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Purification and Characterization of Mouse Hypoxanthine-Guanine Phosphoribosyltransferase*

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SUMMARY

Hypoxanthine-guanine phosphoribosyltransferase (HGPR transferase) (EC 2.4.2.8) has been purified approximately 4500-fold to apparent homogeneity from mouse liver. The procedure involves the use of affinity chromatography and was designed to be readily adaptable to small scale isolations. The enzyme appears to be composed of 3 subunits of identical molecular weight (27,000 per subunit). The subunit molecular weight has also been determined by the analysis of radioactively labeled HGPR transferase immunoprecipitated from wild type and mutant (HGPR transferase) mouse tissue culture cell lines.

The enzyme hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) catalyzes the conversion of hypoxanthine and guanine to their respective nucleotides IMP and GMP. It is surmised that HGPR transferase[1] functions in the intact cell as a salvage enzyme in purine metabolism. A number of features have focused interest on this enzyme. A serious clinical disorder in man, the Lesch-Nyhan syndrome, is caused by a hereditary loss of the enzyme. For somatic cell geneticists, HGPR transferase provides an ideal tool because selection techniques exist for the presence or absence of the enzymatic activity in tissue culture cell lines (1, 2). Our interest in the enzyme arose from the possibility of using this system for the isolation of nonsense suppressor mutants. These studies have been greatly facilitated by acquiring a knowledge of the physical and structural properties of the enzyme. Purified HGPR transferase also permitted the preparation of specific antiserum used in the genetic analysis of altered forms of the enzyme.

For economic reasons, HGPR transferase was isolated from mouse liver rather than mouse L cells grown in culture. The studies reported here show that the enzyme from L cells and mouse liver have identical subunit molecular weights and sedimentation properties. In a separate communication, we show that the two enzymes are immunologically indistinguishable.[2]

We have determined that the Stokes radius of mouse liver HGPR transferase is 36 A by Sephadex gel filtration. This size was also confirmed from studies of the migration of HGPR transferase in polyacrylamide gels of increasing gel concentration according to the methods of Hedrick and Smith (3). The sedimentation coefficient in sucrose gradients is 5 S. These data are most compatible with the assignment of a molecular weight of approximately 80,000 for native HGPR transferase. Analysis of the denatured molecule on calibrated sodium dodecyl sulfate gels indicates a single subunit of molecular weight 27,000. The native molecule therefore appears to be composed of 3 subunits of identical size.

The development of an affinity column which specifically binds HGPR transferase has greatly simplified the rapid purification of this enzyme.

MATERIALS AND METHODS

Commerical Materials—GMP, 5′-phosphoribosyl-1′-pyrophosphate, and MES were purchased from Sigma. $NaBH_4$ was obtained from Ventron, Beverly, Mass. CNBr and reagent grade urea were products of J. T. Baker Chemical Co. [^{3}H]Hypoxanthine was purchased from Schwarz-Mann. Sephadex G-50, G-150, G-200, and CM-50 were obtained from Pharmacia. The technique of Kawata and Chase (4) was used to remove the fines from all of the Sephadex forms except CM-50, giving a more rapid flow rate and greater resolving power. Sepharose 4B was obtained from both Sigma and Pharmacia. Crystalline rabbit serum albumin and Fraction V bovine serum albumin (Sigma) were used without further purification. Horse heart cytochrome *c* type VI (Sigma 99% pure) was further purified by ascending chromatography on Sephadex G-50. Acrylamide and *N*,*N*′-methylenebisacrylamide were obtained from Eastman Kodak. 3,3′-Iminobispropylamine, ethylenediamine, and 1,8-diaminooctane were purchased from Aldrich. Ultrapure sodium dodecyl sulfate was purchased from the Pierce Chemical Co. Ultrapure urea was obtained from Schwarz-Mann. DE81 filter discs were purchased from Whatman.

Cell Lines and Culturing Conditions—Wild type L^+ cells were obtained from J. Littlefield. Mutant cells were those derived by Sharp *et al.* (5). The human cell line D98S was obtained from the American type culture collection (CCL 18.1). The culturing of the L cells was done as previously described (5).

HGPR transferase Assay—In the initial stages of this work, the chromatographic assay of Sharp *et al.* (5) was used. The later phases of the work were done using a DEAE-filter disc assay (6) modified as described below. Unless otherwise stated, the HGPR transferase assays were performed in 50-μl reaction mixtures con-

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[1] The abbreviations used are: HGPR transferase, hypoxanthine-guanine phosphoribosyltransferase; MES, 2-(*N*-morpholino)-ethanesulfonic acid; CRM, cross-reacting material.

[2] G. M. Wahl, S. H. Hughes, and M. R. Capecchi, *J. Cell Physiol.*, in press.

taining 50 mM Tris-Cl, pH 7.4, 7 mM $MgCl_2$, 0.1 mM EDTA, 1 mg per ml of bovine serum albumin, 1.25 mM 5′-phosphoribosyl-1′-pyrophosphate, and 5 μM [^{3}H]hypoxanthine. The reaction mixtures were routinely incubated for 15 min at 37° and the reactions were stopped by the addition of 1 ml of 10 mM Tris-Cl, pH 7.4, and 1.5 mM EDTA. The stopped reaction mixtures were then kept on ice until filtered. The [^{3}H]IMP formed in the reaction was collected by suction filtration through a 2.5-cm DE81 disc. Prior to use, the discs were brought to pH 7.4 by soaking in 1 M Tris-Cl, pH 7.4. The DE81 filter was washed three times with 5 ml of 10 mM Tris-Cl, pH 7.4, the sample was filtered through the DEAE-disc, and the disc was washed four times with 5-ml aliquots of 10 mM Tris-Cl, pH 7.4. The discs were then placed in scintillation vials, the [^{3}H]IMP eluted with 0.5 ml of 3% NaCl and counted in 5 ml of Patterson Greene (5) scintillation fluid.

