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SOLUBLE PROTEINS OF LIVER NUCLEI OF RATS FED 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE*

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Abstract—Nuclei from livers of rats fed semisynthetic diets with and without 3′-methyl-4-dimethylaminoazobenzene were prepared in 2·1 M sucrose supplemented with magnesium. Approximately 50 per cent of the nuclear nitrogen and 84 per cent of bound azo dyes were soluble in hypotonic Tris–chloride buffer containing magnesium. Magnesium-stabilized nuclear extracts differed in column zonal electrophoresis from the unstabilized saline–phosphate extracts studied previously. The stabilized extracts had substantially more of highly acidic and basic and less of weakly acidic and near-neutral proteins. Furthermore, in the magnesium-stabilized extracts bound azo dyes were predominantly in the highly acidic classes of components and *not* in near-neutral classes as in the unstabilized saline–phosphate extracts. These and previous studies indicate that in the liver nuclei of rats fed carcinogenic azo dye some protein–azo dye conjugates are free in solution, but some are in magnesium-dependent association with nuclear ribosomes and some are integral parts of nuclear ribosomes.

THE RIBOSOMES have a high negative charge and consequently an unusual ability to bind various micro- and macromolecules.[1] The association and dissociation of extraneous substances depend strongly on factors like magnesium ion concentration, size of ribonucleoproteins, ionic strength and pH. Many bound substances are separable by electrophoresis.[2] Among substances that have been found to associate with ribonucleoproteins are proteins conjugated with amino azo dyes.[3–8] Some of the protein–azo dye conjugates are removable by deoxycholate,[3, 5, 7, 8] and others remain with ribosomes.[5, 8]

The question arose as to whether the electrophoretic distribution of soluble nuclear proteins in general, and of protein–azo dye conjugates in particular, was affected by the ionic composition of the media, especially by magnesium and phosphate ions. Furthermore, it was of interest to characterize the protein–azo dye conjugates bound by nuclear ribosomes.

In this paper we wish to describe the results of electrophoretic fractionation of soluble nuclear proteins and ribosomes prepared from nuclei isolated in hypertonic sucrose supplemented with magnesium from livers of rats fed carcinogenic azo dye.

METHODS

With few exceptions, materials and methods used in these studies were the same as those described previously.[9] Male, CFN Carworth Farms, Inc. (New City, N.Y.) rats

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weighing 375–425 g were fed the No. 3 diet of Miller *et al.*[10] containing 0·058% 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB). The controls were fed the same No. 3 diet without 3′-Me-DAB. On day 20, diet was removed and 18 hr later the animals were decapitated. Their livers were perfused and rinsed with cold 0·25 M sucrose containing 1 mM $MgCl_2$, homogenized in 2·1 M sucrose containing 1 mM $MgCl_2$, and the nuclei were isolated as before.[9] Nuclei were then extracted 4 times in 10-ml aliquots of Tris–Cl–Mg buffer, pH 7·6. The combined extracts were cleared by a 20-min centrifugation at 22,000 *g* (avg) yielding a supernatant which will be referred to as the nuclear extract.

In one series of experiments (12 rats per experiment) the nuclear extract was filtered through a Sephadex G-25 column which was in equilibrium with veronal–Cl–Mg buffer, pH 8·6.[9] The breakthrough component was concentrated by dialysis against veronal–Cl–Mg buffer containing 40% purified clinical dextran, dialyzed for 9 hr against 2 batches of veronal–Cl–Mg buffer, centrifuged for 20 min at 25,000 *g* and used for column electrophoresis.

In another series of experiments (20 rats per experiment), the nuclear extract was centrifuged for 60 min at 151,000 *g* (avg.) to remove nuclear ribosomes. The postribosomal supernatants were pooled, processed as above, and used for column electrophoresis.

