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# ZONE PAPER ELECTROPHORESIS STUDIES ON RADIO-IODINATED HUMAN SERUM ALBUMIN<sup>1</sup>

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## INTRODUCTION

Iodination of human serum albumin has been investigated considerably. A careful report and review of the literature on physico-chemical aspects was given recently by Hughes and Straessle (1). These authors state: "The experiments reported indicate that considerable amounts of iodine, varying from very small amounts, to amounts greater than the equivalent of the tyrosol residues, may readily be introduced into human serum albumin without detectable changes. . . ." A further discussion of the properties of these iodoproteins is given. Under similar conditions of preparation, radioactive ( $I^{131}$ ) iodinated human serum albumin (IHSA) could be expected to display properties consonant with the above investigation.

A commercial IHSA preparation<sup>4</sup> has become available recently and was used during investigations in this laboratory concerning vascular damage following small doses of ionizing radiation (2). The IHSA disappearance rate from the circulation was established in control rats and compared with that of irradiated animals; the effects of the ionizing radiation were indicated by the altered IHSA disappearance rate from the vascular bed. In these experiments, it was noted that the IHSA disappearance rate in control animals showed irregularities. In the first hour after injection, the disappearance rate was faster than expected. This observation suggested that an in-

vestigation should be made on the physico-chemical properties of the IHSA preparation.

## METHODS

Zone paper electrophoresis was utilized with the apparatus of Kōiw, Wallenius, and Grönwall (3-5). Runs were made using veronal buffer pH 8.6, ionic strength of 0.05, 240 volts, Munktel No. 20 and Whatman No. 1 filter paper. After a varying migration time, wet paper strips were removed, placed in a 70° C. oven to dry for an hour and subsequently stained with bromphenol blue (3).

Radioautography was accomplished by placing the filter paper strip against Kodak Medical X-ray film (tinted safety base, duplitized) and arranging the two strips between pieces of plate glass which were compressed by weights during the period of exposure. Identification marks were pricked through both strips to permit reconstruction of their relative positions after completion of radioautography. The film was developed in Kodak D-19 developer for four minutes, fixed in Kodak Acid Fixer twice the clearing time, and washed in water for one hour.

Measurements of radioactivity were carried out by two different methods. In some experiments the filter paper was cut across into 5 mm. strips which were then cut into three pieces and placed, all with the same side up, in a planchette under a thin mica end-window type Geiger-Mueller tube (TGC-1). All radioactivity was measured with a scaler (Tracerlab Autoscaler) with a counting accuracy of better than  $\pm 2$  per cent.

For measuring the radioactivity in other experiments, a special device designed in this laboratory<sup>5</sup> was employed (Figure 1). A filter paper strip was fixed at both ends to a flat, thin brass plate which was attached to a threaded screw. Rotating a connected circular handle advanced the brass plate, and the paper strip, 0.05 inch per turn. This brass plate and turning screw assembly was bolted to a lead shield (Tracerlab). A separate part, containing an adjustable slit and made of 7 mm. thick iron, was centered with respect to the Geiger-Mueller tube and placed at 1 mm. distance above the brass plate. Width of the slit was 2 mm. and its length equalled the width of the brass plate. This part was also bolted to the lead shield.

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<sup>2</sup> James Purcell Memorial Research Fellow, Department of Surgery.

<sup>3</sup> Luria Foundation Fellow.

<sup>4</sup> Obtained from Abbott Laboratories, North Chicago, Illinois (Lot. Nos. A-254-24, B-21-27, B-210-41, and B-233-1).

<sup>5</sup> Built by Van Collie Studios, Wilton, Connecticut.