A unit of enzyme is defined as that amount required to convert 1 nmol of hypoxanthine to IMP per hour under standard assay conditions.

Buffers—The buffer solutions used in these experiments are listed in Table I. For convenience, they will be referred to by letter. Dithiothreitol was added just before the buffer was used.

Affinity Chromatography Column—The 3,3′-iminobispropylamine-GMP agarose affinity chromatography column used for the purification of mouse liver HGPR transferase was prepared as follows. 3,3′-Iminobispropylamine agarose was prepared from Sepharose 4B using the CNBr technique of Cuatrecasas (7). Sepharose 4B, washed with 10 volumes of deionized water, was activated with CNBr at a ratio of 300 mg of CNBr per ml of packed beads. The activation was terminated by washing with 20 volumes of ice-cold deionized water. The beads were washed briefly with 2 M 3,3′-iminobispropylamine adjusted to pH 10, placed in 1 volume of this solution, and allowed to react overnight at 4°. The trinitrobenzene sulfonic acid color test (7) indicated that a high degree of coupling had been achieved.

A 100-ml packed volume of 3,3′-iminobispropylamine agarose was washed with 5 liters of H_2O, 100 ml of Buffer I, and 100 ml of Buffer J. The washed beads were suspended to a total volume of 130 ml in Buffer J. Other amine agaroses were prepared identically.

Oxidized GMP was coupled to 3,3′-iminobispropylamine agarose using modifications of the methods developed by Gilham for coupling nucleotides to aminoethylcellulose (8). Immediately before the coupling reaction, 3.31 g of GMP (0.1 mmol per ml of beads) was dissolved in 100 ml of deionized water. The pH was 8.2. $NaBH_4$ (118 mg) (1.1 mol per mol of GMP) was added to the GMP solution and allowed to react in the dark at 0° for 30 min. 3,3′-Iminobispropylamine agarose (100 ml) in Buffer J was added to the oxidized GMP solution and stirred gently for 1 hour. Two sequential additions of $NaBH_4$ were then made: the first was 0.83 g dissolved in 75 ml of Buffer J, and the second 1.2 g of solid $NaBH_4$. Each was allowed to react for 1 hour. The reaction was stopped by filtration and washing with 6 liters of H_2O. An appropriate aliquot was poured into a column and washed with >10 volumes of Buffer G, then >2 volumes of Buffer G containing 1 mg per ml of bovine serum albumin, >10 volumes of Buffer H containing 1.5 M KCl instead of 1.2 M KCl, and finally >10 volumes of Buffer G. The 3,3′-aminobispropylamine-GMP agarose prepared in this way was tested on a small scale to ensure that it would retain greater than 10^4 units of HGPR transferase per ml of packed column volume. Each purification required a freshly prepared affinity column.

TABLE I

Buffer compositions

Numbers in parentheses represent millimolar concentrations.

Buffer	Components	pH
A	Tris-Cl (20)-sucrose (200)-NH_4Cl (100)-$Mg(CH_3CO_2)_2$ (5)-dithiothreitol (1)	7.6
B	Tris-Cl (20)-$MgCl_2$ (3)	7.5
C	MES (10)-$MgCl_2$ (2.5)-dithiothreitol (1)	5.8
D	MES (10)-$MgCl_2$ (2.5)-KCl (25)-dithiothreitol (1)	5.8
E	MES (10)-$MgCl_2$ (2.5)-KCl (25)-dithiothreitol (1)	6.2
F	MES (10)-$MgCl_2$ (2.5)-KCl (125)-dithiothreitol (1)	6.2
G	Tris-Cl (50)-$MgCl_2$ (10)-KCl (25)-dithiothreitol (1)	7.4
H	Tris-Cl (50)-$MgCl_2$ (10)-KCl (1200)-dithiothreitol (1)	7.4
I	Tricine[a] (100)-NaCl (500)	8.2
J	Tricine[a] (200)-NaCl (1000)	8.2
K	NaCl (137)-KCl (2.7)-Na_2HPO_4 (8.1)-KH_2PO_4 (1.5)	7.5
L	Tris-Cl (25)-$MgCl_2$ (3.5)-KCl (500)-Triton X-100 (2%)	7.5
M	Tris-Cl (50)-$MgCl_2$ (7)	7.5
N	Tris-Cl (10)-$MgCl_2$ (10)-KCl (30)-Triton X-100 (0.5%)-dithiothreitol (1)	7.4

[a] Tricine is *N*-tris(hydroxymethyl)methylglycine.

Purification of Mouse Liver HGPR Transferase—One hundred Swiss white mice, 6- to 7-weeks-old, were killed by decapitation and their livers rapidly removed. The gall bladders were excised and the livers were washed twice in ice-cold Buffer A. Unless otherwise noted, all subsequent steps were done at 0–4°. Two and one-half volumes of Buffer A were added and the liver cells were broken in a Dounce homogenizer by six strokes with a loose and six strokes with a tight pestle.

The lysate was spun at 10,000 × *g* for 30 min in a Sorvall GSA rotor and the supernatant removed with as little of the overlying lipid as possible. This supernatant (S-10) was centrifuged at 165,000 × *g* for 4 hours in an International A-170 and the supernatant was removed from under the overlying lipid. This material (S-165) could be stored at −20° with little loss of activity for several weeks.