Preparation of nuclear ribosomes. Nuclei from 20 livers, prepared in 2·1 M sucrose containing 1mM $MgCl_2$, were extracted 4 times in 10-ml aliquots of hypotonic Tris–Cl–Mg buffer, pH 7·6, as recommended by Pogo *et al.*[11] The pooled extracts were cleared by a 20-min centrifugation at 22,000 *g* (avg.), layered over 1 ml of 1 M and 1 ml of 0·5 M sucrose solution in Tris–Cl–Mg buffer and centrifuged for 60 min at 151,000 *g* (avg.). The bottom layers (2·5 ml) containing unpelleted ribosomes were pooled, diluted to 0·25 M sucrose with Tris–Cl–Mg buffer, layered over 0·5 M and 1 M sucrose solution in Tris–Cl–Mg buffer, and centrifuged for 60 min at 151,000 *g* (avg) once more. The bottom layers were pooled and filtered through a Sephadex G-25 column which was in equilibrium with veronal–Cl–Mg buffer. The sucrose-free breakthrough fraction was then dialyzed for 9 hr against 2 batches of veronal–Cl–Mg buffer. One portion of the ribosomes was used for free boundary electrophoresis and the other for column electrophoresis.*

Assays. The protein content of the concentrated protein solutions and of column fractions was assayed spectrophotometrically at 284 mμ (A_{284}) in a Beckman DU spectrophotometer and by the biuret reaction. In addition, the protein content of the column fractions was assayed by the ninhydrin reaction.[12] The ribonucleoprotein was assayed by lanthanum chloride precipitation as described elsewhere.[13]

The firmly bound total azo dyes were assyed by the modified formic acid method of Gelboin *et al.*[14] Whole column fractions and aliquots of the starting solution were mixed with 0·5 ml veronal–Cl–Mg buffer containing 10 μg pancreatic ribonuclease (Armor Co., Chicago, Ill.) and incubated for 30 min at 40° in a shaking water bath. Each fraction was then mixed with 0·5 ml veronal–Cl–Mg buffer containing 40 μg pronase (Calbiochem) and placed in a 40° shaking bath for 16–18 hr. In order to test the completeness of protein digestion, tubes containing the highest amount of protein (A_{284}) were heated for 2 min at 100°. If the sample developed turbidity, more pronase

* According to sedimentation in a 15–35% (w/w) sucrose gradient, nuclear ribosomes prepared by this procedure consisted of 72 s (67%), 42 s (24%) and 18 s (9%) components.

was added to each tube and digestion was extended for an additional 4 hr. The digests were then transferred into 25 × 200 mm heavy wall test tubes, shell-frozen, covered with bolting silk (60 mesh), and freeze-dried. The dry powders were dissolved in 0·5- ml aliquots of 88% formic acid, transferred into micro cells of 1 cm light path, and assayed at 400 and 525 mμ in a Beckman DU spectrophotometer. The reference blanks contained the same amount of lyophilized buffer and enzyme. Absorption at 400 mμ determined the interfering color, and measurements at 525 mμ assayed total azo dyes. In addition, complete absorption spectra of the fractions with highest A_{400} and A_{525} and of samples of nuclear extracts were determined in a Beckman DK-2 recording spectrophotometer. This served as additional confirmation for azo dye and for interfering chromogens.

The firmly bound azo dyes in the nuclei, nuclear extract, and column components were also assayed by the extraction–digestion method.[15]

Lactic dehydrogenase (LDH) was assayed spectrophotometrically.[16] The amount of enzyme in each column fraction was expressed as Δ O.D./min × ml.

Electrophoresis. The column electrophoretic fractionation of the 25,000 *g* and 151,000 *g* supernatants of concentrated nuclear extracts was carried out on a 3·1 × 220 cm column and of nuclear ribosomes on a 3·1 × 100 cm column as described by Sorof *et al.*,[17] except for the following modifications: the columns were packed with cellulose which was nitrosylated for 45 min in 0·1 M HCl containing 0·1 M $NaNO_2$, and the veronal–chloride buffer, pH 8·6, was supplemented with 0·5 mM $MgCl_2$. For optimal protein resolution the electrophoresis was conducted with an 80 ma current in the column circuit on the 220 cm column for 122 hr, and on the 100 cm column for 48 hr, respectively.

The free boundary electrophoretic analysis of nuclear ribosomes was carried out in veronal–Cl–Mg buffer in an 11 ml cell of a Spinco model H electrophoresis apparatus at 5·28 V/cm.

Buffers. Tris–Cl–Mg buffer: 0·5 mM $MgCl_2$ and 0·01 M Tris-(hydroxymethyl) aminomethane adjusted with HCl to pH 7·6.