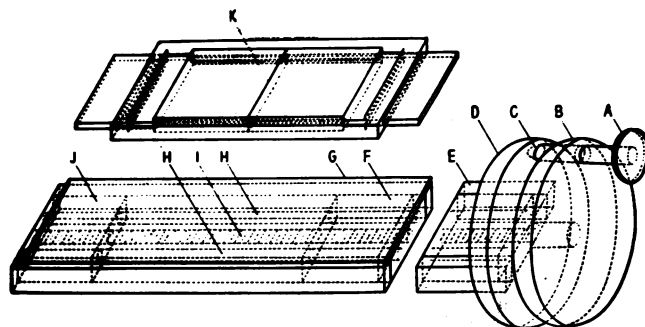


FIG. 1. CONSTRUCTION OF SCANNING DEVICE FOR MEASURING RADIOACTIVITY ON A PAPER STRIP

A—lock handle; B—rotating disk; C—lock notch; D—stable disk; E—frame; F—moving block; G—brass plate; H—stabilizing rods; I—screw; J—stable block; K—adjustable slit.

Protein-bound dye determinations were made, after the radioactivity measurements, by cutting the filter paper strips into 5 mm. sections, placing these strips in test tubes and eluting the dye in 5 ml. of 5 per cent sodium carbonate in 50 per cent methanol. After an hour, the color intensities of the eluates were determined in a Beckman spectrophotometer at 595  $m\mu$ .

#### EXPERIMENTAL

Samples for zone paper electrophoresis were prepared by mixing 1.0 ml. IHSA with 1.0 ml. normal human blood serum and pipetting 0.04 ml. of this mixture onto Munktell No. 20 or Whatman No. 1 filter paper. Figure 2 indicates the results of this experiment. Radioactivity appears increasingly detectable from the "starting point," *i.e.*, the place where the drop was applied, to the albumin fraction. The greatest amount of radioactivity does not coincide with the localization of the normal human serum albumin. In all of the 37 electrophoretic runs using four different batches of IHSA, the peak of radioactivity appeared distinctly ahead of the normal human serum albumin.

In rats, 0.5 ml. IHSA was injected intravenously into two normal animals. Two hours later, blood samples were taken, allowed to clot, and centrifuged to obtain the serum for electrophoresis. Figure 3 shows one of the results. The picture appears identical to the IHSA-human serum experiment in that the IHSA is slightly separated from the rat albumin, and an increasing amount of radioactivity can be noted from the starting point to the albumin peak.

Further experiments were performed to investigate this large amount of radioactivity which continually appeared in the area usually occupied by the globulins. Human blood serum and IHSA were mixed as above, but the drop was pipetted onto the middle of a filter paper strip. During electrophoresis of these samples, the cathode and anode were switched after three hours and the

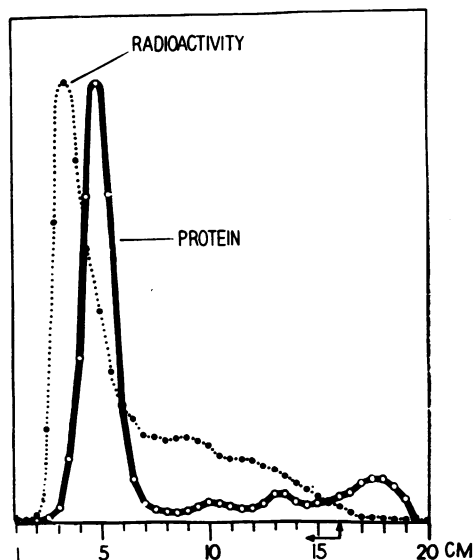


FIG. 2-a. ELECTROPHORETIC PATTERN ON MUNKTELL NO. 20 FILTER PAPER OF NORMAL HUMAN SERUM MIXED WITH IHSA

The arrows represent starting point and direction of migration. Curve labeled "protein" shows concentration of bromphenol blue stain eluted from 0.5 cm. strips. Other curve is radioactivity measured from same strips.

