

Albumin
Macrophage
Formaldehyde

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Proteolysis of Formaldehyde-treated Albumin in Kupffer Cells and Its Inhibition by Suramin¹

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The uptake and breakdown of formaldehyde-treated ¹³¹I- or ¹²⁵I-labeled albumin in liver lysosomes have been measured *in vivo*. Ultracentrifuge studies and gel filtration showed that, in contrast to heat-denatured albumin frequently used in clearance experiments, our formaldehyde-treated protein did not form aggregates. Nevertheless, it was exclusively taken up in the Kupffer cells as demonstrated by autoradiography.

The *in vivo* breakdown of the protein in the Kupffer cells was inhibited by suramin. The results of determinations of the drug in liver fractions were in agreement with a lysosomal localization. Histochemical staining showed a granular accumulation of suramin in Kupffer cells only.

Our results indicate that (1) the hypothesis that denatured serum proteins are rapidly taken up by the reticuloendothelial system because they form aggregates is not tenable; (2) the many reports in literature on intralysosomal breakdown of formaldehyde-treated albumin *in vitro* and its inhibition by suramin apply exclusively to Kupffer cell lysosomes.

Formaldehyde-treated albumin is rapidly taken up from the blood and accumulated in a particulate liver fraction (Mego and McQueen, 1965a). Upon incubation of this fraction *in vitro* degradation products are released into the medium. This system is often used as a model for intralysosomal proteolysis. After preloading the liver with the trypanocide suramin (synonyms: Bayer 205, Germanin), Davies, Lloyd, and Beck (1971) measured a decrease of the release of degradation products in this *in vitro* system.

Liver contains two major types of endocytosing cells, namely, hepato-

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cytes and Kupffer cells, occupying about 87% and 2% of the adult rat liver volume, respectively (Greengard, Federman, and Knox, 1972). The lysosomes of the hepatocytes, which probably form the overwhelming majority of liver lysosomes, differ from the lysosomes of Kupffer cells in relative enzymatic content (Wattiaux *et al.*, 1956). It is, therefore, of interest to know which of the lysosomal populations of liver is represented by the *in vitro* system described above.

Many investigators have presented evidence that aggregates of heat-denatured serum proteins are taken up by cells of the reticuloendothelial system and by polymorphonuclear phagocytes (Benacerraf *et al.*, 1957; Bocci *et al.*, 1968; Kirsch *et al.*, 1972) and Schultze and Heremans (1966) stated that "denaturation presumably favors incorporation because it leads to the formation of aggregates. . . ."

As shown below, the modified albumin used in studies on intralysosomal proteolysis *in vitro* does not form aggregates. We have investigated the cellular and subcellular localization of this nonaggregated protein. In addition we have determined the localization of suramin and studied its effect on the breakdown of the protein *in vivo*.

MATERIALS AND METHODS

Materials

Bayer 205 (symmetrical ureide of *m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-naphthylamino-1-sodium-trisulfonate-4,6,8) was a gift from Bayer, Leverkusen, German Federal Republic. *N*-(1-naphthyl)ethylenediamine dihydrochloride was purchased from Merck, Darmstadt, German Federal Republic. ¹³¹I-Labeled human serum albumin with a specific radioactivity of about 18 μ Ci per mg of protein was obtained from Philips Duphar N.V., Petten, The Netherlands. (¹²⁵I)-iodide was purchased from The Radiochemical Centre, Amersham, England. Human serum albumin (Fraction V) was obtained from Serva, Heidelberg, German Federal Republic. Bovine serum albumin was obtained from Poviet Producten N.V., Amsterdam, The Netherlands. ¹²⁵I-Labeled albumin was prepared by the chloramine-T procedure according to Bocci (1969). The preparations had specific radioactivities of 50–60 μ Ci per mg of protein. Treatment with formaldehyde was carried out by the procedure described by Mego and McQueen (1965a). Before injection labeled protein was exhaustively dialyzed against 0.15 M NaCl. Denaturation by heating and solubilization of the complex was performed as described by Benacerraf *et al.* (1957).