Veronal–Cl–Mg buffer: 0·03 M NaCl, 0·5 mM $MgCl_2$ and 0·02 ionic strength sodium veronal, pH 8·6.

RESULTS

Four consecutive extractions of nuclei in Tris–Cl–Mg buffer removed 48–52 per cent of nuclear nitrogen. The recovery of protein and of ribosomes (A_{284}) in the final concentrated solutions was between 94 and 98 per cent of that present in the original nuclear extracts.

According to the determination of bound azo dyes by the extraction–digestion method, 84 per cent of azo dyes bound by nuclei of 3′-Me-DAB livers was soluble in Tris–Cl–Mg buffer. Furthermore, the concentration of azo dye per unit weight of dry nuclear extract in postribosomal supernatant and nuclear ribosomes was about the same.

In 9 experiments with Tris–Cl–Mg extracts and in 5 experiments with nuclear ribosomes the recovery of protein (A_{284}) in the column eluates was consistently 88 ± 4 per cent.

Column zonal electrophoresis of the 25,000 g *and* 151,000 g *supernatants.* The patterns of the 25,000 *g* and 151,000 *g* supernatants of control and 3′-Me-DAB livers are depicted in Fig. 1 and Fig. 2, respectively. The electrophoretic components in these

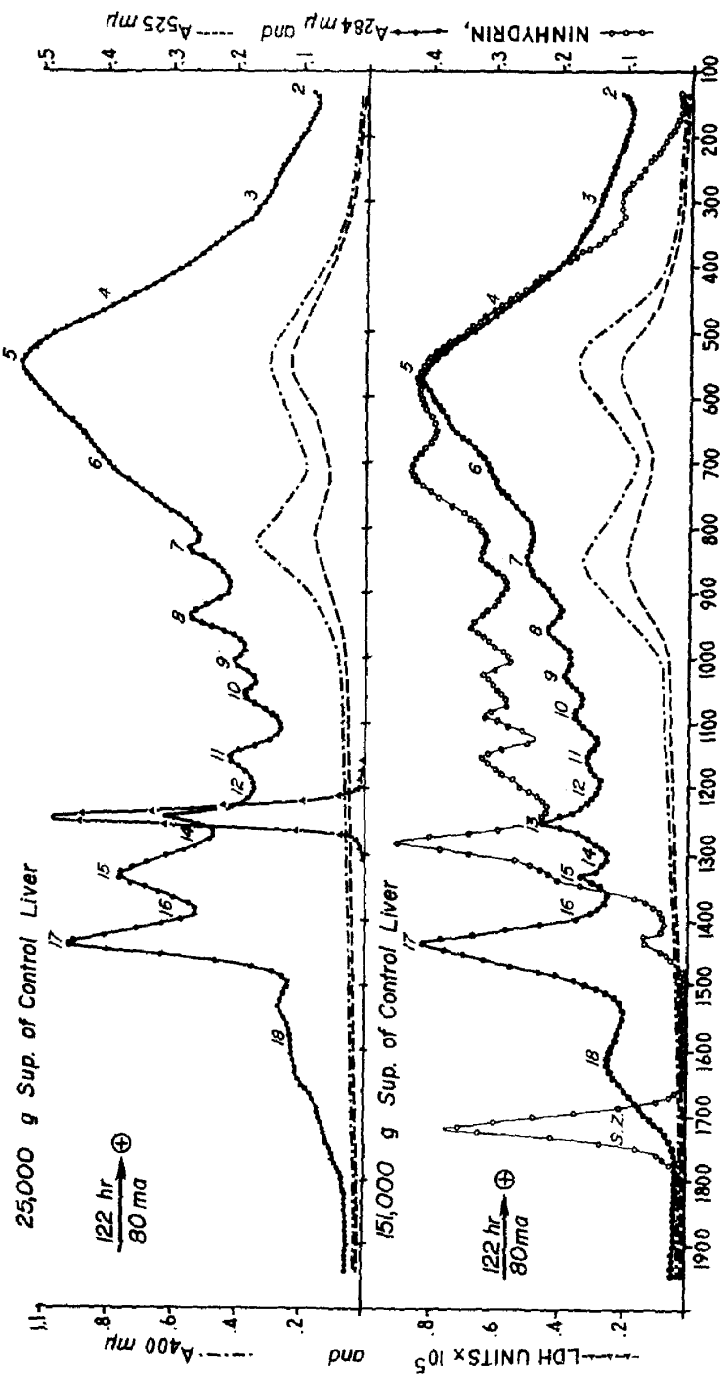

FIG. 1. Column zonal electrophoretic profiles of the 25,000 *g* and 151,000 *g* supernatants of Tris–Cl–Mg soluble nuclear proteins of livers of rats fed the control diet. The protein recoveries in the illustrated profiles were (top) 88 per cent and (bottom) 87 per cent. The recoveries of interfering chromogens were approximately 87 per cent.

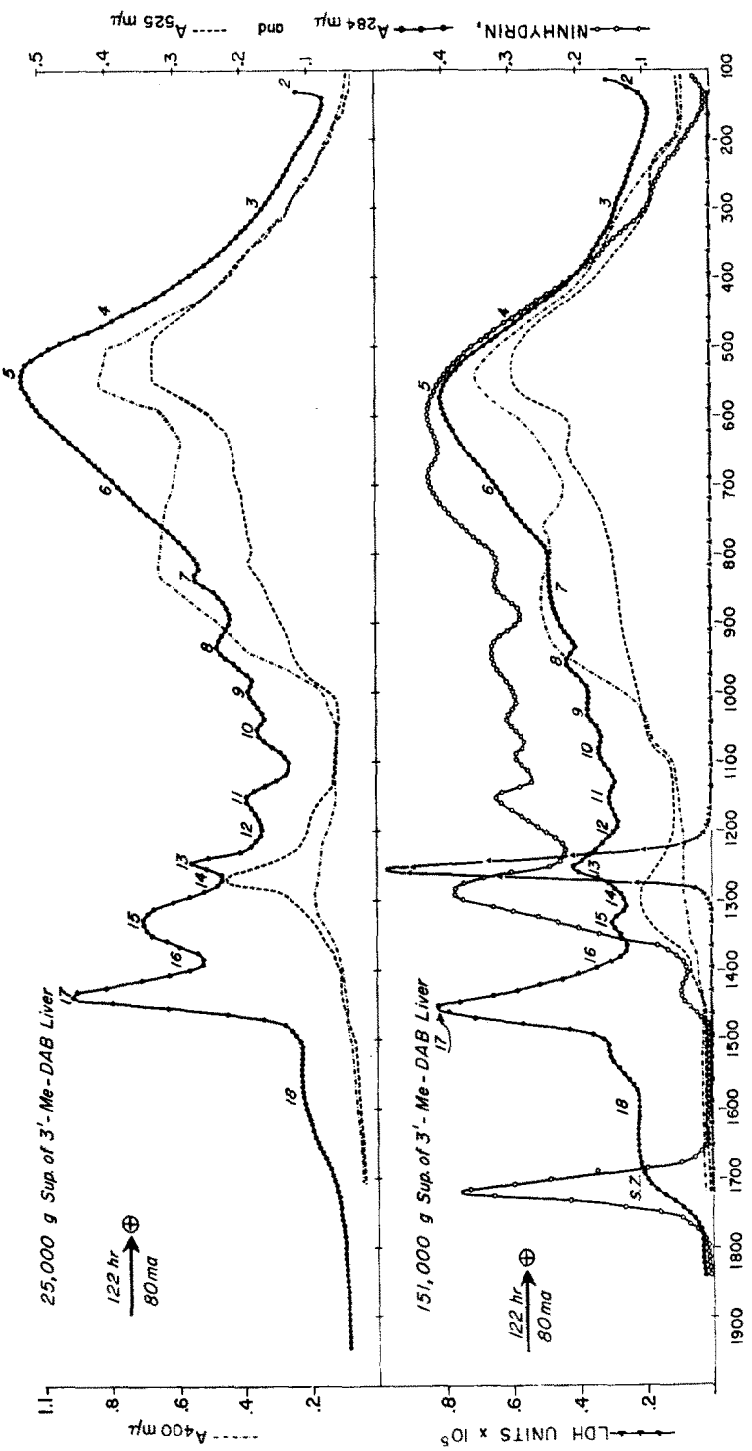

FIG. 2. Column zonal electrophoretic profiles of the 25,000 *g* and 151,000 *g* supernatants of Tris–Cl–Mg soluble nuclear proteins of livers of rats fed the 3'-Me-DAB-containing diet. The protein recoveries in the illustrated profiles were (top) 90 per cent and (bottom) 87 per cent. The azo dye recoveries were approximately 87 per cent.

