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Isolation and Properties of the Principal Liver Protein Conjugate of a Hepatic Carcinogen†

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ABSTRACT: Certain cellular proteins of unknown function are preferred targets of chemical carcinogens during cell transformation to malignancy *in vivo* and in culture. In the present study, the principal species of liver protein–carcinogen conjugate (h_2-5*S* azoprotein) was reproducibly isolated 88–91% pure in 50-mg amounts from livers of rats fed the hepatic azocarcinogen, 3′-methyl-4-dimethylaminoazobenzene. The molecular weight of the azoprotein subunit was determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and by amino acid analysis to be 44,000. The azoprotein consists of two indistinguishable subunits that are not disulfide linked in a molecule of 88,000 molecular weight. The azoprotein molecule contains an average of two bound azocarcinogen residues per two subunits. The limiting amino acids in the protein are tyrosine, tryptophan, and methionine, which are present to the extent of two, two, and five residues per subunit, respectively. This characterization of the principal azoprotein of rat liver provides the basis of its ultimate identification, and for the subsequent determination of the possible importance of the carcinogen–protein interaction in liver carcinogenesis by aminoazo dyes.

The alteration of certain unknown cellular macromolecules by chemical carcinogens is presumed to be essential in carcinogenesis. Particular cellular proteins are preferred targets of chemical carcinogens *in vivo* and in cell culture. During transformation to malignancy caused by aminoazo dyes and by *N*-2-fluorenylacetamide (2-acetylaminofluorene) in rat liver (Sorof *et al.*, 1963, 1969, 1970), and by polycyclic hydrocarbons in mouse skin and cell culture (Tasseron *et al.*, 1970; Kuroki and Heidelberger, 1972), carcinogen derivatives covalently interact mainly with few protein species. The resulting conjugates, which are present in largest amount, are in the cytosols of these organs and cells, and belong to the electrophoretic classes of relatively basic proteins, termed "*h*."

The principal species of conjugate found in liver after the

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ingestion of hepatocarcinogenic aminoazo dyes by rats is the "h_2-5S" azoprotein[1] (Sorof *et al.*, 1963, 1970). It derives from a normal protein (target protein) whose presence in liver cytosol (Sani *et al.*, 1972) and whose nature (Sorof *et al.*, 1973; Sani *et al.*, 1974b) have in part recently been determined, but whose identity and function are unknown. This paper describes in detail the purification and properties of the principal azoprotein conjugate. A preliminary report of part of these studies has appeared (Sorof *et al.*, 1972).

Materials and Methods

Rats, Diet, and Liver Cytosol. Adult CFN rats (Carworth) of both sexes were fed *ad libitum* for 15–18 days a synthetic version of diet 3 of Miller *et al.* (1948) containing 18% casein, 1.0 mg of riboflavine/kg, and 0.058% of the hepatic azocarcinogen, 3′-Me-DAB[2] (Eastman Kodak Co.). Livers were perfused with 0.25 M sucrose at 1–4°, passed through a commercial coarse meat grinder with stainless steel mesh, and then homogenized in 0.25 M sucrose solution (1:1, w/v; Potter-Elvehjem). Liver cytosol was isolated and stored at −60° (Sorof and Young, 1967; Sorof *et al.*, 1972).

Preparation of Column Support Materials. Water used as reagent in this study was redistilled in glass vessels. Prior to each use, 1–1.5 kg of CM-cellulose (Gallard-Schlesinger, Serva, 0.67–0.79 mequiv/g) was washed four times, each time with at least 25 l. of 0.5 M KOH for 5–10 min, followed by rapid filtration using two immersion suction funnels (11 cm, i.d.). The exchanger was then likewise successively washed in at least 10 volumes of water, 1.0 M HCl, and water. If the exchanger had previously been unused, the KOH solution in the last wash cycle contained 0.02 M EDTA. The day prior to use, the suspension was adjusted to pH 7.0 with NaOH, similarly washed three times with the chromatographic starting buffer, packed, and eluted with the same buffer overnight for equilibration; the protein was applied to the column the following day.

Ethanolized cellulose for zonal electrophoresis and Sephadex G-200 for gel filtration were pretreated as previously described (Sorof *et al.*, 1963, 1966, 1970; Sorof and Young, 1967).

Purification of Principal Liver Azoprotein. The purification of the h_2-5S azoprotein was monitored by measurements of protein-bound azo dyes and protein, as described in the section on "assays." Each of three isolations (A, B, and C) was carried out starting with *ca.* 2 l. of liver cytosol containing 76 to 82 g of proteins from 2.3 to 3.9 kg of liver from 291 to 335 rats. The purifications were performed at 1–4° over a period of 35 days, and involved the following four consecutive steps.

Step 1: CM-cellulose Chromatography in Veronal–Chloride Buffer. The cytosols were dialyzed against six successive volumes of 24 l. of 0.002 M sodium veronal buffer (pH 7.0) + 0.003 M NaCl (total 45 hr). The losses attending the dialysis and subsequent centrifugation for clarification reduced the amount of protein to *ca.* 62 to 70 g (Table I). The protein was then applied to a column of CM-cellulose (31 cm, height × 15.5 cm, i.d.) equilibrated with the same veronal–sodium chloride buffer. Elution was carried out with 10 l. of this buffer, fol-

TABLE I: Purification and Recoveries in the Isolation of the Principal Azoprotein (h_2-5S) of Livers of Rats Fed a Hepatic Azocarcinogen.

	Step 1 Chromatography–Veronal			Step 2 Chromatography–Tris			Step 3 Electrophoresis			Step 4 Gel Filtration		
	A	B	C	A	B	C	A	B	C	A	B	C
Protein applied to the column (biuret) (g)	65.7	69.9	61.7	2.72	1.14	1.55	0.63	0.34	0.56	0.27	0.14	0.11
Recovery of total protein (%)	101[a]	91[b]		92[b]	83[b]	89[b]	86[a]	81[a]	101[a]		102[c]	111[c]
Recovery of total azo dye (%)	79	131		100	101	91	104	95	99	102	96	
Recovery of protein in pool (biuret) (%)	4.7	2.3		34	48	56						
Recovery of protein in pool (u.v.) (%)	4.2[a]	1.9[b]	2.6[a]	27[b]	29[b]	41[b]	40[a]	35[a]	15[a]	30[c]	42[c]	60[c]
Recovery of azo dye in pool (%)	17	15	12	53	61	62	89	77	56	53	60	71
Overall dye purification factors[d]	16	29	18	30	54	30	58	104	109	106	146	114
Overall recovery[e] of azo dyes, relative to												
(a) azo dyes applied to column in step 1 (%)	2.2	1.9	2.6									
(b) azo dyes in liver cytosol (%)	1.7	1.6	2.0									