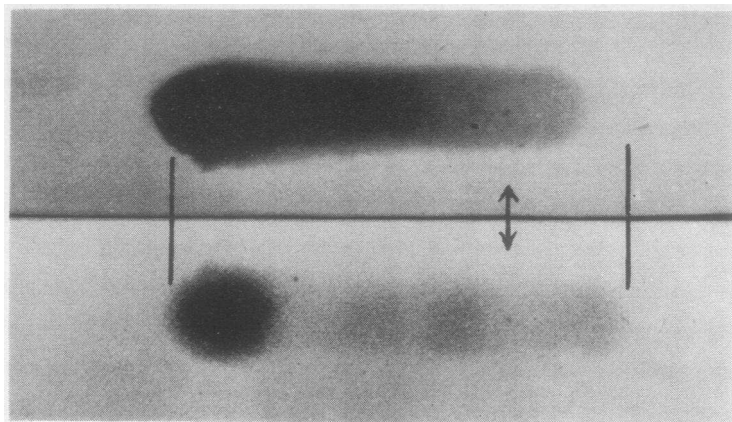


FIG. 2-b. RADIOAUTOGRAPH (UPPER) AND PICTURE (LOWER) OF A SIMILAR EXPERIMENT

Arrows represent starting points. Vertical lines show limits of bromphenol blue staining. Note increased migration and trailing of IHSA on radioautograph.

run was continued for another nine hours. Figure 4 shows the results of this experiment. Whereas the bromphenol blue staining appears as usual, the radioactivity curve is quite different. In this three hour-reverse current-nine hour electrophoresis, there is a peak of radioactivity at each

end of the pattern and radioactivity between the two peaks seems greatly diminished.

Hence, it was apparent that IHSA might contain at least two distinct fractions. This sup-

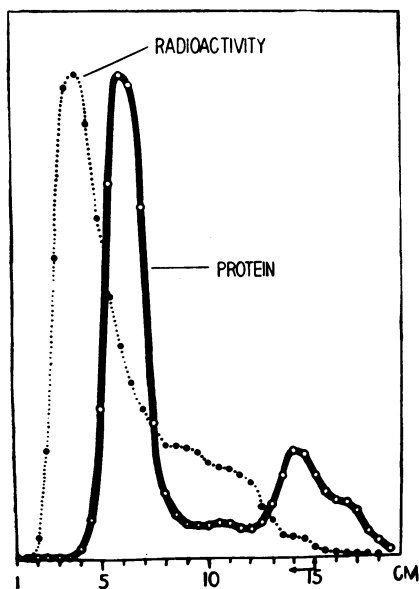


FIG. 3. ELECTROPHORETIC PATTERN ON MUNKTELL No. 20 FILTER PAPER OF RAT BLOOD SERUM

IHSA was injected intravenously two hours before blood sample was taken. Arrows show starting point and direction of migration. For curves labeled "protein" and "radioactivity" see Figure 2-a.

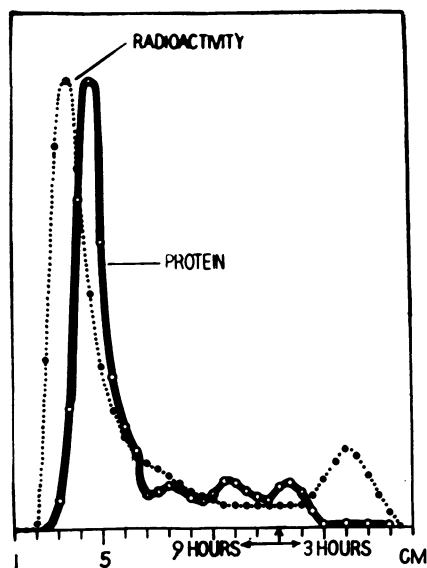


FIG. 4-a. ELECTROPHORETIC PATTERN ON MUNKTELL No. 20 FILTER PAPER OF NORMAL HUMAN SERUM MIXED WITH IHSA

Arrows indicate the starting point, the initial three hour migration and then nine hour migration in the opposite direction. Curve labeled "protein" shows concentration of bromphenol blue stain eluted from 0.5 cm. strips. Other curve is radioactivity measured from same strips.