profiles were identified and designated with the help of the electrophoretic mobility by using the LDH peak and the surface zone (S.Z.) as primary points of reference.[18] The protein profiles of the 25,000 *g* supernatants of control and 3'-Me-DAB livers are basically very similar. The same similarity exists also between the patterns of the 151,000 *g* supernatants of both types of livers. However, protein profiles of the 151,000 *g* supernatants differ from the profiles of the 25,000 *g* supernatants in respect to the relative amounts of near-neutral components.* The 151,000 *g* supernatants contain considerably less of components *13* through *16* than the 25,000 *g* supernatants. Also, the amount of bound azo dyes in components *13* through *16* in the 151,000 *g* supernatants of 3'-Me-DAB livers was significantly diminished.

The examination of column fractions of control livers in 88% formic acid revealed that the 25,000 *g* and 151,000 *g* supernatants of control livers contained chromogenic substances (A_{400} and A_{525}) throughout the whole pattern. Their absorption at 400 mμ was about twice as high as at 525 mμ and they were at highest concentrations in the region of acidic components *5* and *7*.

The assay of column fractions of postribosomal supernatants for ninhydrin-reacting materials has shown that components *2* through *15* contain protein, and components *16* through *18* contain little if any ninhydrin-reactive materials. A similar observation was made with the biuret test in profiles of saline–phosphate extracts prepared from nuclei isolated in 2·1 M sucrose without magnesium.[18] The ninhydrin reaction has revealed exceptionally good detail in the composition between components *6* through *11*, whereas measurements of A_{284} have shown better detail between components *12* through *15*. This indicates that components *6* through *15* differ in regard to the amount of aromatic amino acids and free amino groups.

Because ninhydrin interacts with veronal, the ninhydrin test has helped to locate the S.Z.,† which was marking the void volume of the column and was recognizable by the accumulation of veronal.

The assay of column fractions for ribonucleoproteins by the $LaCl_3$ precipitation reaction[13] revealed that in every profile components *2* through *6* were precipitated quantitatively, components *7* and *8* precipitated partially, while others remained in solution.

Free boundary electrophoresis of nuclear ribosomes. As shown in Fig. 3, after 180 min of electrophoresis at 5·28 V/cm, ribosomes of 3'-Me-DAB-preneoplastic livers were resolved into at least 5 components. Up to 86 per cent of the total area of the pattern in the descending limb was occupied by the highly acidic components *1a* and *2*. The remaining portion of the pattern was made up of two weakly acidic components, *4* and *6*, and one near-neutral component, *15*. The high negative charges of components *1a* and *2* were evident from their high negative mobility.

Column zonal electrophoresis of nuclear ribosomes. A complete column electrophoretic pattern of nuclear ribosomes of livers of rats fed 3'-Me-DAB is illustrated in Fig. 3. The column profile was plotted on a scale which corresponds to the mobility scale of the free boundary electrophoretic pattern shown in the upper portion of Fig. 3. The components in the profile were identified and designated with the help of electrophoretic mobility by using the S.Z. peak and the near-neutral azo dye peak as

* For definition of electrophoretic components, see Bakay and Sorof.[9]

† Surface zone in column zonal electrophoresis represents the accumulation of salts on top of cellulose packing and appears to be quivalent to the salt boundary of free boundary electrophoresis.[17]