[a] Based on absorbancies at 284 nm. [b] Based on absorbancies at 280 nm. [c] Based on absorbancies at 235 nm. [d] Factors are based on the dye concentrations (A_{525}/A_{284}) of the liver cytosol proteins applied to the column in step 1 of the isolation. Purification factors include those of previous steps. Calculations involve use of the values that 47% of the protein-bound dyes of liver cytosol belong to the slow h_2 azoprotein (Sorof *et al.*, 1963), and that 54% of the latter's azo dyes belong to the 5S azoprotein (Sorof *et al.*, 1970). [e] Overall recoveries include losses incurred in ultrafiltrations, assays, etc.

[1] The h_2-5S azoprotein belongs to the slow h_2 electrophoretic class (Sorof *et al.*, 1963) and to the 5S molecular size class (Sorof *et al.*, 1970) of liver cytosol proteins of rats fed azocarcinogen.

[2] Abbreviations used are: 3′-Me-DAB, 3′-methyl-4-dimethylaminoazobenzene; SDS, sodium dodecyl sulfate.

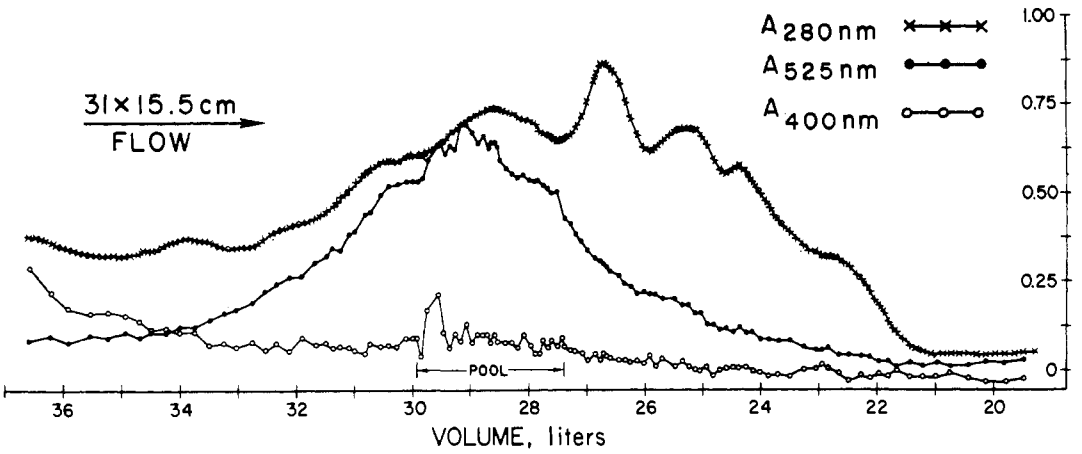

FIGURE 1: Chromatographic profiles of the soluble liver azoproteins eluted from CM-cellulose by Veronal–chloride buffer and a gradient of NaCl (step 1). 70 g of liver cytosol proteins (2225 ml; 31.4 mg/ml) was chromatographed. Absorptions at 525 nm (azo dye) and 400 nm (interfering hemochromogens) are plotted in terms of 3.0 ml of eluate lyophilized and dissolved in 1.0 ml of formic acid (azo dye assay). The fractions pooled for further purification of azoprotein are indicated. Details are described in the text.

lowed by 32 l. of a continuous linear salt gradient starting with that buffer and ending with 0.10 M NaCl in the veronal buffer. Zoning was improved by use of a Hg-sealed Teflon stirrer rotating (10 rpm) immediately above the surface of the cellulose. The main azoprotein fractions, located by assay in the protein profile (Figure 1), were pooled (between *ca.* 0.026–0.041 M NaCl) and concentrated by ultrafiltration (Amicon Corp., UM-10) to 50–70 ml and 22–39 mg of protein per ml.

Step 2: CM-cellulose Chromatography in Tris-Chloride Buffer. The combined fractions were dialyzed against four successive volumes of 4 l. of 0.01 M Tris-Cl buffer (pH 7.0) (total 45 hr), clarified by centrifugation, and applied to a column of CM-cellulose (30 cm × 2.2 cm, i.d.) which had been equilibrated with the Tris-Cl buffer. The column was washed with 100 ml of the buffer, and eluted with 800 ml of a linear gradient of KCl, starting with the Tris-Cl buffer and ending with the buffer containing 0.145 M KCl. The selected pool of the principal azoprotein (spanning *ca.* 0.005–0.063 M KCl) in the protein profile (Figure 2) was ultrafiltered to 5–10 ml and 26–75 mg of protein per ml.

Step 3: Column Electrophoresis. The proteins isolated in step 2 were dialyzed against four successive volumes of 3 l. of 0.02 M sodium veronal buffer (pH 8.6) + 0.03 M NaCl (total 24 hr), and then electrophoresed in a column of ethanolyzed cellulose (95 cm × 3.1 cm, i.d.) at 80 mA for 165 hr (Sorof *et al.*, 1963; Sorof and Young, 1967). The eluted pool of the principal azoprotein (slow h_2) in the protein profile (Figure 3) was adjusted to pH 7.4 with solid Tris-HCl, and ultrafiltered to 4–8 ml which contained 80–268 mg of protein.

Step 4: Gel Filtration. The slow h_2 proteins isolated in step 3 were dialyzed (for ultraviolet spectrophotometry) against three successive volumes of 3 l. of 0.01 M Tris-Cl buffer (pH 7.4) + 0.20 M NaCl (total 24 hr). The proteins were filtered through a column of Sephadex G-200 gel (212–219 cm × 3.4 cm, i.d.) in this buffer (Sorof *et al.*, 1966, 1970; Sorof and Young, 1967). The protein profile contained 1 peak of azoprotein (Figure 4). Its retardation corresponded to a molecular size (60,000–80,000) which was indistinguishable from that previously found with the principal azoprotein of whole liver extract of rats fed azocarcinogen (Sorof *et al.*, 1970). The retardation also corresponded to that of the *5S* component in the molecular size profile of normal rat liver cytosol macromolecules, which mixture was resolved as reference components immediately following each isolation of the h_2-*5S* azoprotein, using the same packed Sephadex G-200 column (Sorof *et al.*, 1966, 1970; Sorof and Young, 1967).