Animals

Male Wistar rats of 200–240 g body weight were used. All injections were given in the caudal veins. Suramin (100 mg per ml in 0.15 M NaCl)

was administered at a dose of 250 mg per kg body weight. Controls received an equal volume of 0.15 M NaCl. The injected labeled albumin solutions contained approximately 0.8 mg of protein in a volume of 0.5 ml 0.15 M NaCl. At the time of injection the ¹³¹I-labeled albumin had a specific radioactivity of about 6 μ Ci per mg of protein.

Liver Fractionation

Livers used for fractionations had been perfused with 0.15 M NaCl until they had become yellowish. Fractionations were carried out as described by Bouma and Gruber (1966).

Measurement of Proteins and Enzymes

Protein was determined by the Lowry method. Cathepsin D was assayed by the method of Anson (1939) with acid-denatured hemoglobin as a substrate. Acid phosphatase was measured according to Gianetto and de Duve (1955) with β -glycerophosphate as a substrate.

Determination of Suramin

Samples of serum, liver homogenates, and liver fractions of suramin-treated rats were hydrolyzed in 6 M HCl at 110°C for 24 hr. The aromatic amines liberated in this way were diazotized and coupled to *N*-(1-naphthyl)ethylenediamine as described by Goldberg and Rutenburg (1958). Standard solutions of suramin were treated in the same way. The absorbance of the products formed was measured at 560 nm. Blank values were obtained using samples from control rats.

Assay of Radioactivity

Radioactivity was determined in serum, liver homogenates, and subcellular fractions, either as total counts or after precipitation with trichloroacetic acid (final concentration 10% (w/v)) in the supernatant fraction as well as in the precipitate. With serum, bovine serum albumin (final concentration 11 mg of protein per ml) was added as a carrier. Precipitates and fractions were completely solubilized in Soluene-100 (Packard Instruments International, Zürich, Switzerland). Samples were counted in a solution of 0.4% 2,5-diphenyloxazole and 0.008% *p*-bis-*o*-methylstyrylbenzene in toluene and ethylene glycol monomethyl ether (3:2 (v/v)) in a Nuclear Chicago Mark I liquid scintillation counter.

Autoradiography; Histochemical Staining of Suramin

For autoradiography liver pieces were fixed in a solution of 2% glutaraldehyde containing 0.1 M Na phosphate buffer, pH 7.4, for 24 hr at 4°C under continuous rotation. Tissue sections were covered with AR-10 strip-

ping film (Eastman Kodak Co., Rochester, NY). After exposure at 4°C for 6 weeks and development (Kodak D 19b developer) for 10 min at 18°C, sections were stained through the film with methylene-pyronin.

For histological demonstration of suramin small pieces of liver were placed in excess of May-Grünwald solution and processed according to Jancsó (1955).

RESULTS

Uptake and Degradation of Formaldehyde-treated Albumin; the Effect of Suramin

After injection of the labeled protein trichloroacetic acid-precipitable radioactivity disappeared rapidly from the blood in both suramin-treated and control rats, resulting in the elimination of about 90% of the dose within the first hour. In the controls as well as in the experimental animals about 30% of the dose was recovered in the liver at 0.5 hr. The radioactivity still present in blood cannot contribute significantly to this value since the livers taken at 0.5 hr had been perfused with 0.15 M NaCl. As Fig. 1 shows, there was a rapid drop in the activity of control livers, whereas an accumulation of modified albumin was found in the livers of the suramin-treated rats. Only after 2 hr did the activity in these livers start to decrease. Even 8 hr after injection of the albumin the quantity of labeled protein in rats pretreated with suramin was still eight times the

