Azoproteins of Liver Nuclei Isolated in an Aqueous or Nonaqueous Medium from Rats Fed an Azocarcinogen

Bohdan Bakay, Sam Sorof, and Günther Siebert

The Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111 [B. B.; S. S.], and Department of Physiological Chemistry, Johannes Gutenberg University, Mainz, Germany [G. S.]

SUMMARY

The proteins and azoproteins of liver nuclei isolated in aqueous or nonaqueous medium from livers of rats fed the hepatocarcinogenic 3'-methyl-4-dimethylaminoazobenzene for 20 days were compared. The two types of nuclear preparation apparently contained similar quantities of saline-soluble and -insoluble protein-bound azo dyes. In aqueous nuclei, the protein-bound dyes represent only 3% of those in liver, were 86% saline-soluble, and were present at a concentration (per mg protein) 43% that in cytoplasm. One-half of the proteins of both nuclei were saline-soluble and were similar according to free boundary and column zonal electrophoresis. In contrast to the situation with aqueous nuclei, which contain sizeable amounts of the specific soluble azoproteins, little of these slow $h_2$-like azoproteins were found in the nonaqueous nuclei. The possible basis of this difference is discussed.

INTRODUCTION

The soluble proteins of liver nuclei isolated in aqueous medium from rats fed the hepatocarcinogenic aminoazo dye 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) are similar electrophoretically to those of cytoplasm (1, 2). This applies particularly to the principal soluble azoproteins. The question arose whether this similarity reflected the situation existing in vivo, or was instead cytoplasmic contamination of the nuclei resulting from the aqueous purification procedure.

Isolation of nuclei in nonaqueous medium according to the method of Siebert appears to minimize nucleocytoplasmic translocation of water-soluble constituents (11, 12). The proteins and azoproteins of such nuclei were therefore compared with their counterparts from aqueous nuclei and cytoplasm.

MATERIALS AND METHODS

Liver Fractions

Adult male rats were fed for 20 days diets lacking or containing 3'-Me-DAB and were fasted 17 hr prior to sacrifice. Details of these aspects, as well as of the isolation of liver nuclei in 2.1 M sucrose solution and of their subsequent subfractionation, have previously been described (1, 2). The recovery of nuclei from the filtered liver homogenate was 71% based on DNA. The following tissue components were prepared by the aqueous method: whole liver, filtrate of liver homogenate, nuclei, nuclear extract, and nuclear residue.

For the nonaqueous isolation of nuclei, 60 male rats (174-188 gm), originally derived from the Sprague-Dawley strain (Wiga Animal Farm, Munich, Germany), were similarly fed the carcinogenic diet for 20 days. After fasting for 17 hr, the animals were anesthetized. The livers were rapidly frozen in situ according to the freeze-stop technic and fractionated as described by Siebert (11). The following fractions were obtained:

Parenchymal Powder. This powder contained the liver pulp (without connective tissue, bile ducts, and blood vessels) and corresponded to the filtered homogenate of the aqueous procedure. The RNA/DNA ratio was 2.82. This fraction served as the starting material in the isolation of nonaqueous nuclei.

Parenchymal Powder after Exposure to the Organic Solvents. This fraction served to evaluate the effects of the various solvents on the tissue components prior to fractionation. The RNA/DNA ratio was 2.90.

Nuclei. The RNA/DNA ratio of the isolated nuclei was 0.28 (11, 13). Recovery of nuclei from the parenchymal powder was 15% based on the DNA. These nonaqueous fractions were then packaged together with silica gel and flown in dry ice to the American laboratory.

The nonaqueous nuclei were homogenized and extracted in 0.12 M NaCl containing 0.01 M sodium phosphate buffer, pH 7.4, yielding the saline-phosphate-soluble nuclear proteins and nuclear residue. Similar subfractions of aqueous nuclei have previously been studied (1, 2).

Assays

Nitrogen and protein contents of nuclear fractions were determined by Kjeldahl and biuret methods respectively. The
protein concentration of column fractions was measured spectrophotometrically at 284 μm. Total azo dyes in column fractions were assayed in 88% formic acid at 525 μm (azo dyes) and at 400 μm (interfering hemochromogens) (5, 15). Firmly bound azo dyes in the liver fractions obtained from the aqueous and nonaqueous procedures were analyzed according to the extraction-digestion method of Miller and Miller (6, 7). The procedure was modified in that aliquots of liver fractions were precipitated in cold 10% trichloroacetic acid, washed successively with cold 1 M sodium acetate buffer at pH 5 and ethanol, and then dried in vacuo over CaCl₂. Duplicate 100-mg samples of each powder were digested for 20 hr at 80°C in 3.2 M KOH and 27% ethanol and extracted twice with a 9:1 ether ethanol mixture. The extracts were dried, dissolved in 7 ml of 4 M HCl containing 45% ethanol, extracted by shaking with 2 ml petroleum ether, and centrifuged 2 min at 12,000 X g. The aqueous fractions were then measured in a Beckman DU spectrophotometer in cells of 1 cm optical depth at 400 μm and 525 μm. The same solutions were used for analyses of total absorption spectrum in a Beckman DU-2 recording spectrophotometer.

