# AUTORADIOGRAPHIC ANALYSIS ON AGAR PLATES OF ANTIGENS FROM SUBCELLULAR FRACTIONS OF RAT LIVER SLICES

W. S. MORGAN, M.D., P. PERLMANN, Ph.D., and T. HULTIN, Ph.D.

From the Wenner-Gren Institute for Experimental Biology, University of Stockholm, Stockholm, Sweden. Dr. Morgan's permanent address is Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts

## ABSTRACT

Slices of rat livers were incubated with $^{14}C$ amino acids, homogenized, and subjected to differential centrifugation. The microsomes were further extracted with the non-ionic detergent Lubrol W and with EDTA. These extracts and the microsome free "cell sap," freed from the pH 5 precipitable fraction, were subsequently reacted with antisera using agar diffusion techniques. The antisera employed were obtained from rabbits injected with different subcellular fractions of rat liver or with rat serum proteins. When the agar diffusion plates were autoradiographed it was found that some of the precipitates were radioactive while others were not. Control experiments indicated that this labeling was due to the specific incorporation of $^{14}C$ amino acids into various rat liver antigens during incubation of the slices rather than to a non-specific adsorption of radioactive material to the immunological precipitates. When the slices were incubated with the isotope for up to 30 minutes, the serum proteins which could be extracted from the microsomes with the detergent were strongly labeled, as were a number of additional microsomal antigens of unknown significance. In contrast, the serum proteins present in the cell sap were only weakly labeled. Most of the typical cell sap proteins, both those precipitable and those soluble at pH 5, seemed to remain unlabeled. No consistently reproducible results were obtained with the EDTA extracts of the ribosomal residues remaining after extraction of the microsomes with the detergent. Incubation of the liver slices for longer periods (up to 120 minutes) led to a strong labeling of the serum proteins in the cell sap as well as to the appearance of labeling in additional cell sap proteins. The results are discussed with regard to the subcellular site of synthesis and the metabolism of the different antigens.

## INTRODUCTION

Experiments reported earlier (20, 21) indicated the feasibility of using a combination of immunological and autoradiographic methods in an analysis of the early pathways in protein metabolism. In essence, it was proposed to take advantage of the findings that soluble proteins derived from subcellular fractions of rat liver homogenates could be characterized immunologically by means of precipitin reactions in agar plates and to combine this method of protein identification with autoradiography in isotope incorporation experiments. The purpose of this report is to give a description of the distribution of radioactive label among the different antigens isolated from rat

liver slices which had been incubated with $^{14}C$ amino acids under various experimental conditions. A preliminary account of some of the results has been given elsewhere (22). A later paper will deal with a similar study made with cell free isotope incorporation systems.

## MATERIALS AND METHODS

ANTISERA: Rabbits were used as a source of antisera. *Microsomes* and soluble fraction (*cell sap*) of rat livers were prepared by homogenization, after perfusion with cold 1.1 per cent KCl, in 2.5 volumes of 0.25 M sucrose medium (see below). The unbroken cells, nuclei, and mitochondria were sedimented by centrifugation at 12,000 *g* for 10 minutes. The mitochondria free supernatant was then layered over 20 ml of 0.5 M sucrose medium and centrifuged for 90 minutes at 79,000 *g*. For removal of remaining microsomes, the supernatant was transferred to smaller tubes and centrifuged for 3 to 5 hours more at 105,000 *g*. The sediment was discarded and the supernatant (cell sap) was used for injection immediately after preparation. The sediment of the first high speed centrifugation (microsomes) was rinsed with medium, pooled, and suspended by homogenization in a few milliliters of sucrose medium. In addition, a *pH 5 precipitable fraction* of the cell sap was prepared essentially by the method of Hoagland *et al.*(10).

Microsomes, cell sap, and pH 5 fraction were emulsified with equal parts of Freund's adjuvant (5). Generally, each rabbit received three intramuscular injections (3 ml per injection) of these emulsions, given at intervals of 3 weeks. If the test sera were satisfactory, the animals were bled to death and the antisera prepared according to standard procedures. The antisera used in this study were each a pool of the serum from five rabbits, the animals of each pool having been injected with the same preparations, and their sera had good individual titers.

Antisera against *rat serum* and against *rat serum albumin* were obtained by a similar procedure with pooled rat serum or with a preparation of lyophilized serum albumin, prepared by ethanol fractionation (8). The preparation of antiserum against unfertilized sea urchin eggs, used for control purposes, has been described elsewhere (7).

ANTIGENS: Unlabeled rat liver antigens were prepared as earlier described (1). The preparation and properties of the extract of the unfertilized eggs of the sea urchin *Paracentrotus lividus* are given elsewhere (7).

The procedure for incubation of rat liver slices and the subsequent preparation of the test solutions were as follows. Male albino rats, fasted overnight, were killed by a blow on the head, and the livers were removed quickly without perfusion and placed in ice cold Krebs-Henseleit medium (14). 2 gm of liver slices, cut at a thickness of 0.3 mm (13), were incubated under 95 per cent $O_2$ + 5 per cent $CO_2$, with 9 ml of Krebs-Henseleit medium, 1 ml of 0.25 M glucose, and 3 $\mu$c each of L-[$^{14}C$]valine (6.5 mc/mmole), L-[$^{14}C$]leucine (6.0 mc/mmole), and L-[$^{14}C$]isoleucine (6.1 mc/mmole). The labeled amino acids had been supplied by the Radiochemical Centre, Amersham, England. Incubation was carried out in a water bath at 37°C with constant shaking. After incubation for various periods of time, the flasks were placed on ice, and all subsequent procedures were performed at 0 to 2°C. The medium bathing the slices was poured off. The slices were rinsed three times with cold Krebs-Henseleit medium and then three times with 0.25 M sucrose containing 0.025 M KCl, 0.01 M $MgCl_2$, and 0.035 M tris buffer (tris[hydroxymethyl]aminomethane), pH 7.8. Next, the slices were homogenized in a motor driven Potter-Elvehjem homogenizer in 2.5 ml of the sucrose medium. The homogenates were freed from debris, nuclei, and mitochondria by centrifugation as described above. They were then layered over 8 ml of 0.5 M sucrose medium and centrifuged for 90 minutes at 105,000 *g*. The supernatant (cell sap) was saved frozen for later use. Before use, the pH 5 fraction (10) was removed by two cycles of precipitation at pH 5.2 and subsequent centrifugations for 20 minutes at low speed. 0.5 ml aliquots of the total homogenates and 1 ml aliquots of the cell saps were removed for determination of the specific radioactivities of the proteins as described elsewhere (13).