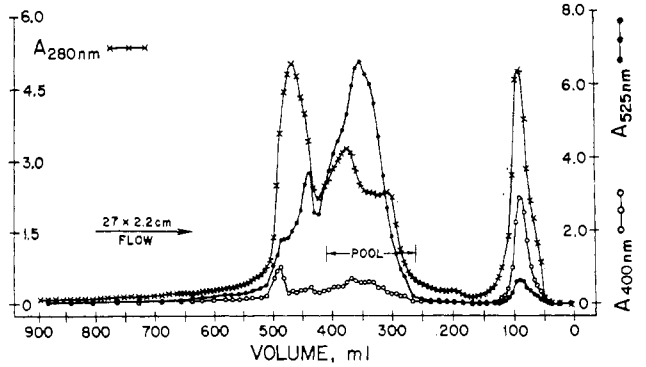

FIGURE 2: Chromatographic profiles of the soluble liver azoprotein fraction eluted from CM-cellulose by Tris-chloride buffer and a gradient of KCl (step 2). The proteins isolated in step 1 (1.1 g in 50 ml) were used as starting material. Absorptions at 525 nm and 400 nm are expressed in the same terms as in the legend of Figure 1. The fractions collected for further purification of azoprotein are indicated (POOL). Details are in the text.

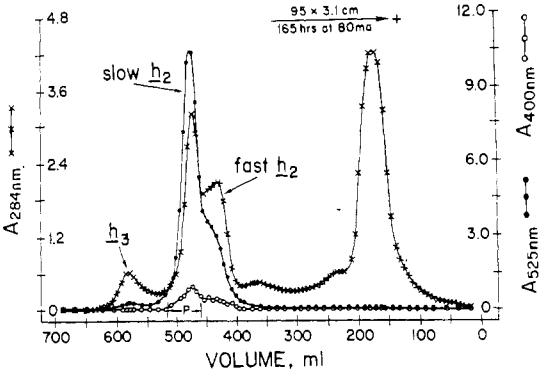

FIGURE 3: Column electrophoretic profiles of the soluble liver azoprotein fraction (step 3). The proteins isolated in step 2 (559 mg in 9.6 ml) were electrophoresed in 0.02 M sodium veronal buffer (pH 8.6) + 0.03 M NaCl and eluted. Components are labeled as belonging to the corresponding electrophoretic classes of liver cytosol. Absorptions at 525 and 400 nm are in the same terms as in the legend of Figure 1. The fractions (P) pooled for further purification of azoprotein are shown. Details are provided in the figure and text.

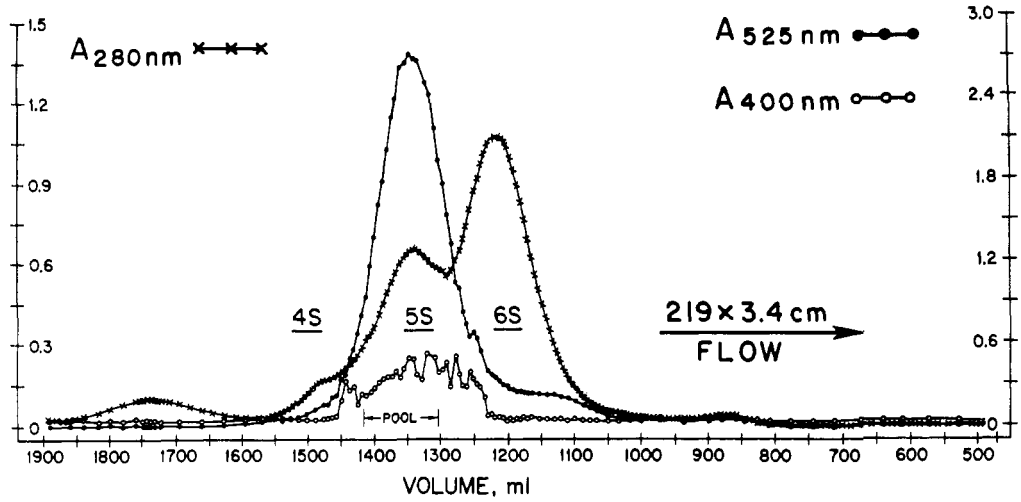

FIGURE 4: Molecular size profiles of the soluble liver azoprotein fraction (step 4). The proteins obtained in step 3 (268 mg in 7.6 ml) were resolved in Sephadex G-200 gel in 0.01 M Tris-Cl buffer (pH 7.4) and 0.20 M NaCl. Components are labeled as belonging to the corresponding molecular size classes of liver cytosol. Absorptions at 525 and 400 nm are as defined in the legend of Figure 1. The purified h_2-*5S* azoprotein was present in the indicated pool of fractions. Details are stated in the text.

The selected pool of azoprotein (Figure 4) was ultrafiltered to 4.5–8.8 ml which contained 52–61 mg of purified h_2-*5S* azoprotein.

Analytical Gel Electrophoresis. The purity and the subunit molecular weight of the h_2-*5S* azoprotein were examined by electrophoresis in 10% polyacrylamide gels containing SDS, according to the procedure of Weber and Osborn (1969).

In method I, the protein was incubated at 37° for 16 hr in 4 M urea (ultrapure), 10 mg/ml of SDS, 1% (v/v) β-mercaptoethanol, and 10 mM $NaPO_4$ (pH 7.0). After the 10% acrylamide and 0.27% bisacrylamide were polymerized for at least 1 hr in 1 mg/ml of SDS, 50 mM $NaPO_4$ (pH 7.0), and 0.67 mg/ml of ammonium persulfate, the gels (90 mm or 140 mm × 5 mm) were prerun (6 mA, 30 min; or 2.75 mA, 50 v, 30 min) to remove persulfate ion. The h_2-*5S* azoprotein was then migrated 3 cm at 6 mA/tube, or 7 cm at 2.75 mA/tube (1200 min).

Method II was like that above, except that the protein was alkylated for improved stability. The protein was reduced in 50 mM β-mercaptoethanol, 8 M urea (ultrapure), and 10 mM $NaPO_4$ at pH 7.0 (30 min, 25°), and then was alkylated in 0.1 M iodoacetamide (30 min, 25°). The excess reagent was consumed with 0.2 M β-mercaptoethanol (>30 min, 25°). SDS (10 mg/ml) was added, and gel electrophoresis was carried out as in method I.