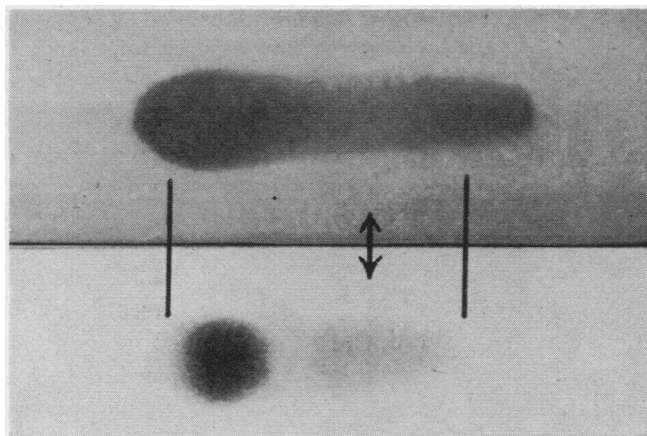


FIG. 4-b. RADIOAUTOGRAPH (UPPER) AND PICTURE (LOWER) OF A SIMILAR EXPERIMENT

Arrows represent starting point. Vertical lines show limits of bromphenol blue staining. Note considerable exposed area of radioautograph to right of bromphenol blue pattern.

position was tested by experimental attempts to separate the two fractions.

To normal human serum, a very small amount of bromphenol blue was added. In minute concentrations, only the albumin absorbs the dye, permitting one to visualize the locus of the albumin during electrophoresis. A large drop of this dye-containing sample was pipetted onto a Munktell No. 20 filter paper strip, and the IHSA-human serum mixture was placed on a similar paper strip. These two different samples were arranged alongside each other between the same pair of buffer-containing cells, and the starting points

were aligned so as to be equi-distant from the cells. After six hours, the filter paper strips were removed, the starting points realigned, and, using the albumin-bromphenol blue migration as a guide, the albumin section of the IHSA strip was

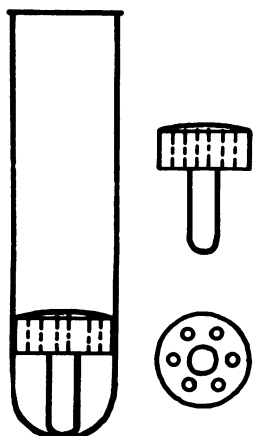


FIG. 5. DEVICE FOR REMOVING FLUID FROM A FILTER PAPER STRIP (SEE TEXT)

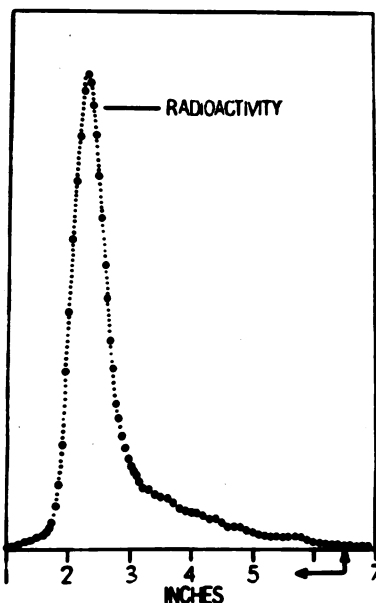


FIG. 6. SECOND ELECTROPHORESIS ON MUNKTELL NO. 20 FILTER PAPER OF FLUID OBTAINED FROM ALBUMIN SECTION OF A FIRST RUN

Arrows represent starting point and direction of migration. Curve is radioactivity as measured with scanning device (Figure 1).

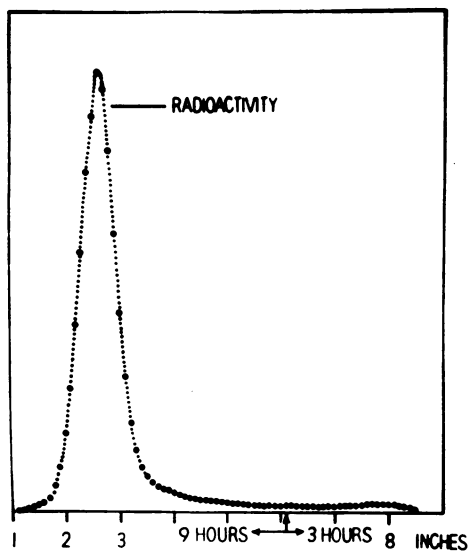


FIG. 7. ELECTROPHORETIC PATTERN ON WHATMAN NO. 1 FILTER PAPER OF NORMAL HUMAN SERUM MIXED WITH IHSA

Compare radioactivity with Figure 4-a.

cut out. Immediately the section was placed in a centrifuge tube containing a mushroom-shaped piece of plastic with six holes through the top (Figure 5). Centrifuging enabled one to separate about 0.5 ml. of fluid from the filter paper. A second electrophoretic run was made on this fluid and the strip was measured for radioactivity using the scanning device (Figure 1). The results of this experiment are indicated in Figure 6. In this attempt to separate the albumin from the "trailing" fraction the albumin was retained, but the trailing fraction was almost eliminated.