The sediments after ultracentrifugation (microsomes) were rinsed with the medium, pooled, and homogenized in 2.5 ml of a 1 per cent solution of the non-ionic detergent Lubrol W (cetyl alcohol polyoxyethylene condensate; see 6, 16). The insoluble portions of the microsomes were separated by centrifugation at 105,000 *g* for 2 hours. The soluble portion, in the following called *Lubrol extract*, was assumed to contain proteins solubilized from the microsomal membranes as well as possible contents of the microsomal vesicles (18, 21).

The pellet remaining after the Lubrol extraction (ribonucleoprotein particles and residual protein) was rinsed with medium and then homogenized in 2.5 ml of a 2 per cent solution of EDTA (ethylenediaminotetraacetate), containing 0.2 M $NaHCO_3$, pH 8.3 (12, 1). After standing in the refrigerator for several hours, it was centrifuged at 105,000 *g* for 2 hours and the soluble portion labeled *EDTA extract*.

Prior to being used in the immunological experiments, samples were taken from all solutions for determination of protein (15). As derived by the procedures described above, the cell sap usually

contained 6 to 9 mg protein/ml, whereas the Lubrol extract and the EDTA extract each contained 2 to 4 mg/ml.

IMMUNOLOGICAL EXPERIMENTS: Antigens and antisera were reacted by means of double diffusion in agar plates according to Ouchterlony (17), and by means of immuno-electrophoresis (9). Before use, the antisera against the various fractions of rat liver were absorbed by addition of lyophilized rat serum. After 2 hours at room temperature and incubation in the refrigerator overnight the precipitate was removed by centrifugation. This procedure was repeated once. Usually, 1 to 2 mg rat serum/ml antiserum were enough for complete absorption of antibodies against the serum proteins.

The methods for the double diffusion experiments have been described earlier (19, 9). However, when radioactive antigen solutions were employed, a special procedure was adopted in order to get as strong precipitates as possible. Thus, the reagents were placed in plastic containers, 1 cm in length and width and 0.5 cm high. The containers had a bottom and were lacking side walls only in those directions where diffusion was supposed to take place. The containers were filled with the reagents and the plates incubated at 37°C. After a few hours, all solutions were removed by suction through a pipette and the containers refilled immediately with fresh antigen solutions and antisera. This was repeated five or six times. In this way, large amounts of antigen and antibodies could be added without at the same time changing their concentrations in the containers. Most of the artifacts otherwise appearing upon repeated fillings of the basins could thus be avoided. After 2 days at 37°C the Petri dishes were kept in the refrigerator for 2 additional weeks. Further processing took place when it was ascertained that the antigen-antibody precipitates had become stationary. In order to remove radioactive contaminations, the plates were washed for 2 days in 0.9 per cent saline, for 4 days in running water, and for 3 additional days in distilled water. The glass plates on the bottom of the Petri dishes, with the agar on top, were taken out and the precipitates were photographed. The agar was then dried to a thin film, firmly adhering to the glass plate.

AUTORADIOGRAPHS were obtained by covering the agar plate with an x-ray film (Ilford "Ilfex") and a second glass plate. The glass plates were held together with clamps, wrapped in alumina foil, and exposed in complete darkness for various periods of time. The autoradiographs were developed in the dark with Gevaert x-ray developer (G 230).

## DESCRIPTION OF THE REAGENTS

THE ANTISERA: As previously described, injection into rabbits of rat liver microsomes or cell sap leads to the production of antisera possessing a fair degree of "fraction specificity" (1, 19, 21). The patterns of precipitates obtained with the antimicrosomal and the anti–cell sap sera of the present study are seen below in Figs. 4 and 5. These figures show that there occurred both antibodies against antigens which were common for microsomal extract and cell sap, and others which were typical for either one of these fractions. None of the precipitates seen in these figures is due to reactions between serum proteins and their antibodies, since the latter had been removed by previous absorption. For further details see references (1, 19, 21).

In addition to these two antisera, one against the pH 5 precipitable fraction of the cell sap was used. This fraction is known to contain the soluble ribonucleic acid and the amino acid–activating enzymes (10, 11). As will be seen from Fig. 1, injection of this fraction into rabbits gives rise to the production of precipitating antibodies against at least four antigens which are specific for this fraction. (Previous findings suggested that

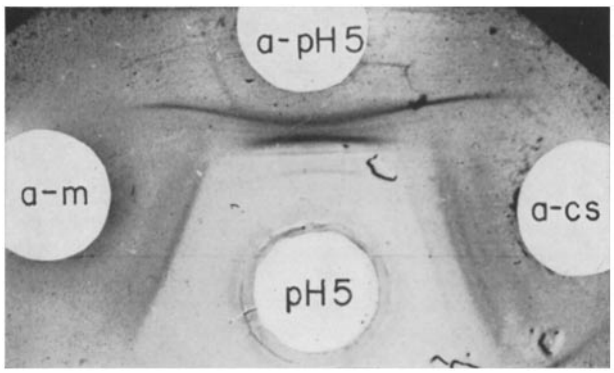


FIGURE 1

Photograph of Ouchterlony plate. *pH 5*, fraction of cell sap precipitated at pH 5.2 and redissolved; *a-pH 5*, antiserum against this fraction; *a-cs*, antiserum against cell sap; *a-m*, antiserum against microsomes. Before use, all antisera were absorbed with lyophilized rat serum.

antisera against the total cell sap did not contain antibodies against antigens of the pH 5 fraction (21).)