Gels were stained for protein with Coomassie Brilliant Blue R-250 (Weber and Osborn, 1969) and scanned at 600 nm with a Gilford spectrophotometer, and the area under absorption peaks measured by planimetry. Likewise, duplicate gels were stained for azo dyes with 5% trichloroacetic acid, and scanned at 520 nm. The determination of molecular weight of the h_2-*5S* azoprotein subunit (treated by methods I and II) was based on a linear plot of the relative mobilities of eight standard purified proteins (treated by method I) in gel electrophoresis. The standard proteins consisted of: crystallized lysozyme (egg white) from Pentex Corp.; twice crystallized trypsin (bovine pancreas), twice crystallized pepsin (porcine stomach), and twice crystallized ovalbumin, all from Worthington Biochemical Corp.; carbonic anhydrase (bovine erythrocytes), crystallized L-glutamate dehydrogenase (bovine liver), and bovine plasma transferrin, all from Schwartz/Mann Laboratories; and crystallized albumin (bovine serum) from Armour Laboratories.

Preparative Gel Electrophoresis. For the purpose of obtaining h_2-*5S* azoprotein for amino acid analysis, the isolated azoprotein was further purified as the subunit to a state of apparent homogeneity by means of preparative gel electrophoresis under conditions of method I as follows. The azoprotein (420 μg of each preparation A and B) was incubated in the solution containing urea, SDS, and β-mercaptoethanol, and loaded on seven gels (90 mm × 5 mm). The yellow band of the azoprotein was allowed to migrate electrophoretically *ca.* 7 cm (440 min), and then was excised. The gel slices were homogenized with 1 ml of electrophoretic buffer containing 1 mg/ml of SDS by means of a number of passes through a 5-ml syringe. The yellow azoprotein in the gel–liquid suspension was then collected by electrophoretic migration through a 10% polyacrylamide gel plug (40 mm × 5 mm) into closed dialysis tubing. The recovery of the azoprotein was 55 and 58% by Folin assay.

Amino Acid Analyses. Protein samples were hydrolyzed in constant-boiling HCl solution at 110° for 72 hr in vials which had been evacuated to less than 50 μ of Hg pressure and hermetically sealed (Moore and Stein, 1963). Samples applied to the amino acid analyzer were equivalent to between 10 and 40 μg of hydrolyzed protein. Cysteine and cystine were determined as cysteic acid in hydrolysates of performic acid oxidized protein (Moore, 1963). Tryptophan was determined on protein samples which were hydrolyzed in constant-boiling HCl solution containing 4% (v/v) mercaptoacetic acid in vials sealed in a vacuum of 25 μ of Hg pressure (Matsubara and Sasaki, 1969). Analyses of amino acid composition were carried out on a Spinco Model 120C machine using a two column chromatographic system (Moore and Stein, 1963). The machine's optical system and recorder measuring circuit were modified for high sensitivity.

Levels of threonine, serine, tyrosine, and cysteic acid were obtained from duplicate analyses of each protein preparation (A and B). Tryptophan was determined in single assays. All other amino acid values were derived correspondingly from quadruplicate analyses. Data for threonine, serine, and tyrosine were extrapolated to zero hydrolysis time, assuming first-order kinetics (Moore and Stein, 1963). The level of methionine sulfone present in the hydrolysate of the formic acid oxidized protein confirmed the methionine content as obtained after hydrolysis of nonoxidized protein. The re-

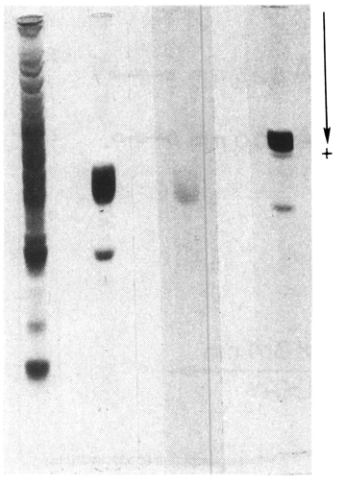

FIGURE 5: Protein purity of the h_2-*5S* azoprotein according to gel electrophoresis in SDS. All samples were reduced with β-mercaptoethanol, not alkylated, and resolved in gels (90 mm × 5 mm) in the direction indicated by the arrow, all as in method I in the text. Gels (a)–(c) were run simultaneously. Gels (a), (b), and (d) were stained for protein with Coomassie Brilliant Blue; gel (c) was stained for azo dyes in 5% trichloroacetic acid: sample (a), 40 μg of liver cytosol proteins of rats fed the azocarcinogen for 17 days; sample (b), 20 μg of h_2-*5S* azoprotein (preparation A); sample (c), 60 μg of h_2-*5S* azoprotein (preparation A); sample (d), 4 μg of h_2-*5S* azoprotein (preparation A) was resolved separately from the others.

covery of tryptophan was assumed to be 90%, in acordance with the results of Matsubara and Sasaki (1969).

Assays. Protein concentration in profiles was measured spectrophotometrically at 280 and 284 nm (Sorof *et al.*, 1963), and in protein pools by biuret or Folin methods. After lyophilization, protein azo dyes were spectrophotometrically assayed in 88% formic acid at 525 nm, and interfering substances were measured at 400 nm (Gelboin *et al.*, 1958; Sorof and Young, 1967). Absorption spectra of the azoprotein and of 3′-Me-DAB were obtained using a Model 15 Cary automatic recording spectrophotometer. The molar ratio of azo dye in h_2-*5S* azoprotein was determined in 88% formic acid using 3′-Me-DAB as reference compound.

The h_2-*5S* azoprotein was analyzed for tightly bound paramagnetic metals and zinc in collaboration with Dr. A. S. Mildvan of this Institute. The h_2-*5S* azoprotein used in nmr measurements was at a concentration of 8.0 mg/ml (91 mM) in 0.2 μM NaCl, 2 mM EDTA, and 10 mM Tris-HCl buffer (pH 7.4). In the determinination of paramagnetic metals, the longitudinal relaxation rate of the protons of water was determined at 22° at 24.3 MHz using a Nuclear Magnetic Resonance Specialties PS60W pulsed nmr spectrometer, as previously described (Scrutton *et al.*, 1966; Mildvan and Engle, 1972). The paramagnetic contribution to the longitudinal relaxation rate was calculated as the difference between the longitudinal relaxation rates of the sample and of the buffer medium. Electron paramagnetic resonance measurements were made on the same solutions at 22° using a Varian E-4 epr spectrometer as described by Mildvan and Engle (1972). The above azoprotein solution was diluted to 1.0 μM with 0.2 M NaCl containing 10 mM Tris-HCl buffer (pH 7.4) and then analyzed for zinc by atomic absorption spectroscopy utilizing the Varian Techtron instrument equipped with a carbon rod.