The S-165 was diluted at 0° with 2 volumes of ice-cold H_2O. The pH was rapidly lowered to 5.0 by dropwise addition of 1 N acetic acid. The resulting precipitate was removed by centrifugation at 10,000 × *g* for 15 min in a Sorvall GSA rotor. The pH of the supernatant was readjusted to 7.6 with 1 N KOH. This step does not give a large purification, but it is known (9) that a number of guanine binding proteins involved in protein synthesis are removed.

The pH 5 supernatant was then heat treated. Aliquots (150 ml) of pH 5 supernatant at 25–30° were diluted with 1 volume of Buffer B preheated to 85°. This mixture was placed in an 85° bath and vigorously agitated. After approximately 2.5 min, the temperature reached 70° and was maintained at 70° by withdrawal and immersion for approximately 2.5 min. The total time was carefully monitored to be 5 min.

The samples were rapidly chilled in an ice salt bath at −15 to −17°. It took approximately 80 s to reach 37° and 3.25 min to reach 15°. The resulting precipitate was removed by centrifugation at 10,000 × *g* for 15 min in a Sorvall GSA rotor. The supernatant was diluted with 1 volume of Buffer C and the pH adjusted to 5.8 with 1 N HCl. The conductivity of the diluted supernatant was always checked and shown to be well below that of Buffer D. The diluted supernatant was then loaded onto a 500-ml CM-50 Sephadex column equilibrated in Buffer D. The column was loaded at its maximal flow rate. After the sample had been loaded, the column was washed with 2 volumes of Buffer D and 2 volumes of Buffer E. The HGPR transferase activity was eluted by 2 volumes of Buffer F, assayed, and pooled.

If the goal is to obtain an enzyme of moderate purity (≤60%) but of higher yield, the following Amicon ultrafiltration and gel filtration steps may be omitted. If a highly purified enzyme is desired, they should be included.

The enzymatic activity was pooled and concentrated to 14.8 ml using an Amicon PM-30 ultrafiltration membrane. This material was loaded on a Sephadex G-150 column (2.5 × 100 cm) equilibrated with Buffer G. The column was eluted in an ascending direction with Buffer G. Five-milliliter samples were collected. The HGPR transferase was located by enzymatic assay and pooled. The pooled material was loaded slowly onto a 30-ml 3,3′-iminobispropylamine-GMP agarose column equilibrated in Buffer G. After the sample had been loaded, the column was washed slowly with Buffer G until no detectable protein eluted. The

enzymatic activity was eluted by the addition of Buffer H and increasing the flow rate to its maximum. The salt front contained a small amount of protein (see Fig. 1), but no HGPR transferase could be detected in these fractions by either enzymatic assay or sodium dodecyl sulfate-urea gel electrophoresis. The enzyme activity eluted behind the salt front in a large volume without detectable A_{280}. HGPR transferase is unstable under these conditions and we were unable to remove the salt by dialysis, Amicon ultrafiltration, Sephadex filtration, or lyophilization without severe loss of enzymatic activity.

The HGPR transferase can be stabilized, however, by the addition of 100 μg per ml of a protein such as rabbit serum albumin, bovine serum albumin, or cytochrome *c*. In the presence of a stabilizing protein, the enzyme can be concentrated and desalted with Buffer G on an Amicon UM-10. Impure samples can be concentrated and desalted on a PM-30, but with highly purified samples activity does pass through a PM-30 filter. Purified HGPR transferase stored with rabbit serum albumin in Buffer G at −80° was found to be stable for at least 6 months.

Sodium Dodecyl Sulfate-Urea Gel Electrophoresis—The stacking gel (3.3% acrylamide and 0.08% *N,N'*-methylenebisacrylamide) contained 125 mM Tris-Cl, pH 6.8, 0.1% sodium dodecyl sulfate, and 6 M urea. The separating gel (11.1% acrylamide and 0.27% *N,N'*-methylenebisacrylamide) contained 150 mM Tris, pH 8.7, 0.1% sodium dodecyl sulfate, and 6 M urea. The running buffer was 25 mM Tris base, 192 mM glycine, 0.1% sodium dodecyl sulfate, and 6 M urea. The sample loading buffer contained 50 mM Tris-Cl, pH 6.8, 1.6% sodium dodecyl sulfate, 5.4 M urea, 18% glycerol, 0.001% bromphenol blue, and 5% β-mercaptoethanol. *N,N,N',N''*-Tetramethylethylenediamine and persulfate were adjusted to give a 10- to 15-min gelling time. Pelleted samples, prepared either by immunoprecipitation or trichloroacetic acid precipitation, were dissolved in a 125-μl sample loading buffer and heated at 100° for 5 min. One hundred microliters of the sample were loaded onto the gel with a Hamilton syringe. The gels (6 × 90 mm) were subjected to electrophoresis at a constant current of 3.5 ma per gel. The bromphenol blue reached the end of a 90-mm gel after approximately 8 hours.