The antiserum against rat serum contained precipitating antibodies against at least fifteen of the rat serum proteins. The antiserum against the serum albumin was heterogeneous. As can be seen from Fig. 2, it contained high concentrations of antibodies against albumin (giving the strong precipitate on the anodic side of the plate) and against one or two $\beta$-globulins. In addition, there were low concentrations of antibodies against at least two $\alpha$-globulins. (The weak precipitates formed by these antibodies are not clearly visible in Fig. 2.)

THE ANTIGENS: The properties of the antigen solutions were those described in earlier papers, although it should be pointed out that the difference between cell sap and microsomal extracts was less sharp than usual, since the homogenates were made in isotonic and not in hypertonic sucrose, and the supernatants were used without

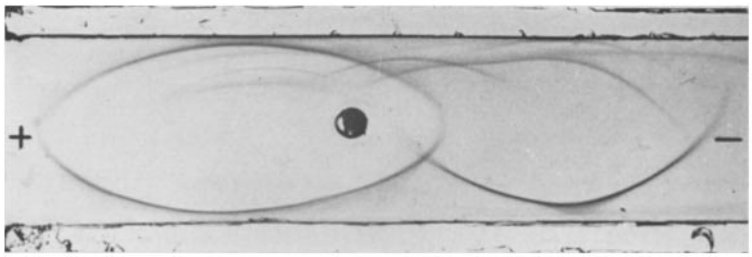


FIGURE 2

Photograph of immuno-electrophoresis of rat serum, diluted 1:2 with physiological saline. After completion of electrophoresis (17 hours, barbiturate buffer pH 8.2, $\mu$ = 0.05, 0.4 ma/cm cross-section, agar layer 2 mm high), antiserum against rat serum was added from the upper longitudinal basin and antiserum against serum albumin (rat) from the lower basin.

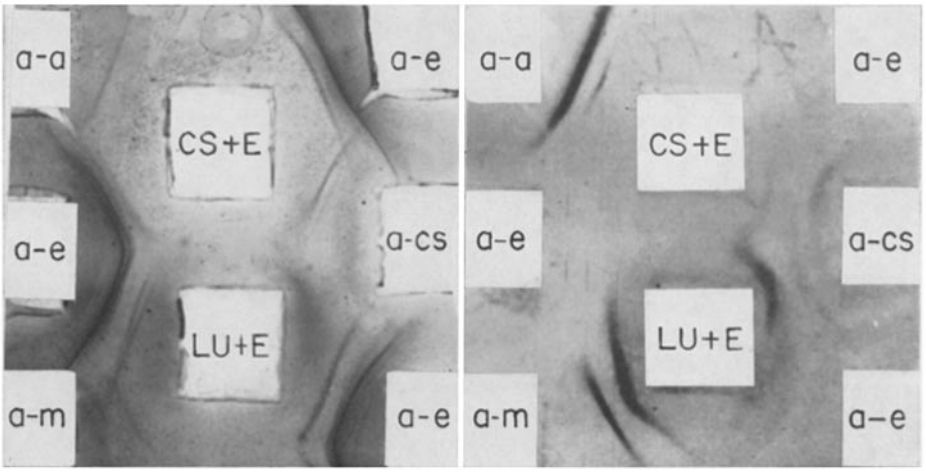


FIGURE 3 *a*

FIGURE 3 *b*

FIGURE 3

Fig. 3 *a* photograph, Fig. 3 *b* autoradiograph of Ouchterlony plate. *CS+E*, cell sap of rat liver and extract of sea urchin eggs, mixed 1:1; *LU+E*, Lubrol extract of rat liver microsomes and extract of sea urchin eggs, mixed 1:1. Cell sap and Lubrol extract were obtained from a homogenate of rat liver slices incubated for 60 minutes with $^{14}C$ amino acids. The radioactivity of the total homogenate was 432 counts/min/mg protein and that of the isolated cell sap was 202 counts/min/mg protein. *a-e*, antiserum against sea urchin eggs; *a-a*, antiserum against rat serum albumin; *a-cs*, antiserum against rat liver cell sap; *a-m*, antiserum against rat liver microsomes. Before use, *a-cs* and *a-m* were absorbed with lyophilized rat serum. Exposure time of autoradiograph, 50 days.

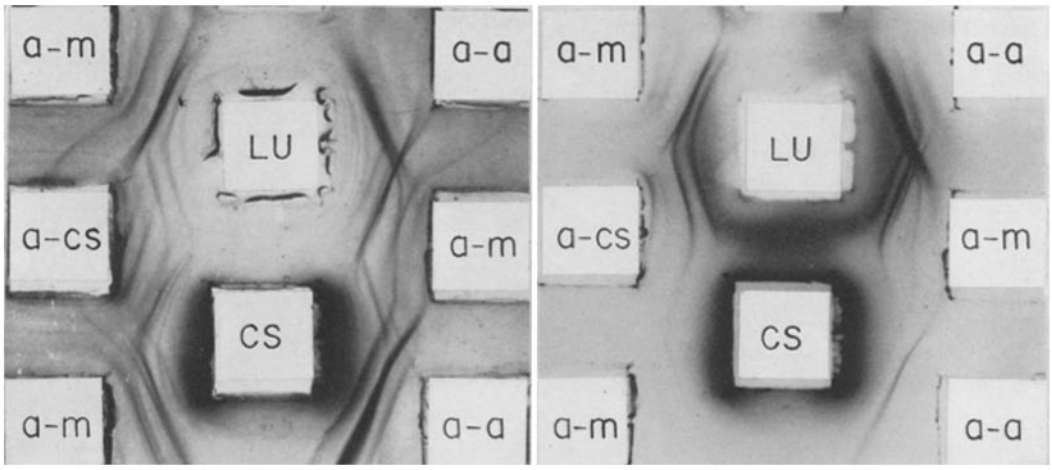


FIGURE 4 *a*

FIGURE 4 *b*

FIGURE 4

Fig. 4 *a*, photograph, Fig. 4 *b* autoradiograph of Ouchterlony plate made with Lubrol extract of microsomes (*LU*) and cell sap (*CS*) of a homogenate of rat liver slices. Prior to homogenization, the slices were incubated for 30 minutes with $^{14}C$ amino acids. The radioactivity of the total unfractionated homogenate was 425 counts/min/mg protein and that of the cell sap 311 counts/min/mg protein. The protein concentration of the Lubrol extract was 2.73 mg/ml and that of the cell sap 6.0 mg/ml. *a-a*, antiserum against rat serum albumin; *a-m*, antimicrosomal serum; *a-cs*, anti-cell sap serum. Before use, *a-m* and *a-cs* were absorbed with lyophilized rat serum. Exposure time of autoradiograph, 40 days.