An experiment was made to determine whether the degree of trailing was consistent with or dependent on the type of filter paper used. When Whatman No. 1 filter paper was used in three hour-reverse current-nine hour electrophoresis, no peak could be noted in the three hour-direction and the trailing in the nine hour direction was almost absent (Figure 7).

#### DISCUSSION

The increased mobility of IHSA is perhaps due to some minute change in the charge or shape of the albumin molecule. The trailing, however, reveals another interesting difference between the IHSA and the human blood serum. In the ex-

periment in which the direction of migration was changed after three hours and the IHSA-normal serum mixture was allowed to move in the opposite direction for another nine hours (Figure 4), no evidence of normal human serum proteins, when measured by the amount of bromphenol blue staining, was demonstrable in the area of the first three hour migration. On the other hand, the IHSA trailing, when measured by its radioactivity, displayed a much different picture. Here a definite peak of radioactivity was noticeable in the three hour direction, and the trailing in the nine hour direction was greatly diminished.

This experiment ruled out the possibility that trailing represents contamination by some radioactive globulins which may have been present in IHSA from the beginning or which may have occurred during the mixing with normal human serum. If some alpha or beta globulins had contained any radioactivity, it should be measurable on corresponding spots of the globulins, but the two peaks of radioactivity were not in globulin areas.

Additional evidence that IHSA contains at least two fractions was obtained in the experiment in which an attempt was made to separate the albumin fraction of the IHSA from the trailing fraction (Figure 6). The albumin peak was still present, but the trailing fraction almost disappeared.

Another implication of this study concerns the interpretation of trailing, *viz.*, a) trailing is an undesirable artifact, b) trailing represents an additional separation. In our experiments it was shown that IHSA trailing on Munktell No. 20 filter paper appears to be a distinct and separable fraction from the albumin. That this trailing was barely evident on Whatman No. 1 filter paper does not necessarily mean that this paper is less effective in separation.

An analogous situation might be found in the work of Kunkel and Slater (6, 7) who reported trailing of beta lipoproteins along the path of migration on Whatman 3 MM filter paper, but they found no trailing to occur when the filter paper was replaced by a starch medium. During some experiments on lipoproteins in our laboratory, it was noticed that Munktell No. 20 filter paper, stained with Sudan Black, also showed trailing.

But it is felt that this represents a subdivision of the beta lipoproteins into two parts, one being the trailing, and the other being, a fraction which migrates in the same manner as beta proteins.

It may now begin to appear that the choice of an electrophoretic medium depends on that which one wants to demonstrate. Use of a starch medium would seem advantageous in many physico-chemical studies. But for the detection of a slight inhomogeneity of a colloidal material, paper electrophoresis may provide much useful information.

The biological meaning of changes in the albumin molecule due to the iodination process may be difficult to evaluate from these electrophoretic studies. Whether or not these results are significant enough to limit the use of the IHSA studied in this paper cannot be definitely determined at this time. However, when it is considered that experiments in this laboratory indicated a disappearance of IHSA in the blood stream of rats which did not seem to follow the expected rate, it may be implied that the IHSA fractions have different metabolic rates.

#### SUMMARY

Commercial iodinated ( $I^{131}$ ) human serum albumin was studied by means of zone paper electrophoresis. In four different samples, at least two fractions were noticed. One showed a slightly higher mobility than noniodinated serum albumin; the other fraction appeared in the form of trailing. The two fractions could be separated, and the trailing consequently eliminated. Hence,

the trailing appears not to be an artifact. The usefulness of paper electrophoresis in the detection of slight inhomogeneities of colloidal materials and the biological significance of the two fractions were discussed.

#### ACKNOWLEDGMENT

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