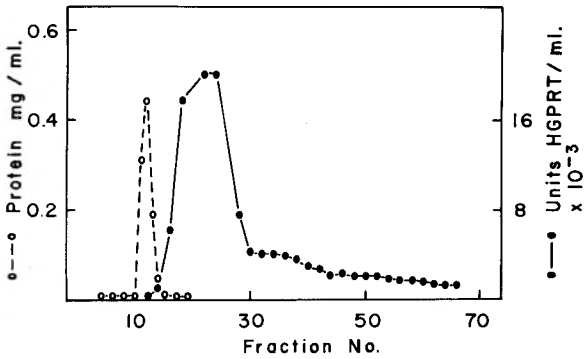

FIG. 1. High salt (Buffer H) elution profile of HGPR transferase from a 3,3'-iminobispropylamine-GMP agarose affinity column. A 30-ml affinity column (11 × 304 mm) was loaded with 76 ml of pooled Sephadex G-150 eluant at a flow rate of 4 drops per min. The column was washed and eluted as described in the text. Three-milliliter fractions were collected. Protein concentrations (○- - -○, mg per ml) and HGPR transferase activity (●——●, units per ml × 10^{-3}) were determined as described under "Materials and Methods."

All of the gels containing radioactively labeled material were internally calibrated with fluorescein isothiocyanate (isomer I)-labeled molecular weight standards. The fluorescein isothiocyanate labeling was done by the methods of Kawamura (10). The position of the molecular weight standards was determined by visual examination under ultraviolet illumination.

Radioimmune Precipitation—Cells were grown in a 60-mm plate in minimal essential Eagle's media plus 10% fetal calf serum to approximately 75% confluency and washed with 5 ml of Buffer K. Minimal essential Eagle's media (1.5 ml) containing [^{3}H]leucine (82 μCi per ml) and [^{3}H]lysine (82 μCi per ml) or [^{35}S]methionine (82 μCi per ml) instead of the respective nonradioactive amino acids was then added. The cells were incubated with the radioactive amino acids for 2.5 hours at 37° followed by two 5-ml washes with Buffer K. The cells were then extracted with 250 μl of Buffer N as previously described (5), followed by centrifugation at 1000 × *g* for 20 min to sediment the cell debris and nuclei. Approximately 3.5 mg of a HGPR transferase$^-$ CRM$^-$ cell extract was added to compete with radioactively labeled non-HGPR transferase protein. Forty-two micrograms of anti-HGPR transferase serum were added followed by incubation at 4° for 12 hours. Goat anti-rabbit γ-globulin (1.5 mg) was then added and incubation continued for an additional 4 hours at 0°. The resulting immunoprecipitates were sedimented by centrifugation at 1000 × *g* for 20 min. The supernatants were removed and the precipitates were washed twice with 400 μl of Buffer L, with centrifugation at 1000 × *g* for 20 min after each washing. The precipitates were washed for a final time with 400 μl of Buffer M and the pellets were sedimented as before. The pellets were then processed and subjected to electrophoresis as described above.

Nondenaturing Gels—The methods and buffers of Hedrick and Smith (3) were used except that all of the electrophoreses were done at 4°. The marker gels were fixed and stained with Coomassie brilliant blue. The HGPR transferase-containing gels were frozen at −20° and cut into 2-mm slices for enzyme assay. The slices were eluted overnight at 4° followed by 30 min at 37° in 100 μl of Buffer G containing 1 mg per ml of bovine serum albumin.

Sucrose Gradient Velocity Sedimentation—Linear gradients of 10 to 30% sucrose, buffered by 50 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 100 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol, were run in the International SB-283 at 4° for 40 hours at 283,000 × *g*. Seven-drop fractions were collected through a needle inserted at the bottom of the tube.

Protein Concentrations—Protein concentrations were measured by the techniques of Warburg and Christian (11) unless otherwise stated.

RESULTS

Purification—The purification of HGPR transferase from mouse liver is summarized in Table II. The method includes a series of batch steps, Sephadex gel filtration, and affinity chromatography. The over-all yield of 17% results in a homogeneous enzyme as judged by electrophoresis on sodium dodecyl sulfate-urea polyacrylamide gels (see Fig. 2).

TABLE II

Tabulation of the purification data for mouse liver HGPR transferase

Stage	Volume	Protein	Total activity	Specific activity	Yield	Purification
	ml	*mg*	*units*	*units/mg*	%	*-fold*
S-10	330	26,400	10.5×10^5	40		
pH 5	820	8,849	8.4×10^5	95		2.4
Heated	1640	3,316	8.4×10^5[a]	253	100	6.35
CM-50	550	214.5	7.2×10^5	3364	86	84.5
G-150	760	55.5	7.1×10^5	14100	83	350
Affinity column	350	≤0.8	1.46×10^5	$\geq 1.8 \times 10^5$	17	≥4500

[a] Yield from the heat step in this particular preparation is lower than usual. The activation of mouse liver and L$^+$ cell HGPR transferase by heating routinely results in a yield of 110 to 120%.