prolonged ultracentrifugation. Moreover, it will be noted that the cell saps contained considerable amounts of serum proteins. In previous experiments, when the livers were fractionated immediately after perfusion, the cell sap seemed to contain only relatively small amounts of these proteins (1, 21). In the present case, the homogenates were made from slices of unperfused livers incubated for various periods of time. This procedure apparently led to a higher content of serum proteins in the cell sap (see however 24). The EDTA extracts of the ribonucleoprotein part of the microsomes were similar to those previously tested. They contained small amounts of contaminating proteins, belonging to the Lubrol extract, and a number of typical antigens which could only be visualized when the extracts were reacted with antiserum against the ribosomal part of the microsomal fraction (1, 21).

## THE SPECIFICITY OF THE LABELING OF THE PRECIPITATES

As previously reported, autoradiographs made from Ouchterlony plates obtained with radioactive antigen solutions show a distinct labeling of the precipitates (20). The question then arises whether this label represents a true radioactivity of the antigens in the various precipitates, or whether it merely indicates that radioactive material has been adsorbed non-specifically to the precipitates. As a rule, dilution of the slices after homogenization with an excess of unlabeled amino acids did not change the results. However, other workers have observed that radioactive material, occurring in an homogenate of the present type, may become adsorbed non-specifically to unrelated antigen-antibody precipitates, when the precipitin reactions are performed in tubes (2, 4, 23). However, previous addition of unrelated precipitin systems did not noticeably change the radioactivity of the precipitates formed subsequently with the homologous antisera, when the precipitin reactions were performed in agar plates.

The specificity of the labeling of precipitates is also illustrated by the experiment of Fig. 3. In this experiment, extracts from radioactive liver slices were mixed with an unrelated antigen solution, an extract of unfertilized sea urchin eggs. This extract contained a great number of protein antigens (7) which did not cross-react with antibodies against rat antigens. In the agar

plate of Fig. 3 *a*, this mixture was allowed to react either with anti-sea urchin serum or with the various anti-rat sera. As can be seen, the labeling of the precipitates was highly specific. However, in a few controls, where the specific radioactivity of the proteins in the antigen solutions was unusually high, a nonspecific labeling of unrelated precipitates was noted exceptionally. This non-specific label was always so weak that it could not be made visible in the photographic print. In any event, this means that one has to be careful in drawing conclusions from precipitates which carry a weak label in the autoradiograph, particularly if the specific radioactivity of the total protein in the solutions is high.

The pH 5 precipitable fraction of the cell sap from incubated liver slices has a fairly high isotope content. This fraction contains unstable components which readily precipitate around the basins with cell sap, when the Ouchterlony plate is incubated at 37°C. The radioactive precipitate

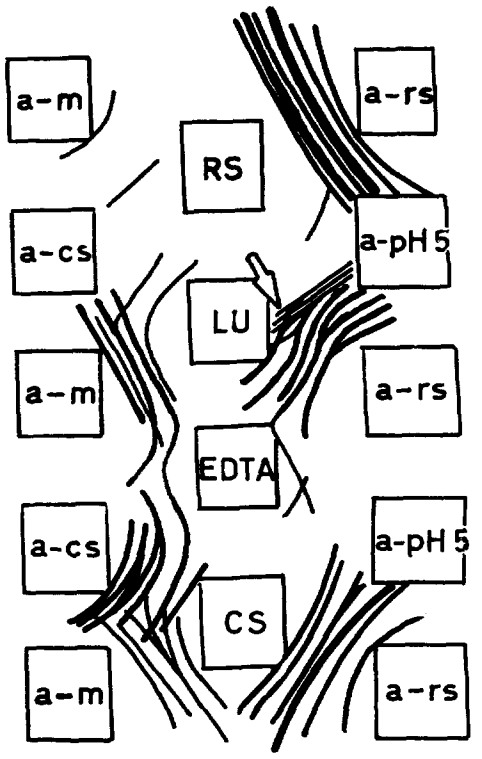


FIGURE 5

Diagram of typical Ouchterlony plate made with the fractions of a homogenate of rat liver slices. *RS*, rat serum, diluted 1:10 with physiological saline; *LU*, Lubrol extract of microsomes; *EDTA*, EDTA extract of ribonucleoprotein part of the microsomes; *CS*, cell sap; *a-rs*, antiserum against rat serum; *a-pH 5*, antiserum against pH 5 precipitate of cell sap; *a-m*, antimicrosomal serum; *a-cs*, anti-cell sap serum. Before use, all antiliver sera were absorbed with lyophilized rat serum. For further explanations see text and legends to Figs. 6 *a to d*.

FIGURE 6

Autoradiographs of Ouchterlony plates made with fractions of homogenates of rat liver slices. Before homogenization, the slices were incubated with $^{14}C$ amino acids for various periods of time. For explanation of symbols see legend to Fig. 5.

FIG. 6 *a*. Incubation, 15 minutes. Radioactivity: total unfractionated homogenate, 135 counts/min/mg protein; cell sap, 68 counts/min/mg protein. Protein: *LU*, 3.20 mg/ml; *CS*, 4.60 mg/ml; *EDTA*, 2.15 mg/ml. Exposure time of autoradiograph, 90 days.