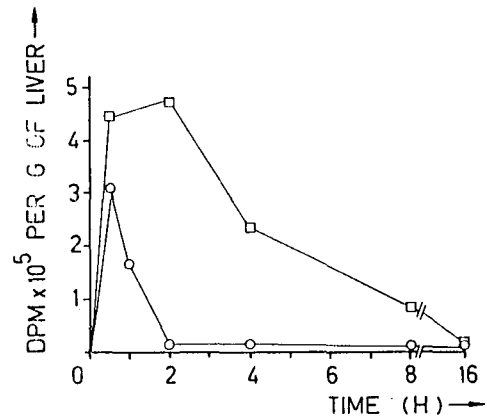


FIG. 1. Uptake and disappearance of injected formaldehyde-treated albumin in livers of control (O) and suramin-treated (□) rats. Abscissa: time after injection of 10^7 dpm (0.8 mg) of formaldehyde-treated 125 I-labeled human serum albumin. Ordinate: trichloroacetic acid-precipitable radioactivity (dpm $\times 10^5$ per g fresh weight of liver). Experimental animals had received an injection of 250 mg suramin per kg 24 hr before injection of the albumin. Each point is the mean of at least three separate experiments.

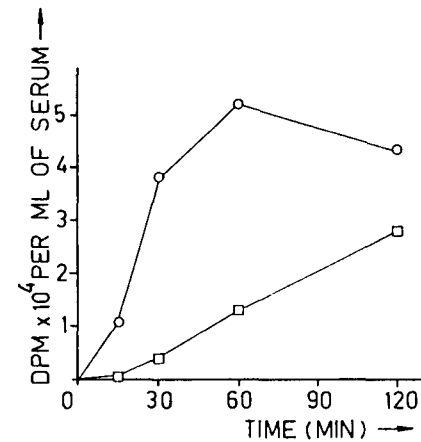


FIG. 2. Appearance of degradation products of injected denatured formaldehyde-treated albumin in serum of control (O) and suramin-treated (□) rats. Abscissa: time after injection of 10^7 dpm (0.8 mg) of formaldehyde-treated 125 I-labeled human serum albumin. Ordinate: trichloroacetic acid-soluble radioactivity. Experimental animals had received an injection of 250 mg suramin per kg 24 hr before injection of the albumin. Each point is the mean of five separate experiments.

quantity present in control livers. The same course of events was found for the spleen.

At 0.5 hr after the injection of albumin, trichloroacetic acid-soluble radioactivity was significantly higher in the livers and spleens of control animals than in those of treated rats. The appearance of acid-soluble activity in serum is shown in Fig. 2. Its increase is much greater in control rats than in treated rats. These data clearly reflect a decreased digestion of formaldehyde-treated albumin in the organs of the treated rats, especially in the liver and the spleen.

The results described above were obtained with labeled human serum albumin. For bovine serum albumin the same rapid uptake from the blood was observed.

Characterization of Formaldehyde-treated Albumin by Ultracentrifugation and Gel Filtration

As mentioned earlier, many investigators have presented evidence that aggregates of heat-denatured serum proteins are rapidly cleared from the blood by cells of the reticuloendothelial system. Thus, the rapid disappearance of formaldehyde-treated albumin described in the previous section might have been due to the formation of aggregates during the formaldehyde treatment, which causes the formation of methylene bridges between different amino acid side chains in proteins (Means and Feeney, 1971). It seemed quite possible that, under the conditions used by Mego and