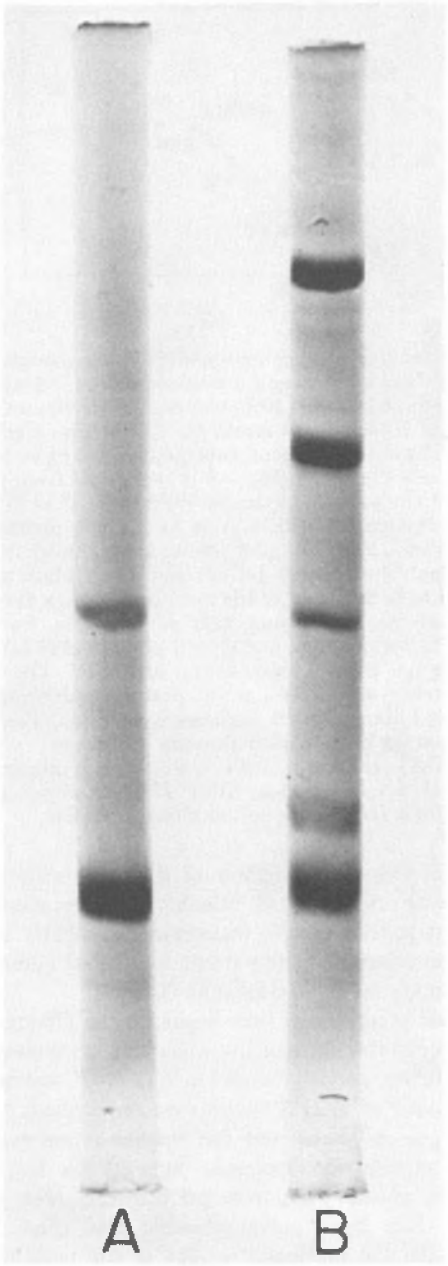

FIG. 2. Sodium dodecyl sulfate-urea gels of purified HGPR transferase. The purified mouse liver HGPR transferase used for this gel was stabilized by the addition of cytochrome *c* (see text). The gels were prepared as described under "Materials and Methods" and stained with Coomassie brilliant blue. In *A*, the *top band* is HGPR transferase (10 μg) and the *bottom band* is cytochrome *c*. In *B*, we show purified HGPR transferase with a series of protein markers of known subunit molecular weight. The *bands*, starting from the *top*, correspond to bovine serum albumin, aldolase, HGPR transferase, myoglobin, and cytochrome *c*. The wires mark the position of bromphenol blue.

The scheme can be adapted readily to small scale isolations because only the Sephadex G-150 filtration step requires monitoring of HGPR transferase-specific activity. If this gel filtration is omitted, the enzyme obtained is approximately 60% pure with an over-all yield of 25 to 30%. The purification of HGPR transferase was facilitated by the use of affinity chromatography as the last step.

Properties of Affinity Column—The details of preparing the 3,3′-iminobispropylamine-GMP agarose column are given under "Materials and Methods." Table III shows that the ability of the column to bind HGPR transferase is very dependent on the nature of the arm linking the oxidized GMP to the agarose. The ethylenediamine-GMP-agarose column has a much lower capacity for binding HGPR transferase than does the 3,3′-iminobispropylamine-GMP agarose column. 3,3′-Iminobispropylamine agarose does not bind HGPR transferase unless it is reacted with oxidized GMP. The choice of an appropriate arm, however, is dependent not only on the length, but also on the chemical composition. For example, 1,8-diaminooctane agarose was found to have the unfortunate property of binding many proteins before or after coupling with oxidized GMP. Because this effect was not observed with ethylenediamine or 3,3′-iminobispropylamine, we have attributed it to the increased hydrophobicity of the long hydrocarbon chain. The binding specificity of 3,3′-iminobispropylamine-GMP agarose is demonstrated by its ability to retain HGPR transferase from mouse, rabbit, goat, and pig, but not the related enzyme adenine phosphoribosyltransferase.

TABLE III

Elution of HGPR transferase as function of affinity column arm composition

Column	Activity eluting in low salt (Buffer G)	Activity eluting in high salt (Buffer H)
	%	
—N(H)—CH_2—CH_2—N(H)—GMP	27	73
—N(H)—$(CH_2)_3$—N(H)$(CH_2)_3$—N(H)—GMP	0	80–100
—N$(CH_2)_3$—N$(CH_2)_3$—N(H)	100	0

The yield of HGPR transferase from the affinity column was dependent on the purity of the loaded sample. Affinity chromatography of crude supernatant fractions results in a 100-fold purification of HGPR transferase with quantitative recovery of activity. On the other hand, the passage of highly purified samples such as the G-150 eluant through the column affords lower yields (see Table II).

Physical Properties of Undenatured HGPR Transferase—Before undertaking a study of mutant forms of HGPR transferase, some of the physical properties of the wild type enzyme were determined. This included measurements of the enzyme's behavior on sucrose gradients, Sephadex G-200 gel filtration, and nondenaturing polyacrylamide gels of increasing gel concentrations.

As observed in Fig. 3, HGPR transferase from both mouse liver and L cells sediments slightly faster than horse alcohol dehydrogenase (mol wt = 83,000, (13)) with an $s_{20,w}$ of 5 S. The position of the enzymes was determined by activity assays.

Mouse liver HGPR transferase was run on a Sephadex G-200 column with proteins of known Stokes radii (see Fig. 4). The enzymatic activity eluted just ahead of bovine serum albumin at a position indicating a Stokes radius of 36 A, precisely the same value reported for human HGPR transferase (15). Care must be taken in such experiments because at high dilution and low ionic strength the enzyme will interact with Sephadex and be retarded from this position.

Because the elution position of a protein from Sephadex is very sensitive to shape as well as molecular weight (16), we have also examined the electrophet icicmigration of HGPR trans-

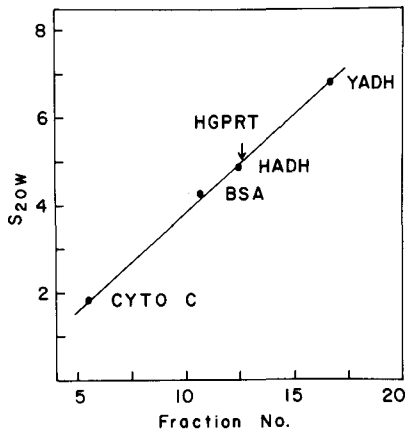

FIG. 3. Sedimentation of HGPR transferase on calibrated sucrose gradients. Linear gradients of 10 to 30% sucrose were run as described under "Materials and Methods." Cytochrome *c* and bovine serum albumin were assayed by absorption at 550 nm and 280 nm, respectively. Horse and yeast alcohol dehydrogenases (*HADH* and *YADH*) were assayed by the method of Vallee and Hoch (12). HGPR transferase was assayed as described under "Materials and Methods."