FIG. 6 *b*. Incubation, 30 minutes. Radioactivity: total unfractionated homogenate, 402 counts/min/mg protein; cell sap, 280 counts/min/mg protein. Protein: *LU*, 2.90 mg/ml; *CS*, 7.80 mg/ml; *EDTA*, 4.04 mg/ml. Exposure time of autoradiograph, 42 days.

FIG. 6 *c*. Incubation, 60 minutes. Radioactivity: total unfractionated homogenate, 478 counts/min/mg protein; cell sap, 380 counts/min/mg protein. Protein: *LU*, 4.80 mg/ml; *CS*, 4.36 mg/ml; *EDTA*, 1.96 mg/ml. Exposure time of autoradiograph, 42 days.

FIG. 6 *d*. Incubation, 120 minutes. Radioactivity: total unfractionated homogenate, 2190 counts/min/mg protein; cell sap, 1510 counts/min/mg protein. Protein: *LU*, 2.00 mg/ml; *CS*, 4.08 mg/ml; *EDTA*, 3.74 mg/ml. Exposure time of autoradiograph, 21 days.

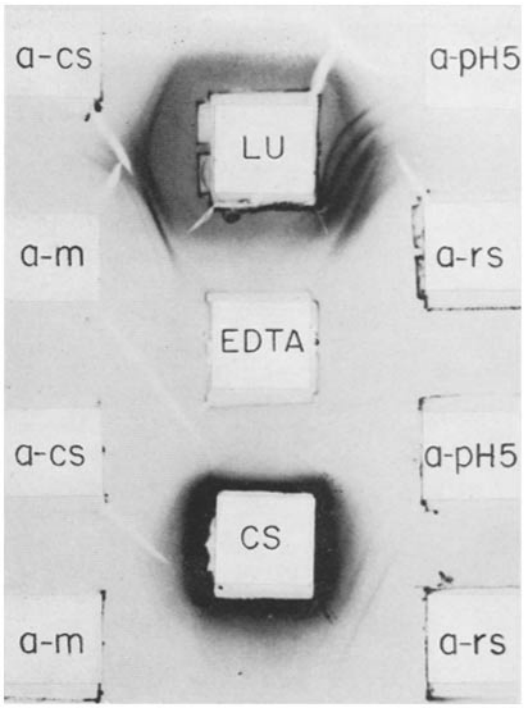


FIGURE 6 *a*

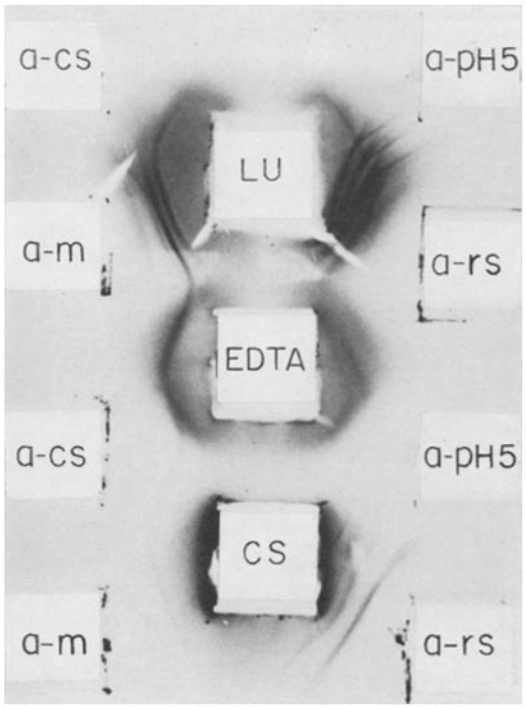


FIGURE 6 *b*

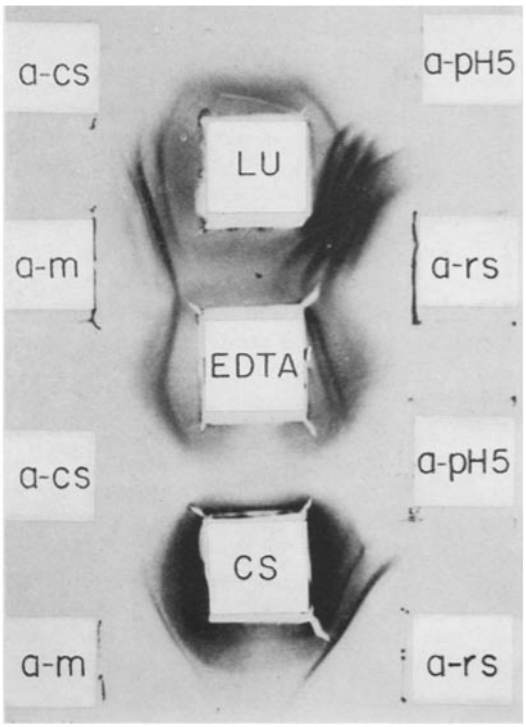


FIGURE 6 *c*

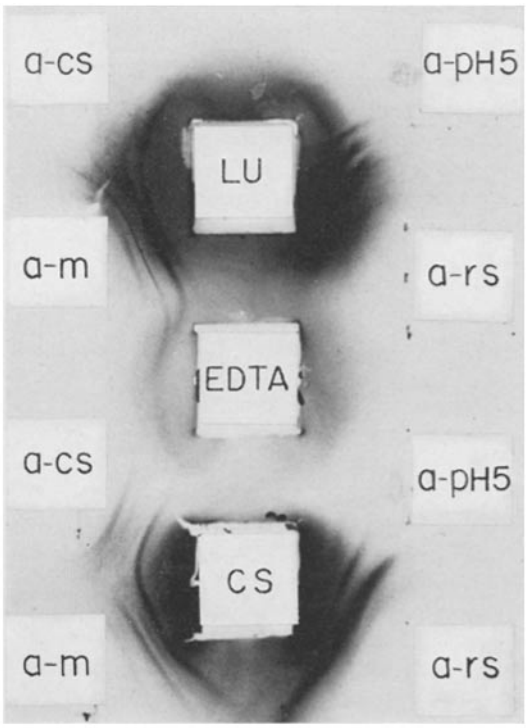


FIGURE 6 *d*

cannot be properly washed out from the agar. An unspecific labeling of precipitation lines may therefore take place in this zone, and the results may also be otherwise obscured. Therefore, the pH 5 fraction was always removed in advance by repeated precipitation at pH 5.2. In this way, the unspecific blackening of the x-ray film around the cell sap basins was considerably reduced. When the pH 5 fraction was resuspended and reacted with its homologous antiserum, the precipitates which appeared were only weakly labeled.