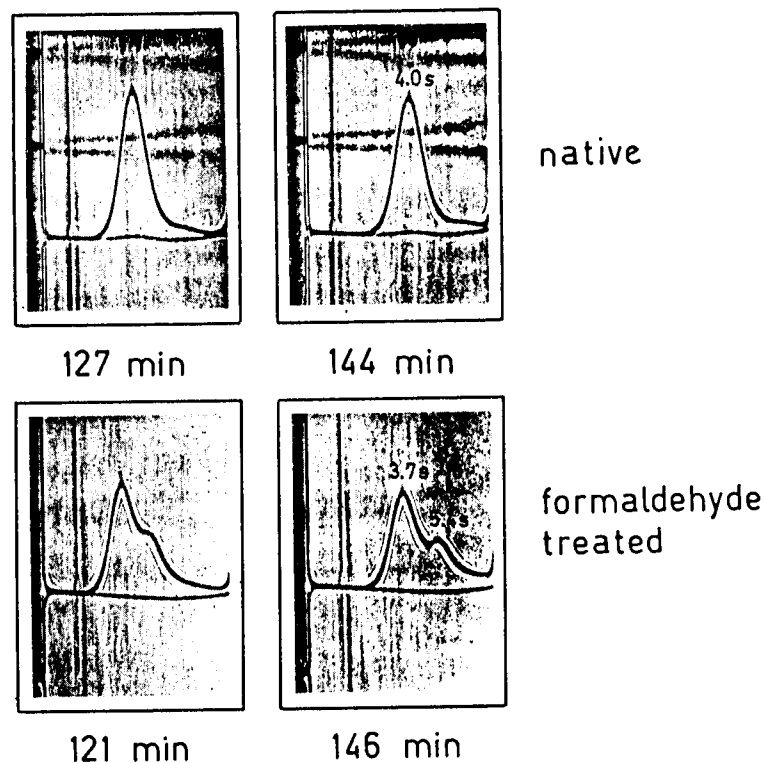


FIG. 3. Sedimentation patterns of native and formaldehyde-treated bovine serum albumin in 0.15 M NaCl containing 6.7 mM sodium phosphate (pH 7.4). $c = 6$ mg/ml. Experiments were performed at 52,640 rpm and 20°C in a Spinco Model E ultracentrifuge equipped with phase-plate Schlieren optics.

McQueen (1965a) and by us, intermolecular methylene bridges would be formed resulting in large aggregates.

Figure 3 shows that the behavior of formaldehyde-treated albumin in the ultracentrifuge is similar to that of the native protein, although the sedimentation constant is slightly decreased. The sedimentation pattern of the native protein shows a shoulder which is probably due to the presence of a dimer. A considerably higher shoulder is seen for the treated protein. No aggregates were observed when formaldehyde-treated protein was centrifuged in rat serum at 38°C after 30-min preincubation. Under the same conditions solubilized heat-denatured albumin prepared according to Benacerraf *et al.* (1957) sedimented as large complexes with a sedimentation value around 30S.

Both native and formaldehyde-treated albumin were also analyzed by chromatography on a calibrated column of Sephadex G 200. The elution

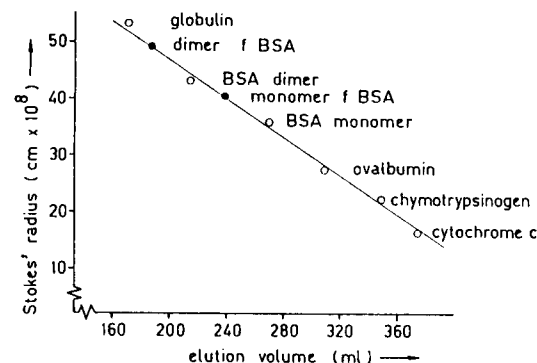


FIG. 4. Estimation of Stokes' radius of formaldehyde-treated bovine serum albumin (fBSA). Gel filtration was performed on a Sephadex G-200 column (50×3.2 cm) with a Tris-buffered NaCl solution (0.1 M NaCl, 0.05 M Tris, pH 8.2). Stokes' radii for standard proteins are taken from Andrews (1970).

profile showed an increase in the dimer fraction of the modified albumin, thus confirming the results obtained in the ultracentrifuge. Figure 4 shows that in comparison with mono- and dimer fractions of native albumin, the corresponding fractions of the formaldehyde-treated protein have a somewhat larger Stokes' radius. The combination of a lower sedimentation constant and a larger Stokes' radius can only be explained by a partial unfolding of the formaldehyde-treated molecule.