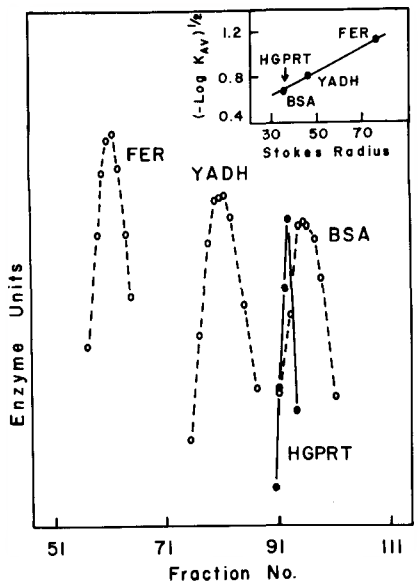

FIG. 4. Elution profile of HGPR transferase on a calibrated Sephadex G-200 column. The column, which had previously been calibrated, was run with the internal protein markers ferritin (*FER*), yeast alcohol dehydrogenase (*YADH*), and bovine serum albumin (*BSA*). *FER* and *BSA* were measured by A_{550} and A_{280}, respectively. HGPR transferase was assayed as described under "Materials and Methods." Yeast alcohol dehydrogenase was assayed by the method of Vallee and Hoch (12). The Stokes radius was calculated according to the method of Siegel and Monty (14). The retardation constant (K_{av}) was calculated from the formula $K_{av} = (Ve - V_0)/(Vt - V_0)$ where Ve is the elution volume of the protein of interest, V_0 is the void volume of the column determined by blue dextran, and Vt is the total volume of the gel bed determined by the elution volume of $^{32}P_i$.

ferase in nondenaturing polyacrylamide gels of increasing gel concentrations. This method allows an estimate of molecular size to be made from a comparison of the behavior of the enzyme relative to protein markers of known size (3). The data were analyzed both by the methods of Hedrick and Smith (3) and Rodbard and Chrambach (16). The former gives an estimate of molecular weight, whereas the latter gives the radius, R, of

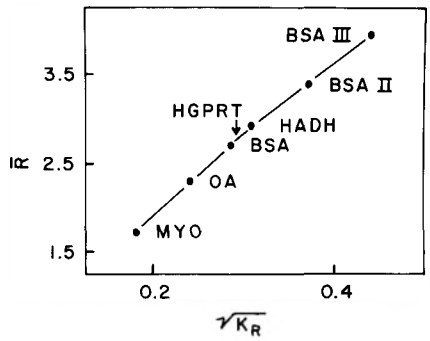

FIG. 5. Molecular size determined from nondenaturing polyacrylamide gels of increasing gel concentrations. A series of nondenaturing gels (5%, 7.5%, 10%, and 12.5%) were run according to the method of Hedrick and Smith (3) except that electrophoresis was at 4°. The data have been analyzed according to the methods of Rodbard and Cranbach (16). $\bar{R}$ is calculated from the molecular weight of the protein in the native state. $\bar{R} = [3(\text{mol wt})\,\bar{v}/4\pi N]^{1/3}$, $\bar{v}$ is assumed to be 0.74, N is Avogadro's number. K_r was calculated from a Ferguson plot (16) of log R_F (migration relative to bromphenol blue) *versus* gel concentration (data not shown). K_r is defined to be the slope of the line obtained in a Ferguson plot. HGPR transferase-containing gels were frozen for 1 hour at −20°, cut into 2-mm slices, and eluted into 100 μl of Buffer G containing 1 mg per ml of bovine serum albumin. The calibrating gels were marked with a wire at the position of bromphenol blue, then fixed and stained with Coomassie brilliant blue. Separate gels were used for each marker protein. The markers are: *MYO*, myoglobin; *OA*, ovalbumin; *BSA*, bovine serum albumin; *HADH*, horse alcohol dehydrogenase; *BSA II*, bovine serum albumin dimer; and *BSA III*, bovine serum albumin trimer.

an equivalent unhydrated sphere of the same surface area (see Fig. 5). Both methods of calculation gave similar results showing that native HGPR transferase is slightly larger than bovine serum albumin. This result is in good agreement with the size estimate made on Sephadex G-200.

It has been pointed out that Sephadex gel filtration is much more sensitive to the shape of the molecule than is electrophoresis on nondenaturing polyacrylamide gels (16). Consequently, the similar behavior of HGPR transferase on Sephadex and polyacrylamide gels indicates that the enzyme is not highly asymmetrical. Because the molecule appears to be reasonably spherical, the elution data from gel filtration (Fig. 4) and the retardation data from polyacrylamide gels (Fig. 5) can be correlated with the molecular weight of the protein. In both systems, HGPR transferase exhibits a molecular weight of slightly under 80,000. On the other hand, the behavior of HGPR transferase on sucrose gradients is that of a molecule slightly larger than 80,000. We feel that these data indicate that HGPR transferase is not highly asymmetrical, is of greater than normal density, and has a molecular weight of 80,000 ± 4,000.

Subunit Structure of HGPR Transferase—The subunit structure of purified mouse liver HGPR transferase was investigated by sodium dodecyl sulfate and sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis. As observed in Fig. 2, the purified protein migrated as a single band on such gels. Comparison of the electrophoretic mobility of this band with proteins of known subunit molecular weight used as internal standards indicates that it has a molecular weight of 27,000 ± 1,000 (see Fig. 6).