## THE LABELING OF ANTIGENS IN DIFFERENT FRACTIONS

When fractions derived from liver slices, incubated as described above, are reacted with antisera and autoradiographed, an over-all pattern of the isotope distribution among the immunological precipitates is obtained. Fig. 4 may serve as an example. Fig. 4 *a* represents a photograph of an Ouchterlony plate, made with cell sap and the Lubrol extract from the homogenate of slices, incubated with isotope for 30 minutes, and subsequently reacted with various antisera.

It is apparent from Fig. 4 that both the Lubrol extract and the cell sap contained antigens reacting with the anti-albumin serum. Fig. 4 *a* indicates that the microsomal extract formed at least four precipitates with this serum. Immuno-electrophoretic evidence indicated that the precipitate closest to the antigen container was that of the $\beta$-globulin, followed by the albumin. (Although, in the print, these two precipitates seem to form one line, they appeared as two distinct lines in the original plate.) Two precipitates formed by $\alpha$-globulins were closer to the antiserum basin. They were very weak and can hardly be seen in the print (Fig. 4 *a*). Only three of these precipitates appeared between the cell sap and an aliquot of the same antiserum. As is seen from Fig. 4 *a*, the precipitates of the $\beta$-globulin and the albumin from the Lubrol extract joined with those from the cell sap. Fig. 4 *b* shows that all four precipitates of the serum protein–like antigens from the Lubrol extract were distinctly labeled. In fact, the precipitates of the two $\alpha$-globulins appear much stronger in Fig. 4 *b* than in Fig. 4 *a*. In contrast, only one of the three precipitates formed by the serum protein–like antigens of the cell sap, that of the serum albumin, carried a clearly distinguishable label. The blackening of this line was less than that of the corresponding Lubrol precipitate. (This could be seen in the original autoradiograph but is not quite so apparent in the print.) The other two were very weak and could hardly be seen in the original autoradiograph.

Fig. 4 *a* also demonstrates that the Lubrol extract and the cell sap contained both antigens in common and a number of "fraction specific" antigens, immunologically different from one another and from the serum protein–like antigens described above. In regard to the Lubral extract, the autoradiograph (Fig. 4 *b*) reveals that at least three of the microsomal precipitates were strongly labeled. It should be noted that the blackening of the lines in the autoradiograph is not correlated with the density of the precipitates in Fig. 4 *a*. In contrast again, the precipitates formed by the cell sap, although numerous and heavy, carried only a very weak label or none at all.

## THE LABELING OF THE PRECIPITATES AS A FUNCTION OF THE TIME OF INCUBATION

In the next series of experiments, the fractions from four different homogenates of slices, incubated with isotope for various periods of time, were reacted, on separate agar plates, with aliquots of a number of antisera. The protein concentrations of the corresponding antigen solutions were about equal and the immunological reactions were performed under closely similar conditions. For identification of the serum protein–like antigens, an antiserum against total rat serum was used in this series. For control, an unlabeled rat serum was added from a separate container on each agar plate.

Fig. 5 represents a diagram of the Ouchterlony reactions obtained with the fractions of one of the homogenates (homogenate incubated for 15 minutes; see Fig. 6 *a*). This diagram is typical for the whole series. The small differences which existed between the patterns of the different plates are of no significance for the present discussion. It should be noted again that the Lubrol extract of the microsomes, in addition to its specific antigens, contained a considerable number of serum protein–like antigens. The serum proteins, diffusing from the adjacent receptacle containing rat serum, caused a certain bending of the precipitates and thus facilitated their identification. The spurs indicated by the arrow

in Fig. 5 were probably formed by serum proteins which were present in the serum, but absent in the Lubrol extract. The cell sap gave a smaller number of precipitates with this antiserum. It reacted well with the antimicrosomal serum and also contained a good number of antigens reacting only with antibodies in the anti-cell sap serum. The EDTA extract of the microsomes gave two precipitates with the antimicrosomal serum and only a few, faintly visible precipitates with the other sera.

Figs. 6 *a* to 6 *d* are prints of the autoradiographs obtained from the four plates just described. Since the precipitates obtained with the unlabeled rat serum did not appear in the autoradiograph, they have been omitted from these figures. It is evident from Fig. 6 *a* that the serum protein-like antigens in the Lubrol extract, as well as other microsomal antigens, already carried a heavy label after 15 minutes of incubation with the isotope, whereas the radioactivity of the cell sap and the EDTA-extracted antigens was hardly detectable. The picture obtained after 30 minutes of incubation (Fig. 6 *b*) is essentially the same as in the experiment shown in Fig. 4 *b*. It can be seen that a great number of labeled spurs were here formed by the precipitates of the serum protein-like antigens of the Lubrol extract. This indicates that these four or five precipitates were highly heterogeneous, each consisting of several antigen-antibody systems superimposed upon one another. It should be mentioned that these spurs could not be seen in any of the original agar plates. In contrast, the spurs formed by the unlabeled serum proteins (designated with arrow in Fig. 5) are not visible at all in the autoradiographs. After 60 minutes and 120 minutes of incubation (Figs. 6 *c* and 6 *d*), a greater number of more strongly labeled precipitates appear in all fractions. Thus, while strongly labeled precipitates of the serum protein-like antigens occur in all four Lubrol extracts, correspondingly labeled precipitates of these proteins now also appear in the cell sap. Moreover, a sufficiently long period of incubation also leads to a labeling of cell sap antigens, not visible at all in Figs. 6 *a* and 6 *b*. A thorough inspection of the original autoradiographs revealed that several of these labeled precipitates were