**Electrophoreses**

Soluble proteins of nonaqueous nuclei were analyzed by free boundary electrophoresis in a Spinco Model H apparatus in 0.02 ionic strength sodium veronal buffer, pH 8.6, containing 0.03 M NaCl. After analysis, the protein solution was recovered, concentrated to 2.5% protein, dialyzed in the veronal-chloride buffer containing 0.5 mM MgCl₂ (veronal-Cl-Mg buffer), and resolved by column zonal electrophoresis (2, 14, 15). These results were compared with those of aqueous nuclei in the previous reports (1, 2).

**RESULTS**

**Partitions among Subcellular Fractions**

The saline-phosphate extract of nonaqueous nuclei contained 50% of the total nuclear nitrogen. An essentially identical amount (49%) was obtained previously with aqueous nuclei (1, 2).

The azo dyes firmly bound to aqueous nuclei were determined with the accuracy afforded by multiple analyses and correction with data derived from rats fed control diet (see Table 1). It is seen in Table 1 that the nuclear dyes represented only 3% of all those in rat liver (0.109 x 0.07 x 100/0.244). It is of interest that in the present study the concentration (per mg protein) of bound dyes in aqueous nuclei was as much as 43% of that in the cytoplasm (0.109 x 0.93 x 100/0.244-0.0076). Eighty-six ± 8% of the azo dyes of the aqueous nuclei was bound to extracted proteins (0.192 x 0.50 x 100/0.109). By independent analyses, 12 ± 11% was associated with the nuclear residue after the exhaustive extractions (0.027 x 0.50 x 100/0.109).

The nonaqueous and aqueous nuclei of rats fed 3'-Me-DAB appeared to contain similar quantities of protein-bound azo dyes (Table 1). The similarity is evident from a comparison of the dye analyses without adjustment for control diet experiments (see column under 3'-Me-DAB). Based on such uncorrected determinations, both types of nuclei had approximately 5% (cf. above) of the dye contents of their starting liver materials, i.e., the filtered liver homogenate and parenchymal powder (aqueous: 0.223 x 0.07 x 100/0.291, and nonaqueous: 0.201 x 0.07 x 100/0.258). These estimations include the factors reflecting that the nucleus comprises approximately 7% of liver cellular matter (10). Likewise, the nuclear soluble proteins are calculated without control correction to contain 63% (86% after correction) and 50% of the dyes in the aqueous and nonaqueous nuclei respectively (0.287 x 0.49 x 100/0.223, and 0.243 x 0.50 x 100/0.201). Corresponding independent values for the nuclear residues are 35% and 39% (0.159 x 0.49 x 100/0.223, and 0.157 x 0.50 x 100/0.201). With the qualifications imposed by the lack of the control correction and the single nature of the nonaqueous experiment, it appears that both nuclei possess similar amounts of soluble and insoluble protein-bound azo dyes.

**Free Boundary Electrophoresis**

Free boundary electrophoresis of the saline-phosphate extract of nonaqueous nuclei yielded the pattern shown in Chart 1. The overall profile resembled that of the extract of the aqueous nuclei at low electrophoretic resolution (1). Present were a discrete, fast-migrating component of strongly polyanionic constituents (1%), a broad unresolved group of acidic components (82%), and a slow family of near neutral and basic components (17%). At the equivalent resolution of extracts of aqueous nuclei, analogous regions comprise 1%, 67%, and 32% respectively. However, there were also finer differences discernible between the two types of nuclear extract. In terms of the previously used nomenclature of components and compared to aqueous nuclei (1, 2), the nonaqueous nuclei yielded more of the acidic Components 2 through 5 and correspondingly less of the regions consisting of the weakly acidic Components 6 through 8, the near-neutral Component 11, and the basic Component 17.

**Zonal Electrophoresis**

Chart 2 shows the column zonal electrophoretic profile of the soluble proteins of nuclei isolated in nonaqueous medium from livers of rats fed the 3'-Me-DAB diet. The minor differences revealed by free boundary electrophoresis are also evident here. Recoveries in the column eluate were 76% in the case of the proteins (A₂₈₄ μm), 72% of the azo dyes (A₅₂₅ μm, uncorrected for controls), and 77% of the interfering hemochromogens (A₄₀₀ μm, uncorrected).