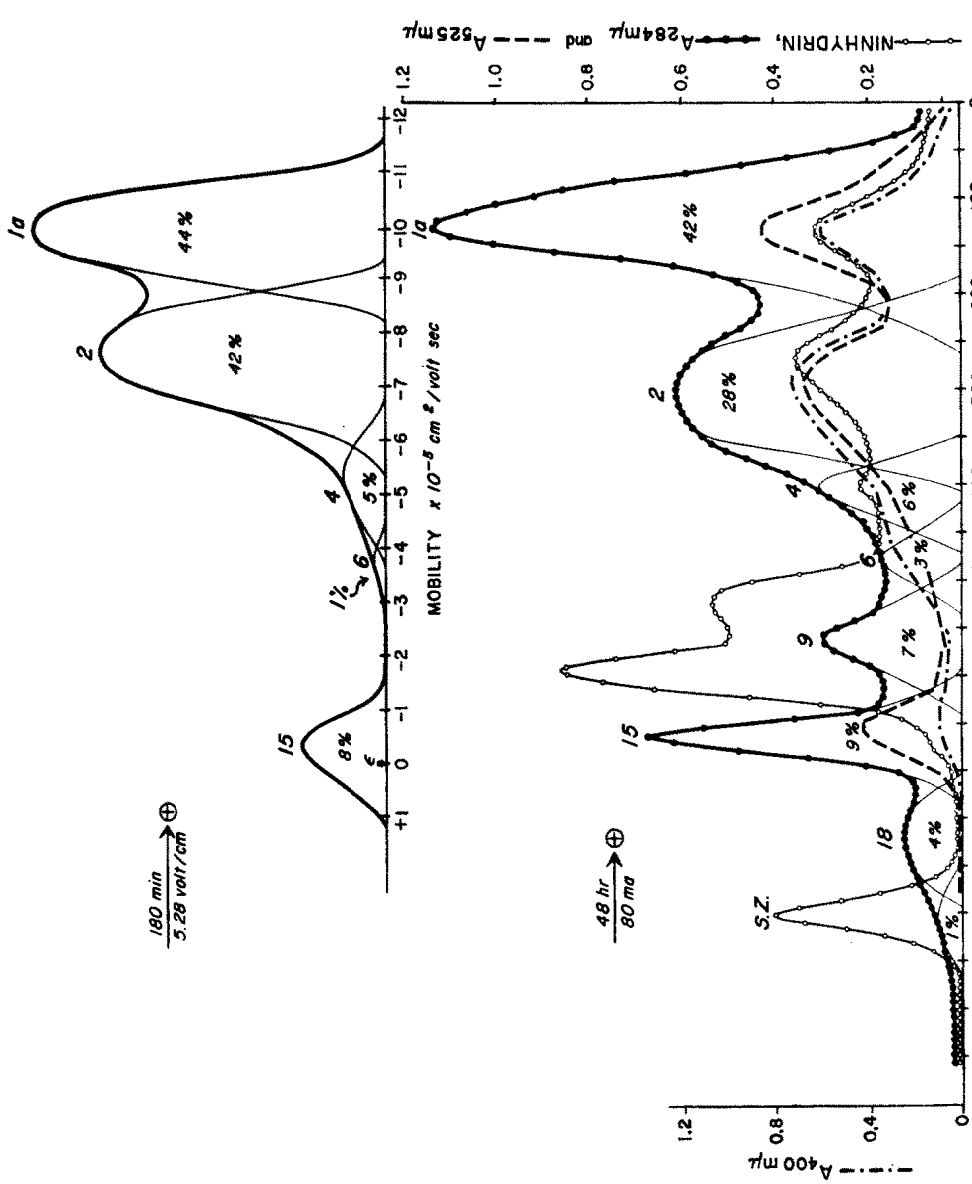

FIG 3. (Top) Free boundary electrophoretic patterns of the nuclear ribosomes of 3'-Me-DAB liver. Buffer, veronal–Cl–Mg, pH 8·6; protein concentration, 0·56%; phase plate angle, 17°. (Bottom) Column zonal electrophoretic profiles of the nuclear ribosomes of livers of rats fed the 3'-Me-DAB-containing diet. Buffer, veronal–Cl–Mg, pH 8·6; protein concentration, 2·6%. The protein recovery in column eluates was 83 per cent.

primary points of reference.[18] According to the spectrophotometric assay of protein at 284 mμ, nuclear ribosomes were resolved into at least 7 components. Of these, 6 were ninhydrin-positive and 1 was ninhydrin-negative. Components *1a* and *2* were completely precipitable with 0·01 M $LaCl_3$, while others were not. When submitted to a 60-min centrifugation at 151,000 *g* component *1a* sedimented quantitatively while 70–80 per cent of component *2* remained unsedimented.