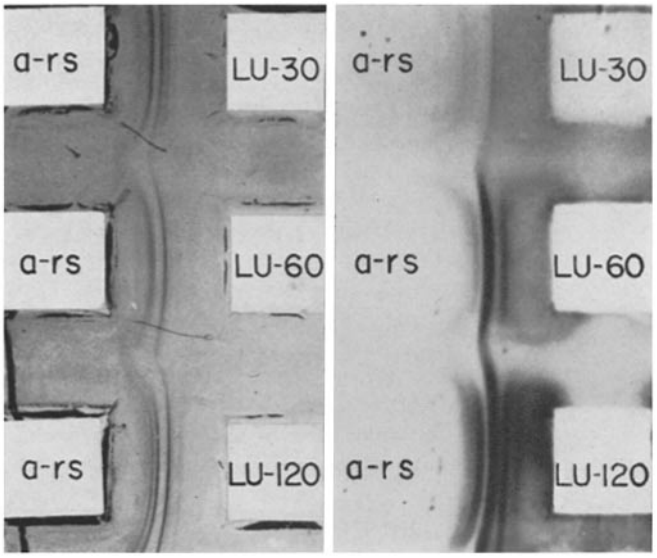


FIGURE 7 *a* FIGURE 7 *b*

FIGURE 7

Fig. 7 *a* photograph, Fig. 7 *b* autoradiograph of Ouchterlony plate made with Lubrol extracts of microsomes from three homogenates of rat liver slices, and antiserum against rat serum (*a-rs*). *LU-30*, *LU-60*, *LU-120*, extracts from slices incubated with $^{14}C$ amino acids for 30, 60, and 120 minutes, respectively. The radioactivities of the corresponding unfractionated homogenates were 340, 690, and 2090 counts/min/mg protein, respectively. The protein concentrations of the Lubrol extracts were 2.58, 1.74, and 2.54 mg/ml, respectively. Exposure time of autoradiograph, 40 days.

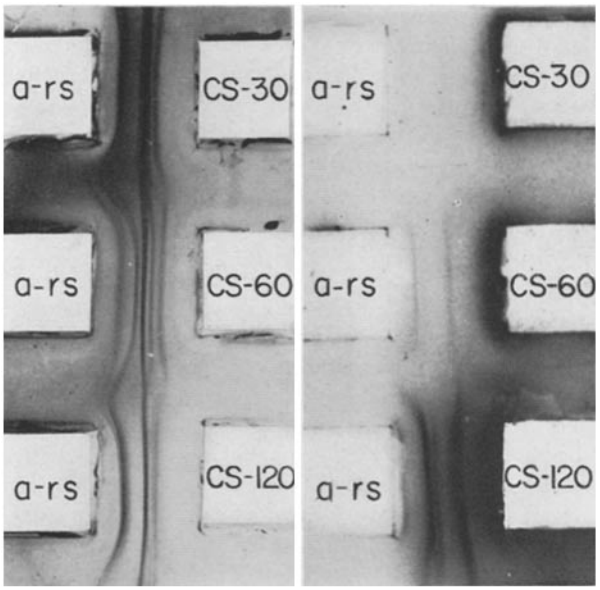


FIGURE 8 *a*

FIGURE 8 *b*

FIGURE 8
Fig. 8 *a* photograph, Fig. 8 *b* autoradiograph of Ouchterlony plate made with cell saps of the three homogenates of Fig. 7, and antiserum against rat serum (*a-rs*). *CS-30, CS-60, CS-120,* cell saps from slices incubated with $^{14}C$ amino acids for 30, 60, and 120 minutes, respectively. The radioactivities of the cell saps were 220, 348, and 1715 counts/min/mg protein, respectively. The protein concentrations of the cell saps were 8.07, 7.20, and 7.35 mg/ml, respectively. Exposure time of autoradiograph, 40 days.

formed by antigens which only reacted with antibodies in the anti-cell sap serum, crossing the precipitates formed between the cell sap and the antimicrosomal serum (see also Fig. 5). These details cannot be seen in the print of this autoradiograph (Fig. 6 *d*).

In some of the autoradiographs, the EDTA extracts showed a number of labeled lines which appeared much more distinct than in the original agar plates. However, this phenomenon was not always observed, and the labeled precipitates were very likely formed by antigens not satisfactorily removed by the extraction of the microsomes with detergents, or were present due to unspecific adsorption occurring during fractionation. The labeled antigen reacting with antibody in the anti-pH 5 serum seems to be a microsomal antigen, different from the serum proteins. Its precipitate was so faint that it could not be distinguished in the original agar plates (Fig. 5).

In this series, the time of exposure of the autoradiographs had been varied inversely with the time of incubation of the slices with isotope. In another series of exposures, autoradiographs of the same agar plates were made by exposing them all for 120 days. The number of lines appearing in these autoradiographs was the same as that shown in Fig. 6. However, the high degree of blackening, both of the lines and of the background, of the autoradiographs from the homogenates of Figs. 6 *c* and 6 *d*, made them unsuitable for reproduction.

The more intense blackening of lines after long incubations, as compared with that after short incubations, was apparently not related to an increase in concentration of the antigens in question. Thus, in the series of Fig. 6 the position of most of the precipitates between antigen and antibody containers was very nearly the same for any given system, regardless of the time of incubation. In order to investigate this point further, additional experiments were set up as illustrated in Figs. 7 and 8. Slices incubated for three different periods of time were used in these experiments. Fig. 7 shows the photograph and the autoradiograph obtained when the three Lubrol extracts were reacted on an agar plate with aliquots of antiserum against rat serum. Fig. 8 shows the corresponding prints obtained with the cell saps from the same homogenates. The parallel arrangement of the containers allowed a better estimation of the relative concentrations of the individual antigens. Model experiments, made with dilution series of unlabeled extracts, showed that, in the concentration ranges valid here, an increase of the original concentration of an antigen by a factor of 2 resulted on the average in a displacement of 1.5 mm of its precipitate toward the antiserum container. As will be seen from the autoradiographs, there was no correlation between

the blackening of the lines and the concentration of the antigens in each of the three Lubrol extracts or cell saps. The photographs of the original agar plates of these experiments also indicate that the density of corresponding precipitates was very similar in all three Lubrol extracts and all three cell saps. It can be assumed that the intracellular concentration of the serum protein–like antigens is maintained at an approximately constant level through their continuous secretion to the medium (24).