Results

Isolation of the h_2-5S Azoprotein. The four successive steps in the fractionation yielded the eluent profiles of protein and azoproteins which are shown in Figures 1–4. The fractions pooled for further purification and as final product are indicated.

FIGURE 6: Azo dye distribution of the h_2-*5S* azoprotein as resolved by gel electrophoresis in SDS. Sample (c) of Figure 5 was migrated from position S, and stained for azo dyes in 5% trichloroacetic acid, and scanned spectrophotometrically at 520 nm.

Table I lists the quantitative data pertaining to the starting amounts and recoveries of protein and protein-bound azo dyes in the three preparations, A–C. The recoveries of protein and of protein-bound dyes in each of the profiles of the four steps were 79% or more. The overall recovery of bound azo dyes in the final azoprotein pool of step 4 was 1.9–2.6% relative to the bound dyes of the starting mixture of step 1, and 1.6–2.0% relative to those of the original liver cytosols. In addition, the purification factors of the h_2-*5S* azoprotein, based on the content of bound azo dyes at the start of step 1, are presented in Table I for each step of the three preparations. The overall purification of the h_2-*5S* azoprotein was 106- to 146-fold relative to its content in liver cytosol. Inasmuch as *ca.* 40% of the liver proteins are soluble in isotonic sucrose, the purification from liver was *ca.* 265- to 365-fold, assuming no h_2-*5S* azoprotein to be present in other subcellular fractions.

Purity of the h_2-5S Azoprotein. Gel electrophoresis of the azoprotein (2–60 μg) in SDS, after reduction with β-mercaptoethanol without alkylation (method I), revealed the presence of one main component (h_2-*5S* azoprotein subunit) and small amounts of two contaminants (Figure 5b and d). This degree of purity is to be compared to the considerable protein heterogeneity displayed in a parallel analysis of the whole liver cytosol from which the h_2-*5S* was isolated (*cf.* Figure 5a and b). That the main protein is the azoprotein subunit was established by its content of azo dyes, as evidenced by the pink coloration at the position of the main component in a parallel run when the gels were soaked in 5% trichloroacetic acid (*cf.* Figure 5b and c). A spectrophotometric scan at 520 nm confirmed the homogeneity of the acid-stained azoprotein component (Figure 6). The similar detection of the azo dyes of the h_2-*5S* azoprotein in the liver cytosol was not possible, inasmuch as it would have required an appreciable protein overloading of the gel, with consequent obliteration of discernible resolution.

Without reduction by mercaptan, the h_2-*5S* azoprotein subunit exhibits a microheterogeneity. This microheterogeneity can be lessened by prior reduction with 1% (v/v) β-mercaptoethanol for 1 hr at 37°, further lessened by an additional incubation with this reagent for 8 days at 25°, and almost eliminated if the reduction is followed by alkylation with iodoacetamide, both according to method II. The identical subunit size of the h_2-*5S* azoprotein without and with reduction by β-mercaptoethanol indicates that the subunits are not disulfide linked.

The isolated azoprotein was determined to be 88–91% pure according to disc gel electrophoresis in SDS gels. The reduced and alkylated protein, stained with Coomassie Brilliant Blue, also contained a "minor" component to the extent of 4–8%, and a "trace" component of 3–4% (Figure 7). The high degree of purity of the h_2-*5S* azoprotein following reduction and alkylation was also confirmed by disc gel electrophoresis in

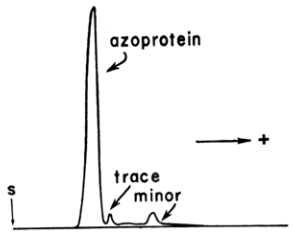

FIGURE 7: Protein profile of h_2-5S azoprotein as resolved by gel electrophoresis in SDS. A 10-μg sample of reduced and alkylated protein was resolved from the starting position S according to method II as described in the text. Proteins were stained with Coomassie Brilliant Blue and scanned spectrophotometrically at 600 nm.

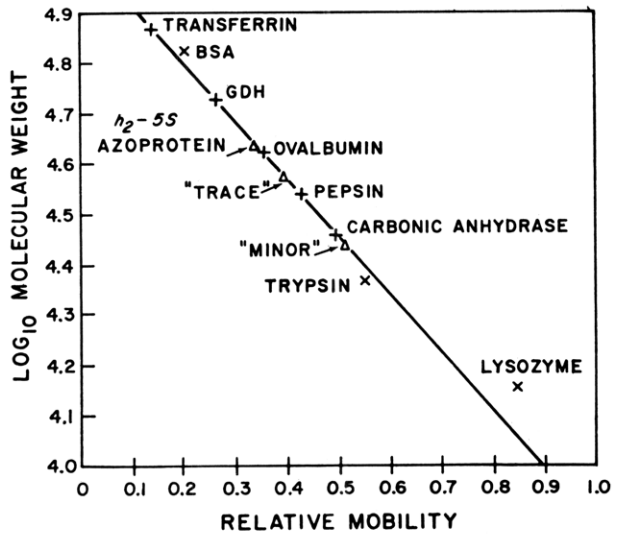

FIGURE 8: Linear plot in the estimation of the molecular weight of the h_2-5S azoprotein subunit by SDS–polyacrylamide gel electrophoresis. Mobilities of marker proteins were calculated relative to the migration of the tracking dye, Bromophenol Blue. The arrows indicate the relative mobilities of the azoprotein subunit, and of the trace and minor contaminants.

acetic acid and urea according to the method of Panyim and Chalkley (1969).

Molecular Weight of the h_2-5S Azoprotein Subunit. The molecular weight of the h_2-5S azoprotein subunit after reduction and alkylation was found by SDS gel electrophoresis to be 44,000 ± 1000 (S.D.). This size is the mean of six assays of the three h_2-5S azoprotein preparations (A–C), and is based on six separate plots of the relative migration rates of eight standard proteins.[3] A sample plot is shown in Figure 8. When the azoprotein was not reduced and alkylated prior to electrophoresis, slightly smaller molecular weight values (*ca.* 40,000) were obtained, possibly due to conformational differences caused by intramolecular disulfide linkages.