Cellular Localization of the Albumin, and Suramin Storage

Figure 5 shows the results of an autoradiographic examination of livers of controls and treated rats at 0.5 and 2 hr after injection of formaldehyde-treated ^{125}I -labeled human albumin. The grains resulting from radioactive radiation are always located above the Kupffer cells only. At 0.5 hr, but not at 2 hr, grains could also be detected above the blood vessels. A remarkable loss of grains occurred in control livers between 0.5 hr and 2 hr, whereas livers of treated rats still showed considerable activity at 2 hr. These results are in agreement with the quantitative measurements described above. Formaldehyde-treated ^{125}I -labeled bovine serum albumin was also localized exclusively in Kupffer cells at 0.5 hr after injection.

Figure 6 shows that 24 hr after injection of 250 mg per kg body weight suramin is localized as large granules in Kupffer cells. In control animals no granules could be detected.

Subcellular Localization of the Albumin, and Suramin Storage

Wattiaux *et al.* (1956) have shown that the lysosomal enzyme acid phosphatase is relatively more abundant in the hepatocytes, whereas

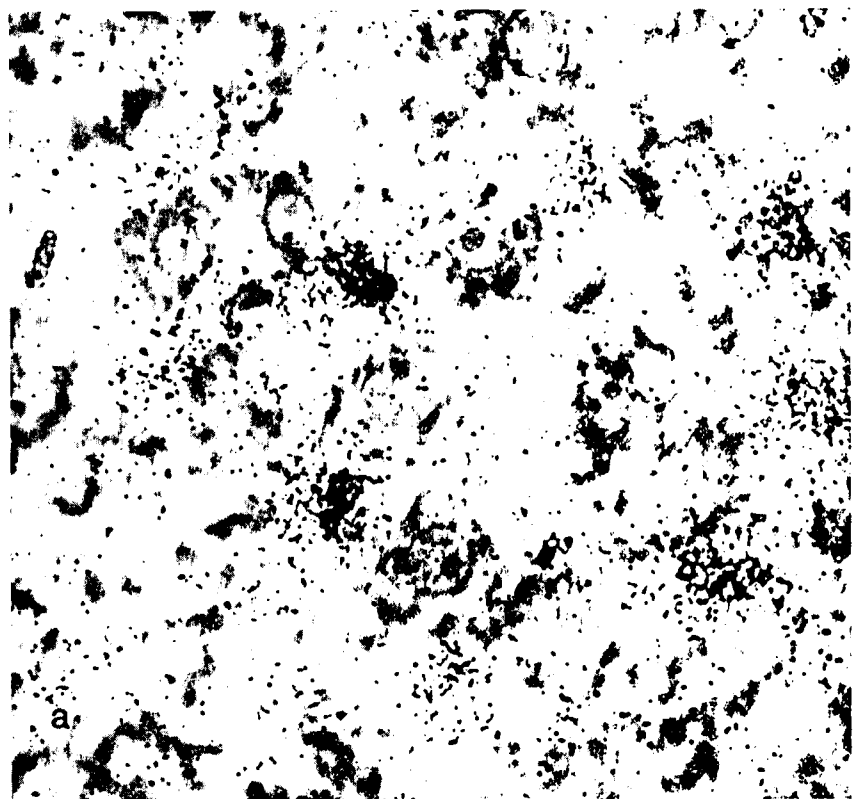


FIG. 5. Autoradiograms of liver sections of rats injected with formaldehyde-treated ^{125}I -labeled albumin. (a) liver of control rat 0.5 hr after injection of labeled protein. (b) liver of suramin-treated rat 0.5 hr after injection of labeled protein. (c) liver of control rat 2 hr after injection of labeled protein. (d) liver of suramin-treated rat 2 hr after injection of labeled protein. All sections have been exposed for the same time. For details see Materials and Methods. $\times 400$.