## DISCUSSION

Within certain limits, the blackening of a given area in an autoradiograph of an Ouchterlony plate may be assumed to be proportional to the radioactivity in the corresponding area of the agar film. However, no direct conclusions with regard to the specific radioactivity of an antigen can be drawn from autoradiographs of its precipitates. Even if an element of non-specific adsorption of labeled components to the precipitates can be excluded, the blackening of individual lines in an autoradiograph of an Ouchterlony plate is a function of (*a*) the specific radioactivity of the antigens, (*b*) the concentration of the antigen in the precipitate, and (*c*) the amount of precipitate in the area under consideration. Evidently, these parameters will be widely different for different precipitates, depending on such variables as the chemical nature and the turnover rates of the antigens, their antibody combining ratios, the immunological homogeneity of the precipitate, its physical nature, the total amount of antigen and antibody added, and the ratio of their initial concentrations.

In spite of these limitations, certain conclusions can be drawn regarding immunologically and chemically identical antigens occurring in different subcellular fractions of the same homogenate and reacted in a single plate with aliquots of the same antiserum. Thus, serum albumin from microsomes, cell sap, or rat serum gives a single precipitate without spurs when tested with anti-albumin serum in an Ouchterlony plate. This suggests that the albumins from different sources are immunologically identical and combine with the antibodies in an identical fashion. (They can be shown to be chemically similar; see 3, 4, 24.) In the experiment of Fig. 4 *a*, the position of the albumin precipitate between antigen and antiserum containers indicated that the albumin occurred in a somewhat higher concentration in the cell sap than in the microsomal extract. In addition, the corresponding precipitate of the cell sap manifested a typical broadening due to a certain antigen excess. One would expect, therefore, that in the agar plate the actual amount of albumin was at least as large in the albumin precipitate of the cell sap as in that of the microsomal extract. Despite this, the albumin precipitate of the microsomal extract gave rise to a much more intense blackening in the autoradiograph than did that of the cell sap (Fig. 4 *b*). This strongly suggests that, under the conditions of this experiment, the serum albumin solubilized from the microsomes had a higher specific radioactivity than that in the cell sap.

The experiments with antiserum against rat serum (Figs. 6 *a* to 6 *d*) confirmed these results and showed that the same was true for a great number of additional serum proteins. It should be noted that, in this series, the time of exposure of the autoradiographs had been varied inversely with the time of isotope incorporation of the slices. In spite of this, the precipitates of the serum protein–like antigens from the cell sap appearing after long-time incubation gave a much stronger blackening of the autoradiographs than did those of the short-time experiments. This suggests that the appearance of radioactive serum proteins in the cell sap was connected with a considerable time lag. There was no evidence of such a lag in the microsomal extracts (see 23, 24).

The results are consistent with the interpretation that the specific radioactivity of the serum proteins in the soluble fraction increases when freshly synthesized molecules are transferred from the microsomes to this fraction. However, the lower specific activity of serum albumin and other serum proteins in the cell sap could also be due to a larger pool size of these proteins in the latter fraction. A rough estimate of the *original* concentration of serum albumin in the cell sap used in the experiment of Fig. 4 *a* showed that it contained 3 to 5 times more albumin than the correspondingly diluted microsomal extract. Model experiments suggested that this difference was not enough to account for the differences which were observed in the autoradiographs. This speaks against the idea that differences in pool size might be responsible for the difference in the labeling pattern of albumin in the microsomal and cell sap fractions.

Essentially similar results were obtained with a number of additional antigens extracted from the microsomes. The nature of these is unknown. Some may very well be proteins which are secreted by the liver. This may be valid particularly for one of the antigens which was common to microsomal extracts and cell sap and which was strongly labeled in all Lubrol extracts but in the cell sap only after a long period of incubation (Fig. 6).

In contrast, there occur a great number of precipitates, particularly certain ones derived from the cell sap, which appear not to be labeled at all after a short period of incubation. That these antigens are proteins is known from various chemical experiments (1, and unpublished experiments). However, when the isotope incorporation is studied as a function of time, it is seen that the specific radioactivity of certain cell sap antigens increases upon sufficiently long incubation of the slices. Their precipitates then appeared darker in the autoradiographs although their concentrations in the solutions remained essentially unchanged. It would be tempting to interpret this as meaning that the incorporation of $^{14}C$ amino acids into these antigens proceeds at a slower rate than that into the serum proteins. It must be emphasized, however, that such a conclusion cannot be strictly substantiated from the experimental evidence.

So far, attempts to obtain radioactive precipitates with the pH 5 precipitable fraction of the cell sap have been without success. This may partly be due to the great lability of this fraction. However, it could also be expected that most of the radioactive amino acids present in this fraction were not incorporated into its antigenic proteins. Some of these are supposed to correspond to amino acid–activating enzymes (unpublished experiments).

As already pointed out, the labeled precipitates which were sometimes formed by the EDTA extracts were most likely due to the presence therein of contaminating antigens typical for the Lubrol fraction. Although proteins such as the serum proteins are supposed to be synthesized by the ribosomes, freshly formed molecules probably do not remain attached to the particles long enough to become detectable in the EDTA extracts, or can only be removed from the particles by other means. It should be pointed out, however, that the total trichloracetic acid precipitable protein of these extracts often was of a high specific radioactivity. This indicates that the highly labeled proteins in the ribosomal extracts are of low antigenic activity. So far, no labeling has been detected of the antigens considered typical for the EDTA extracts (1). The nature of these antigens is unknown.

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