As is evident from Fig. 3, all of the components that were present in the free boundary electrophoretic pattern were also present in the column pattern. In addition, in the column pattern there was one more weakly acidic component, *9*, and one basic component, *18*. In the free boundary pattern, component *2* constituted 42 per cent of the area, while in the column pattern it constituted only 28 per cent. At the same time, the amounts of components *4*, *6* and *15* in the column pattern were substantially greater. There was a small amount of component *14* present in the column profile, but it was masked by component *15*.

Essentially identical profiles (not shown) were also obtained in the free boundary and column zonal electrophoresis for nuclear ribosomes of control liver.

Determination of the bound azo dye (A_{525}) in the column fractions of nuclear ribosomes of 3′-Me-DAB livers revealed that most of the carcinogen was associated with the acidic components *1a* and *2* and the near-neutral component *14*. The weakly acidic components *6* and *9* had relatively little bound dye. The presence of azo dye in components *1a*, *2* and *15* was also confirmed by the absorption spectral analysis. Each of these components exhibited characteristic maxima at 512–520 mμ.

DISCUSSION

The presence of the same amounts of soluble nitrogenous constituents and bound azo dyes in the extracts prepared in Tris–Cl–Mg buffer from nuclei isolated in sucrose supplemented with magnesium as was found in the saline–phosphate extracts of nuclei isolated in sucrose without magnesium[18] and the saline–phosphate extracts of nuclei isolated in nonaqueous media[19] showed that all three types of nuclear extracts had similar gross compositions. According to column zonal electrophoresis, the nuclear extracts prepared in Tris–Cl–Mg buffer from magnesium-stabilized nuclei resembled the saline–phosphate extracts of non-aqueous nuclei. In contrast, they were vastly different from the extracts prepared in saline–phosphate from nuclei isolated in magnesium-deficient sucrose. The patterns of the 25,000 *g* and 151,000 *g* supernatants of extracts prepared in Tris–Cl–Mg buffer had a greater amount of the highly and weakly acidic components *2* through *5*, less of the weakly acidic components *6* through *12*, considerably less of component *13*, and two to three times more of the basic components *15*, *16* and *17*. There was little difference in the relative amount of component *14*.

Changes in the distribution of proteins (A_{284}) were paralleled by changes in the distribution of protein-bound azo dyes (A_{525}) and interfering chromogens (A_{400}). In the 25,000 *g* supernatant of saline–phosphate extracts, components *14*, *15* and *16* contained more than 50 per cent of the total bound azo dyes.[18] In contrast, the same components of the 25,000 *g* supernatants of the Tris–Cl–Mg extract contained about 20 per cent of all bound azo dye. The same components of the 151,000 *g* supernatants of Tris–Cl–Mg extracts contained less than 10 per cent of all bound azo dye. The bulk of bound azo dye was localized in the highly and weakly acidic com-

ponents *2* through *5*. Described differences appear to be due mostly to magnesium-dependent binding of proteins by the ribonucleoproteins. Such binding was first observed in the column electrophoretic profiles of the 25,000 *g* and 151,000 *g* supernatants of the Tris–Cl–Mg extracts studied here. The ribosome-free 151,000 *g* supernatants had a considerably smaller amount of the near-neutral components *13* through *16* and of the bound azo dye in component *14* than the 25,000 *g* supernatants containing ribosomes.

A clearer picture of the binding of proteins by nuclear ribosomes was obtained by electrophoresis of the nuclear ribosomes. Three extraneous substances, separated from ribonucleoproteins by free boundary electrophoresis, comprised 14 per cent of the pattern while in the column pattern there were five extraneous components which comprised 30 per cent of the pattern. Among the substances that were separated by column electrophoresis, there were also the near-neutral protein–azo dye conjugates *14* and *15*. Judging by the decrease in the amount of acidic components *1a* and *2* and by the increase of other less acidic constituents in the column profile, component *2* was binding the extraneous substances almost exclusively. The fact that component *1a* was sedimentable at 151,000 *g* and component *2* was not suggests that component *2* was a smaller form of ribonucleoprotein. Thus, binding of extraneous substances by the ribonucleoproteins of the nuclei depends on Mg^{2+} and on the size of the ribonucleoprotein. This is in complete agreement with the observation of Petermann and Pavlovec.[2]