In order to prove that this band was derived from HGPR transferase, we have used sodium dodecyl sulfate-urea gel electrophoresis to analyze the radioactively labeled material precipitated from wild type and mutant L cell extracts by

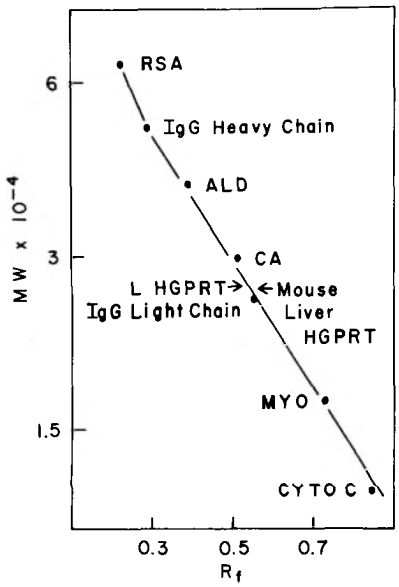

FIG. 6. Analysis of mouse liver and L cell HGPR transferase on sodium dodecyl sulfate-urea gels. The mouse L cell HGPR transferase was isolated from cell extracts by radioimmunoprecipitation with antiserum directed against purified mouse liver HGPR transferase as described under "Materials and Methods." The protein markers are: *RSA*, rabbit serum albumin; *IgG heavy chain*, goat γ-globulin heavy chain; *ALD*, aldolase; *CA*, carbonic anhydrase; *IgG light chain*, goat γ-globulin light chain; *MYO*, myoglobin; and *CYTO C*, cytochrome *c*. The position of migration is given relative to bromphenol blue (R_F). The interpolated mobilities of mouse liver and L^+ cell HGPR tarnsferase indicate a subunit molecular weight of 27,000 ± 1,000.

HGPR transferase-specific antiserum. In experiments to be reported in detail elsewhere,[2] mutant extracts known to lack enzymatic activity were assayed for the presence of inactive HGPR transferase molecules by immunological techniques. These experiments tested for the presence of material which would cross-react with anti-HGPR transferase serum and prevent the wild type enzyme from being immunoprecipitated. Cell lines having this material are designed as HGPR transferase$^-$ CRM$^+$. Fig. 7 shows sodium dodecyl sulfate-urea electrophoresis patterns of the immunoprecipitated radioactivity from [^{3}H]lysine, [^{3}H]leucine-labeled extracts of (*A*) wild type L^+, (*B*) wild type L^+ to which purified unlabeled mouse liver HGPR transferase was added prior to immunoprecipitation, (*C*) an HGPR transferase$^-$ CRM$^-$ L cell mutant, and (*D*) an HGPR transferase$^-$ CRM$^+$ mutant. The ^{35}S-labeled material in Fig. 7, *C* and *D* is wild type L^+ HGPR transferase added as an internal marker. It is apparent that the peak of radioactivity precipitated by HGPR transferase-specific antiserum is absent in the HGPR transferase$^-$ CRM$^-$ cell extracts and can be isotopically diluted with purified mouse liver enzyme. The reduction of radioactivity in the peak as a result of adding purified mouse liver HGPR transferase (compare Fig. 7, *A* and *B*) correlates well with the amount of added enzyme relative to the endogenous level found in the extracts. These experiments confirm genetically that the 27,000-dalton band (*peak*) is the HGPR transferase subunit.

The subunit molecular weight of mouse liver and L cell HGPR transferase does not agree with the reported subunit molecular weight of human HGPR transferase (15). To test this discrepancy, we have compared the electrophoretic mobility on sodium dodecyl sulfate-urea gels of HGPR transferase immunoprecipitated from human D98S and mouse L cells.