cathepsin D occurs predominantly in Kupffer cells. Since our autoradiographic results showed that formaldehyde-treated albumin is localized exclusively in Kupffer cells, we compared the distribution of the labeled albumin in subcellular fractions with those of the lysosomal enzymes mentioned above. A comparison of Fig. 7a with Fig. 7c and d shows that the distribution of radioactivity in rats not treated with suramin is clearly lysosomal and resembles more closely the distribution of cathepsin D than that of acid phosphatase as far as the relative concentrations in the nuclear, mitochondrial, and microsomal fractions are concerned. The relatively high radioactivity in the supernatant fraction might be caused by the incomplete removal of serum by the perfusion with saline.

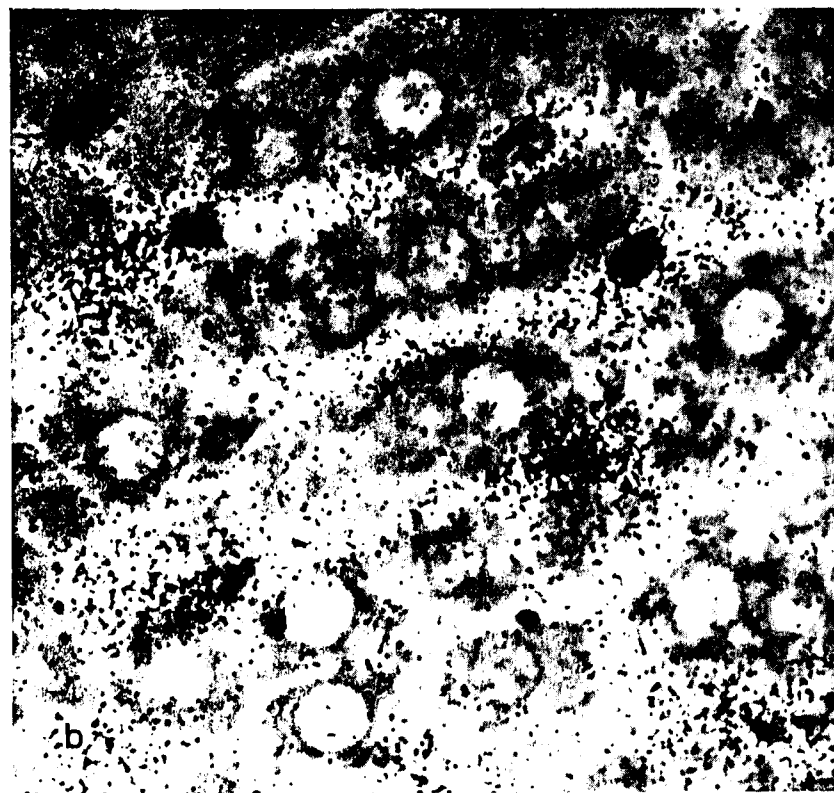


FIG. 5b.

Figure 7b shows the distribution of radioactivity in liver fractions of rats treated with suramin. A reliable determination of lysosomal enzymes in these animals was not possible. The enzymes were inhibited by suramin present in the homogenates. In addition there was no linearity between the residual enzyme activities and the concentration of the tissue fractions. However, the similarity between the distribution patterns of the radioactivity in experimental and control rats showed that in the suramin-treated rats the distribution of the radioactivity is also lysosomal. The shift toward the heavier "mitochondrial" fraction is probably due to the loading of lysosomes with suramin.

Between 1.8 and 2.2% of the dose corresponding to about 1 mg of suramin was present in the livers used for fractionations. The distribution of the drug in the subcellular fractions could not be determined with great accuracy owing to high blank values. A significant difference in absorbancy between experimental and control samples could be found in the "mito-

