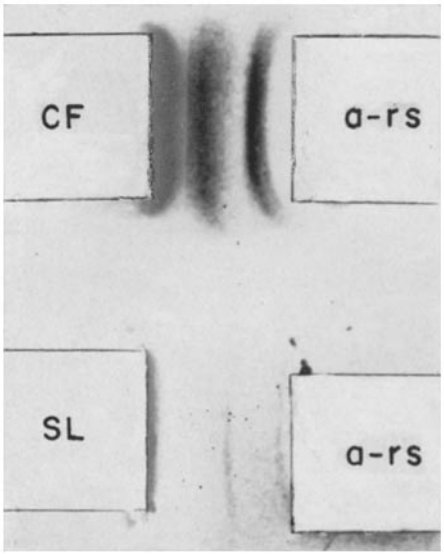


FIGURE 4

Autoradiograph of Ouchterlony plate, made with EDTA-bicarbonate extracts of ribonucleoprotein particles from rat liver. *a-rs*, antiserum against rat serum. *CF*, extract of particles previously incubated for 60 minutes with ^{14}C amino acids. Radioactivity of *incubation mixture* (*particles + cell sap*), 274 counts/min/mg protein. *SL*, extract of particles isolated from microsomes of liver slices, previously incubated for 60 minutes with ^{14}C amino acids. Radioactivity of *total unfractionated homogenate*, 190 counts/min/mg protein. Time of exposure of autoradiograph, 30 days.

ribonucleoprotein particles under the present conditions is largely dependent on an energy-requiring enzymatic mechanism (8, 14).

SUMMARY

Mitochondrial supernatants of rat liver were incubated with ^{14}C amino acids and supplied with energy. The incorporation into individual proteins was analyzed by means of immuno-diffusion techniques in agar, combined with autoradiography. High labeling was observed in serum protein–like antigens extracted from the isolated microsomes with detergents. Less radioactivity was found in the precipitates formed by other microsomal antigens. No radioactivity could be detected in the antigens present in the cell sap, except in connection with disintegration of the microsomes, after prolonged incubation. In similar systems containing isolated *ribonucleoprotein particles* and cell sap an incorporation into several serum protein–like antigens was also observed.

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