The h_2-5S azoprotein thus appears to be a dimer with two subunits of essentially identical size of 44,000. This conclusion derives from the presence of only one species of subunit, whose size is approximately one-half of the estimated molecular weight of the whole molecule as determined previously by gel filtration (60,000–80,000) (Sorof *et al.*, 1970).

Amino Acid Composition. The h_2-5S azoprotein, further purified especially for amino acid analysis, was examined by SDS–polyacrylamide gel electrophoresis according to method I. The h_2-5S azoprotein was homogeneous by this criterion. A single protein band and absorption peak was

[3] The molecular weight of the trace component was 38,000 ± 600, and that of the minor component was 27,000 ± 1100.

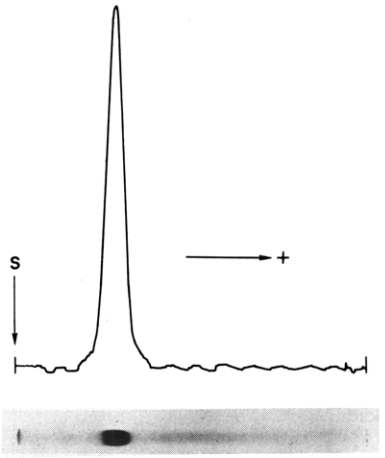

FIGURE 9: Test of purity of the h_2-5S azoprotein used for amino acid analysis. The h_2-5S azoprotein, at 88–91% purity, was further purified by preparative gel electrophoresis, as described in the text. The product was then analyzed electrophoretically in SDS in polyacrylamide gels according to method I. Protein in gels was stained with Coomassie Brilliant Blue R-250: sample (a), 5 μg of purified h_2-5S azoprotein (preparation B); sample (b), 9 μg of purified h_2-5S azoprotein (preparation A). In juxtaposition is a spectrophotometric scan of the stained protein in this gel as measured at 600 nm.

obtained when the gels were stained for protein and scanned at 600 nm (Figure 9).

The amino acid composition of the homogeneous h_2-5S azoprotein is presented in Table II. The amino acid data for

TABLE II: Amino Acid Composition of the Principal Azoprotein (h_2-5S) of Livers of Rats Fed a Hepatic Azocarcinogen.

Amino Acid	Preparation A mol %	Preparation B mol %	Average mol %	Average Residues per subunit
Lys	7.0	7.4	7.2	31.1
His	2.3	2.4	2.4	10.4
Arg	3.0	3.1	3.0	12.9
Asp	7.7	6.3	7.0	30.2
Thr[a]	6.2	5.6	5.9	25.5
Ser[a]	6.7	6.0	6.4	27.6
Glu	6.8	5.4	6.1	26.3
Pro	6.6	6.6	6.6	28.5
Gly	13.0	15.3	14.2	61.3
Ala	9.8	9.3	9.6	41.4
Val	9.0	10.2	9.6	41.4
Met	1.2	1.0	1.1	4.7
Ile	6.6	6.7	6.6	28.5
Leu	7.6	7.3	7.4	31.9
Tyr[a]	0.45	0.49	0.47	2.0
Phe	2.7	3.4	3.0	12.9
Try[b]	0.45	0.59	0.52	2.2
$^1/_2$-Cystine as cysteic acid[c]	3.0	2.8	2.9	12.5

[a] Data from 72-hr hydrolysate were corrected to zero hydrolysis time, using the first-order correction described by Moore and Stein (1963). [b] Tryptophan was determined in constant-boiling HCl containing 4% mercaptoacetic acid (Matsubara and Sasaki, 1969). [c] Performic acid oxidized protein (Moore, 1963) was hydrolyzed and analyzed for cysteic acid.

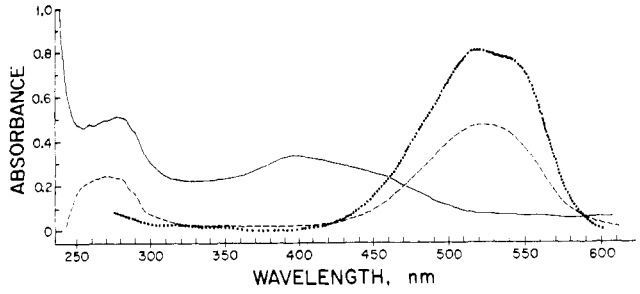

FIGURE 10: Absorption spectra of the h_2-*5S* azoprotein and the azocarcinogen, 3′-Me-DAB: (—) 750 μg/ml of h_2-*5S* azoprotein in 0.1 M NaCl + 0.01 M $NaPO_4$ (pH 7.4); (- - -) 547 μg/ml of h_2-*5S* azoprotein in 88% formic acid; (· · ·) 2 μg of 3′-Me-DAB per ml of 88% formic acid.

the two preparations A and B were in reasonable agreement. No evidence was obtained for the presence of a unique amino acid derivative which might reflect covalently bound azo dye. The azo dye apparently did not survive the hydrolysis in the hot HCl.

The limiting levels of amino acid residues in the azoprotein were those of tyrosine, tryptophan, and methionine, which occurred in the ratio of approximately 2:2:5, respectively. Accordingly, if the azoprotein subunit contains two tyrosine, two tryptophan, and five methionine residues, the corresponding subunit molecular weight based on amino acid composition has a calculated value close to 43,600. This result is in good agreement with the above observed subunit molecular weight of 44,000.

Absorption Characteristics of the h_2-5S Azoprotein. The h_2-*5S* azoprotein exhibits absorption properties due to its bound azo dye (Figure 10). In 88% formic acid, the azoprotein has a characteristic absorption maximum at 522 nm, which agrees with that at 520 nm of the 3′-Me-DAB that was fed to the rats. In 0.1 M NaCl + 0.01 M sodium phosphate buffer (pH 7.4), the azoprotein has a maximum at 397 nm due to the bound azo dye, and a maximum at 278 nm due to its aromatic amino acids.