The presence of bound dye in the highly acidic components *1a* and *2* after electrophoresis is indicative of the strong bonding, perhaps chelating action of magnesium. This deduction is based on the fact that in the nuclear extracts prepared in magnesium-deficient media protein-bound azo dyes were localized predominantly in the near-neutral components *14*, *15* and *16*.[18] There was no protein-bound azo dye in the region of the highly acidic components.

When the above and other[18, 19] evidence concerning the distribution of bound azo dyes in the soluble nuclear proteins of livers of rats fed 3′-Me-DAB were taken into consideration, the following conclusions were reached: in the nuclei, one portion of the protein–azo dye conjugates was free in solution, the second portion was in loose, magnesium-dependent association with nuclear ribosomes, and the third portion was an integral part of ribosomes. The remainder of the bound azo dyes was in the nuclear residue. A similar distribution of azo dye was also observed in the cytoplasm of rat livers.[3, 5, 6, 8] It is possible, however, that in the sucrose-isolated nuclei part of the protein–azo dye conjugates were of cytoplasmic origin, perhaps representing contamination. This conclusion was reached on the basis of experiments with nuclei isolated in organic solvents.[19] Whether or not nuclear protein–azo dye conjugates are the same components as cytoplasmic protein–azo dye components remains to be explored. Binding of azo dyes by the proteins of nuclear ribosomes might be of great significance to the cell, since it might affect the synthesis of nuclear proteins leading to malfunction of the genome. However, at the present state of knowledge all comments that could be made regarding this question would be nothing more than speculation and it remains for future research to provide the correct answers.

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REFERENCES

1. M. L. PETERMANN, *The Physical and Chemical Properties of Ribosomes*. Elsevier, New York (1964).
2. M. L. PETERMANN and A. PAVLOVEC, *J. biol. Chem.* **236**, 3235 (1961).
3. J. C. ARCOS and M. ARCOS, *Biochim. biophys. Acta* **28**, 9 (1958).
4. R. W. BALDWIN and C. R. BARKER, in *Cellular Control Mechanism and Cancer* (Eds. P. EMMELOT and O. MUHLBOCK). Elsevier, New York (1964).
5. T. HULTIN, *Expl Cell Res.* **10**, 71 (1956).
6. T. HULTIN, *Expl Cell Res.* **10**, 697 (1956).
7. T. HULTIN, *Expl Cell Res.* **13**, 47 (1957).
8. T. YAMADA, M. MATSUMOTO and H. TERAYAMA, *Expl Cell Res.* **29**, 153 (1963).
9. B. BAKAY and S. SOROF, *Cancer Res.* **24**, 1814 (1964).
10. E. C. MILLER, J. A. MILLER, B. E. KLINE and H. P. RUSCH, *J. exp. Med.* **88**, 89 (1948).
11. A. O. POGO, B. G. T. POGO, W. C. LITTAU, V. G. ALLFREY and A. E. MIRSKY, *Biochim. biophys. Acta* **55**, 849 (1962).
12. S. MOORE and W. H. STEIN, *J. biol. Chem.* **211**, 907 (1954).
13. B. BAKAY, L. B. KIRSCHNER and G. TOENNIES, *Biochem. biophys. Res. Commun.* **2**, 259 (1960).
14. H. V. GELBOIN, J. A. MILLER and E. C. MILLER, *Cancer Res.* **18**, 608 (1958).
15. E. C. MILLER and J. A. MILLER, *Cancer Res.* **7**, 468 (1947).
16. G. WEBER and A. CANTERO, *Cancer Res.* **19**, 763 (1959).
17. S. SOROF, E. M. YOUNG, M. M. MCCUE and P. L. FETTERMAN, *Cancer Res.* **23**, 864 (1963).
18. B. BAKAY and S. SOROF, *J. Pharmac. exp. Ther.* in press.
19. B. BAKAY, S. SOROF and G. SIEBERT, *J. Pharmac. exp. Ther.* in press.