The only evident localization of azoproteins in the profile was at the near-neutral Component 14 (Chart 2). Only there did the profiles of absorptions at 525 μm and 400 μm substantially differ. The ratio A₅₂₅ μm/A₄₀₀ μm of the peak fraction of Component 14 exceeded 2, while elsewhere it hovered around 0.5. Azoproteins were detected throughout the entire span of Components 2–15 by the presence of characteristic absorption maxima at 512 μm through 520 μm. Similar low levels of nonspecifically bound azo dyes have previously been found throughout the electrophoretic profiles of the soluble
proteins of aqueously isolated nuclei and cytoplasm (2, 15). In contrast, no dye was detected at Components 16, 17, and 18, consistent with the absence of protein in these components in extracts of aqueous nuclei (2). The individual species of the heterogeneous assortment of nonspecific azoproteins at Components 2–13 and 15 were present in smaller quantities than were the specific azoproteins at Component 14. Collectively, however, the former accounted for 96% of the total absorption at 525 mµ (uncorrected) in the profile. To what extent this absorption represented azo dyes rather than hemochromogens is unknown because of the lack of equivalent data in control diet experiments. However, in profiles of aqueous nuclei, where the control data are available (2), the comparable uncorrected value is 75%. If the soluble proteins of both types of nuclei are assumed to contain the same relative amount of protein in aqueous medium were generally similar. With both nuclear preparations, one-half of all the proteins were extracted in saline-phosphate buffer. Their soluble proteins exhibited overall analogous electrophoretic distributions. In addition, the two types of nuclei contained like quantities of protein-bound azo dyes, and further, the partition of bound azo dyes between soluble and insoluble fractions in both types of nuclei were similar. However, there were fine quantitative differences. The extracts of nonaqueously isolated nuclei contained relatively less near-neutral and more acidic proteins than did the extracts of aqueously prepared nuclei. Further, the nonaqueous nuclei yielded much smaller amounts of specific azoproteins belonging to near-neutral Component 14 than did the aqueous nuclei.

There is agreement that liver nuclei of rats fed azocarcinogen contain protein-bound azo dyes (2, 3, 8) and metabolites thereof (4, 9). Price et al. (8) reported that after feeding 3′-Me-DAB for 4 weeks, liver nuclei purified in isotonic sucrose contained 4–7% of all liver protein-bound dyes. Likewise, in the present two studies corresponding values were approximately 3–5% in both types of liver nuclei isolated in the hypertonic sucrose or nonaqueous medium. Further, the extractable azoproteins contain the bulk of the nuclear protein bound azo dyes (this study; 2).

The observation that there may be much smaller amounts of specific near-neutral azoproteins at Component 14 in the charge profile from nonaqueous nuclei than in those from aqueous nuclei deserves additional study. The finding that both types of nuclei have closely similar quantities of soluble and insoluble protein-bound azo dyes weakens explanations involving cytoplasmic contamination in aqueous nuclei and subcellular relocation. In addition, it indicates that extraction of perhaps a minor population of nonaqueous nuclei containing only 15% of the liver DNA (the yield in the nonaqueous procedure) is as representative as in the case of aqueous nuclei. One plausible conjecture assumes that in the extract from nonaqueous nuclei the specific near-neutral azoproteins are complexed with polyanions, and hence belong to weakly acidic components. Detection of the possible presence of azoproteins there is hindered by the considerable amount of interfering chromogens present at that location (Chart 2). Another hypothesis suggests precipitation of the specific azoproteins during electrophoresis as a consequence of the nonaqueous isolation on nuclei. The incomplete recovery of protein-bound azo dyes (72%) in the column eluate is compatible with such loss. Such behavior would be consistent with the thus far experienced lability of the apparently equivalent slow $h_2$ azo-
Chart 1. Free boundary electrophoretic pattern of the soluble proteins of nonaqueously isolated nuclei of livers of rats fed 3'-methyl-4-dimethylaminoazobenzene. Sodium veronal buffer, 0.02 ionic strength, pH 8.6, containing 0.03 M NaCl. Protein concentration, 1.7%. Phase plate angle, 27°. Duration and potential gradient are shown.

Chart 2. Zonal electrophoretic profiles of the soluble proteins and azoproteins of nonaqueously isolated nuclei of livers of rats fed 3'-methyl-4-dimethylaminoazobenzene. Sodium veronal buffer, 0.02 ionic strength, pH 8.6, containing 0.03 M NaCl and 0.5 mM MgCl₂.
proteins of liver cytoplasm. Known factors may be involved in either assumed mechanism. Nonaqueous nuclei have more Cl\(^-\), Na\(^+\), K\(^+\), and Mg\(^{++}\) ions (12), and also presumably the macromolecules therein have altered conformations and less hydrophobically held constituents than have aqueous nuclei. These alterations could conceivably labilize certain proteins. These factors individually and collectively may affect the specific azoproteins and thus alter their electrophoretic behavior.

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