Content of Bound Carcinogen in the h_2-5S Azoprotein. The amount of azo dyes bound in a mole of h_2-*5S* azoprotein was determined spectrophotometrically at 520 nm using the carcinogen, 3′-Me-DAB, as reference compound. Two preparations (A and B) of the azoprotein, measured at concentrations[4] of 302 and 404 μg/ml in 88% formic acid, had absorbancies of 0.450 and 0.502. Based on an observed molar absorbance of 3′-Me-DAB of 62,000 in the same medium, and the molecular weight of the h_2-*5S* azoprotein of 88,000, the ratio of moles of azo dye per mole of protein was determined to be 1.76 and 2.10, with an average of 1.93, corresponding to an average distribution of 1 residue of bound azo dye per subunit.

Bound Metals in the h_2-5S Azoprotein. The protons of water in a solution of the azoprotein at 8 mg/ml (91 μM) showed a longitudinal nuclear relaxation rate of 0.453 sec^{-1}, which on addition of 2 mM EDTA decreased to 0.428 sec^{-1}, a value only slightly greater than that of the buffer, 0.424 sec^{-1}. Hence, the paramagnetic contribution to the relaxation rate in the absence of EDTA was used to estimate the total paramagnetic metal concentration, while this parameter in the presence of EDTA was used to estimate the concentration of paramagnetic metals tightly bound to the protein (Scrutton *et al.*, 1966; Mildvan and Engle, 1972). From the value in the absence of EDTA, it was calculated that the azoprotein contains less than stoichiometric quantities of Fe^{3+} (≤0.027 Fe/mol), Mn^{2+} (≤0.038 Mn/mol), Cu^{2+} (≤0.36 Cu/mol), but could contain ≤1.53 Co^{2+}/mol if the entire effect were due to Co^{2+}. In the presence of EDTA, all of these limiting values were reduced to 14% or less of the above levels. Further, these residual effects observed with EDTA could be due to the viscosity of the protein solution. Hence, the azoprotein contains less than stoichiometric amounts of tightly bound paramagnetic metals. This point is further supported by the absence of detectable epr signals of the azoprotein over that seen with the buffer, an observation that argues against the presence of Cu^{2+} or Mn^{2+}.

The presence of Zn^{2+}, a diamagnetic metal, was not excluded by the above measurements, and was therefore assayed by atomic absorption spectroscopy. A solution of the azoprotein (1.0 μM) in 0.2 M NaCl containing 10 mM Tris-HCl buffer (pH 7.4) had 0.99 ± 0.10 μM Zn. Correction for the Zn content of the buffer (0.36 ± 0.05 μM) yielded a value of 0.63 ± 0.11 Zn atom/mole of azoprotein.

Discussion

This report describes the isolation of a principal species of carcinogen–protein conjugate from liver undergoing transformation to malignancy. The h_2-*5S* azoprotein has been reproducibly isolated to 88–91% purity in 50-mg amounts from livers of rats fed the hepatic azocarcinogen, 3′-Me-DAB. The way is open to the further characterization of this conjugate, and to the identification of the normal target protein from which the conjugate derives. This information is prerequisite to the determination of whether or not the alteration of the normal protein resulting from the interaction with carcinogen is involved in liver carcinogenesis by aminoazo dyes.

The carcinogen–protein conjugate which has been purified is the principal azoprotein, *i.e.*, the h_2-*5S* azoprotein, of liver cytosol of rats fed azocarcinogen. The necessity for its positive identification as such stems from the existence also of a variety of azoproteins which are present in minor amounts in liver cytosol (Sorof *et al.*, 1963, 1970). That the isolated azoprotein is the principal azoprotein (h_2-*5S*) is supported by the following. (a) The purification procedure involved the isolation of the h_2 proteins, from which the *5S* azoprotein was subsequently isolated. (b) Specific antiserum prepared against the purified azoprotein precipitates only an h_2 protein in electrophoretic profiles of liver cytosol, and only a *5S* protein in corresponding molecular size profiles (Sani *et al.*, 1974b).

The observed size of 44,000 for the molecular weight of the h_2-*5S* azoprotein subunit is in good agreement with the value of its minimum molecular weight as based on amino acid composition. The subunit size is also compatible with the molecular weight of 60,000–80,000 for the whole molecule, as previously estimated by gel filtration (Sorof *et al.*, 1970). Collectively, the evidence indicates that the h_2-*5S* azoprotein has a molecular weight of 88,000, and is composed of two similar subunits that are not disulfide linked. The stoichiometry of two azo dye residues in the two subunits of the azoprotein of molecular weight 88,000 is further supportive of the assembled data. On the other hand, the possibility exists that the purified h_2-*5S* azoprotein may

[4] The determination of the concentration of h_2-*5S* azoprotein incorporated two corrections of the Folin protein assay: one indicated by the amino acid analyses of pure h_2-*5S* azoprotein; the other allowed for the 88 and 91% purity of the h_2-*5S* azoprotein in preparations A and B, respectively. The first correction resulted in a greater molar ratio of azo dye per molecule than the value (1.4) reported preliminarily (Sorof *et al.*, 1972).

actually be a mixture of azoprotein with more than two azo dye residues, and of its normal target protein. This surmise arises from the closely similar, if not identical, molecular charge and size of the azoprotein and normal target protein (Sani *et al.*, 1974b). Inasmuch as the azoprotein was separated on the basis of these properties, and since liver cytosol of rats fed azocarcinogen likely contains both forms of the protein, the isolated azoprotein may likewise.

It is of interest that the three amino acids, tyrosine, tryptophan, and methionine, which are limiting in amount in the h_2-*5S* azoprotein (this report), are 3 of the 4 previously known target amino acids of the azocarcinogens (Miller and Miller, 1969; Miller, 1970). If it will be found that one (or more) of these three is the target of azocarcinogen, then the low content of both the carcinogen and of that amino acid in the conjugate will be of considerable assistance in determining their locations in the primary structure of the azoprotein. On the other hand, the presence of 12 half-cystine residues per subunit increases the likelihood that cysteine may be the target amino acid in the h_2-*5S* azoprotein, Indeed, cysteine is the target in the interaction *in vivo* of azocarcinogen with the liver protein, ligandin (Ketterer and Christodoulides, 1969).

Ligandin is the only specific protein target of carcinogens *in vivo* which has thus far been identified. Ligandin is a minor target protein of the azo dyes (Ketterer *et al.*, 1967; Litwack and Morey, 1970), and probably is also a target of the carcinogenic polycyclic aromatic hydrocarbons (Tasseron *et al.*, 1970; Singer and Litwack, 1971). At present, the role of ligandin in cells and the consequences, if any, of its interaction with carcinogens are unknown, although ligandin has been suggested to serve as a sequestering agent, and as an intracellular transporting agent of hydrophobic molecules including certain steroids and carcinogens (Litwack *et al.*, 1971). In addition, the identity of the principal protein target of azocarcinogens in rat liver has not been established. Arginase was formerly speculated to be the target protein from which the h_2-*5S* azoprotein derives (Sorof *et al.*, 1967). This suggestion was rejected when it was learned that liver arginase and the azoprotein have different molecular weights (Sorof *et al.*, 1967) and subunit sizes (Sorof *et al.*, 1972). In addition, the amino acid composition of the h_2-*5S* azoprotein, as determined in the present study, differs from that of rat liver arginase (Hirsch-Kolb and Greenberg, 1968), and also incidentally from that of rat liver ligandin (Ketterer *et al.*, 1967; Morey and Litwack, 1969).

Liver alcohol dehydrogenase has also been proposed to be the principal protein target from which the h_2-*5S* azoprotein stems. Because "nothing dehydrogenase" and the pink color of the *h* azoproteins in acid overlapped in electrophoretic profiles in starch gels, Murray *et al.* (1972) suggested that liver alcohol dehydrogenase is a target of the azocarcinogens. It is difficult to evaluate the significance of the data inasmuch as "nothing dehydrogenase" activity is associated with three or more closely migrating components, *i.e.*, slow h_2, fast h_2, and slow h_1 (S. Sorof and E. M. Young, unpublished). In addition, Tokuma and Terayama (1973) recently isolated an azoprotein from the livers of rats given a single dose of azocarcinogen. The azoprotein preparation exhibited low alcohol dehydrogenase activity, and had an amino acid composition similar to those of horse, rat, and human liver alcohol dehydrogenases. On the basis of the similarity of the molecular and subunit sizes of liver alcohol dehydrogenase, of their isolated azoprotein, and of the h_2-*5S* azoprotein, they proposed that the principal protein target of the azocarcinogens in rat liver is alcohol dehydrogenase. Comparison of the amino acid composition of the h_2-*5S* azoprotein, as determined in the present study, with those of rat, horse, and human liver alcohol dehydrogenases (Jörnvall and Markovič, 1972) indicates that there is an overall similarity among them, although minor differences are evident. In addition, the presence of 0.63 ± 0.11 Zn atom in the h_2-*5S* azoprotein molecule (this report) may conceivably reflect in part the residue of four Zn atoms in liver alcohol dehydrogenase. On the other hand, our specific antiserum against the principal liver azoprotein (h_2-*5S*) fails to give any detectable precipitin band with horse liver crystalline alcohol dehydrogenase in gel double immunodiffusion, whereas the purified h_2-*5S* azoprotein does. This is so despite the approximate 80% homology in the amino acid sequences and peptides of the alcohol dehydrogenases of horse and rat livers (Jörnvall and Markovič, 1972), and the known immunological cross-reactivity of the two dehydrogenases in gel double immunodiffusion (Fuller and Marucci, 1972). Furthermore, while our specific antiserum precipitates sizeable amounts of the h_2-*5S* azoprotein from liver cytosol of rats fed azocarcinogen, the antiserum fails to precipitate any protein with alcohol dehydrogenase activity. Liver alcohol dehydrogenase thus appears not to be the principal protein target of azocarcinogens (Sani *et al.*, 1974a).

Two approaches to the identification of the target protein are feasible as a result of the present study. In the first, represented in part by this and other studies (Sani *et al.*, 1972, 1974b; Sorof *et al.*, 1973), both the conjugate and the target protein have been characterized, in order to limit the field of candidates as possible target protein. In the second, specific antiserum against the azoprotein has been used to determine: (a) the content of the target protein in various normal tissues (Sani *et al.*, 1972) and liver tumors (Mott *et al.*, 1973); and (b) the influence of the specific antiserum on the biochemical activities of known protein receptors and enzymes, in search of the functional role of the target protein (Sani *et al.*, 1974b). It is evident that the evaluation of the possible importance of the carcinogen–protein interaction in liver carcinogenesis by amino azo dyes must await the identification of the target protein, and the determination of the functional consequences, if any, of its interaction with carcinogen.

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3-(3-Amino-3-carboxypropyl)uridine: a Novel Modified Nucleoside Isolated from *Escherichia coli* Phenylalanine Transfer Ribonucleic Acid†

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ABSTRACT: An unknown modified nucleoside located in the extra-region of *Escherichia coli* $tRNA^{Phe}$ has been characterized as 3-(3-amino-3-carboxypropyl)uridine (4abu^{3}U). Its identity was established unambiguously by its ultraviolet absorption spectrum, thin-layer chromatographic and electrophoretic mobilities, chemical reactivity, mass spectra, and nuclear magnetic resonance spectrum, and by comparison with those of a chemically synthesized authentic sample.

The primary sequence of *Escherichia coli* $tRNA^{Phe}$ was previously reported by Barrell and Sanger (1969). It was shown that an unknown modified component designated as X was located in the extra-region. The exact chemical structure of X, hereafter designated as N*,[1] has not been determined. We observed that phenylalanine acceptor activity of *E. coli* $tRNA^{Phe}$ was extensively inactivated by chemical modification with cyanogen bromide, suggesting that a component other than 4-thiouridine, possibly N*, had reacted with cyanogen bromide (Saneyoshi and Nishimura, 1971). It was necessary to determine the structure of N* in order to understand its chemical reactivity, function, and biosynthesis.

We present evidence that N* is 3-(3-amino-3-carboxypropyl)-uridine (4abu^{3}U) (I). In order to characterize the structure of N*, the nucleoside was isolated in large scale from purified *E. coli* $tRNA^{Phe}$, and its properties were thoroughly examined. For final characterization, 4abu^{3}U was chemically synthe-

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[1] Abbreviations used are: 4abu^{3}U, 3-(3-amino-3-carboxypropyl)-uridine; N*, unidentified modificd nucleoside located in the extra-region of *E. coli* $tRNA^{Phe}$ and characterized as 4abu^{3}U; N**, a derivative of 4abu^{3}U found in small amount in *E. coli* $tRNA^{Phe}$; m^{7}G, 7-methylguanosine; s^{4}U, 4-thiouridine; Bz, benzoyl group; A_{260} unit, the amount of material giving an absorbance of 1.0 at 260 nm when dissolved in 1 ml of water and measured in a cell of 1-cm light path; M, molecular ion.