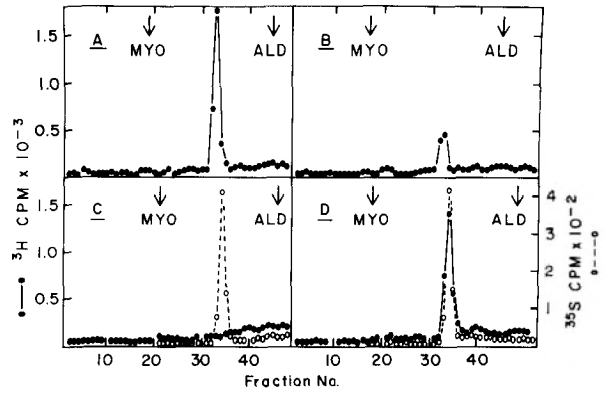

FIG. 7. Analysis of HGPR transferase from L^+ and mutant (HGPR transferase$^-$) cell extracts by sodium dodecyl sulfate polyacrylamide gel electrophoresis. L^+ and HGPR transferase$^-$ mutants were labeled with radioactive amino acids as described under "Materials and Methods." The cell extracts were then prepared, precipitated with antiserum prepared against purified mouse liver HGPR transferase, and subjected to electrophoresis as described under "Materials and Methods." The gels were frozen, cut into 1-mm slices, eluted with 0.5 ml of 0.1% sodium dodecyl sulfate, and counted in a liquid scintillation counter. Each gel was internally standardized with fluorescein-labeled myoglobin (*MYO*), aldolase (*ALD*), and bromphenol blue. The fluorescein-labeled proteins were shown to co-migrate upon electrophoresis with their unlabeled counterparts. *A* shows the pattern of immunoprecipitable product isolated from L^+ extracts. The experiment shown in *B* is as in *A* except that 600 units of unlabeled purified mouse liver HGPR transferase were added to the L^+ extracts prior to immunoprecipitation. *C* shows the result of co-precipitation of [^{3}H]leucine- and [^{3}H]lysine-labeled HGPR transferase$^-$ CRM$^-$ cell extract (●——●) and [^{35}S]methionine-labeled L^+ extract (○- - -○). The protein ratio of the extracts (^{35}S:^{3}H) was 1:20. The experiment in *D* is as in *C* except that a [^{3}H]leucine- and [^{3}H]lysine-labeled HGPR transferase$^-$ CRM$^+$ extract was used instead of a HGPR transferase$^-$ CRM$^-$ extract. The peak of radioactivity corresponds to a molecular weight of 27,000. The CRM activity of the mutants was determined by a standard assay measuring the prevention of immunoprecipitation of L^+ HGPR transferase activity as a function of added mutant extract.[2]

The co-electrophoresis of human and mouse subunits seen in Fig. 8 leads us to conclude that they have identical molecular weights.

DISCUSSION

Hypoxanthine-guanine phosphoribosyltransferase has proven to be an important tool for the genetic analysis of mammalian cells in culture. Strong selection methods for isolating mammalian cell lines that have lost or regained HGPR transferase activity are available (1, 2). Analysis of HGPR transferase$^-$ cell lines and their HGPR transferase$^+$ revertants have demonstrated that structural gene mutants can be isolated from mammalian cells in culture (5, 6). Detailed analysis of such cell lines will permit the study of the mutagenic process in tissue culture cells. Because the enzyme is X-linked in man (17) and probably also in hamsters (18), HGPR transferase mutants can serve as markers for the study of X-chromosome regulation (19). HGPR transferase is one of the common markers used for the selection of hybrid cell lines (2). In addition, resolution of the current controversy over the importance of genetic *versus* epigenetic events in the production of altered phenotypes could come through the study of cell lines defective in HGPR transferase.

As a prerequisite for the detailed analysis of mutant forms of HGPR transferase, we developed a purification method suitable

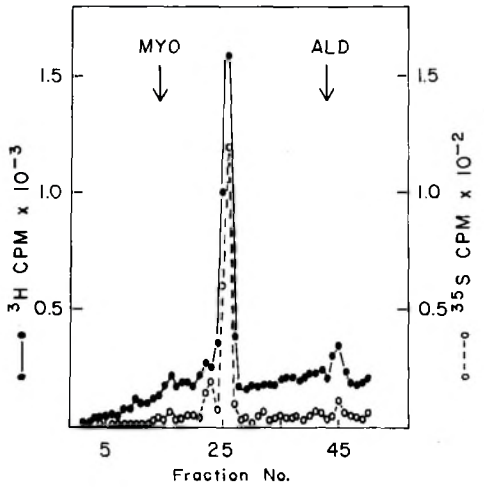

FIG. 8. Comparison of the electrophoretic mobilities of human and mouse HGPR transferase subunits on sodium dodecyl sulfate-urea gels. Human D98S cells were labeled with [^{3}H]leucine and [^{3}H]lysine. Mouse L$^+$ cells were labeled with [^{35}S]methionine. Processing of the cell extracts and immunoprecipitation were done as described under "Materials and Methods," except that a ratio of 1 μg of antiserum to 10 μg of cell extract protein was used to compensate for the lower affinity of the anti-mouse HGPR transferase serum for human HGPR transferase. The ratio of cell extract protein was again 1:20 (^{35}S:^{3}H). (^{3}H, ●——●; ^{35}S, O- - -O). The higher background seen in this experiment relative to the ones depicted in Fig. 7 resulted from the increased amount of anti-HGPR transferase serum used.

for microscale enzyme isolations. Characterization of the physical properties of the wild type enzyme was also required.

The key to the isolation procedure is the affinity column. It can be used to purify HGPR transferase approximately 100-fold from crude supernatants. The "arm" linking the GMP to the agarose was carefully chosen in order to obtain a column which bound HGPR transferase strongly and selectively. Both length and chemical composition of the arm were found to be important. The methodology used to build the 3,3′-iminobispropylamine-GMP agarose column should be applicable to the construction of any nucleotide affinity column. The procedure is rapid and does not demand extensive organic synthesis.

Our studies of the physical characteristics of undenatured HGPR transferase have led us to the conclusion that it has a molecular weight of 80,000 ± 4,000, is not highly asymmetrical, and is probably of greater than average density. The shape of the enzyme was deduced from the observation that HGPR transferase behaves similarly (in terms of size) on both Sephadex G-200 gel filtration and nondenaturing polyacrylamide gel electrophoresis. If HGPR transferase were highly asymmetrical, these two techniques would not be in agreement due to the greater sensitivity of gel filtration to molecular shape (16). On both Sephadex G-200 and polyacrylamide gels, HGPR transferase behaves as a molecule smaller than 80,000. In contrast to these results, the enzyme sediments slightly faster than a molecule of 80,000 on calibrated sucrose velocity gradients. These data suggest that the molecule is of greater than average density.

A combined biochemical and genetic analysis has been used to determine the subunit structure of HGPR transferase. Electrophoresis of the purified mouse liver enzyme on calibrated sodium dodecyl sulfate and sodium dodecyl sulfate-urea gels resulted in a single band migrating at a position corresponding to 27,000 ± 1,000 daltons. Genetic evidence indicates that this band is the HGPR transferase subunit. Extracts of wild type L cells (HGPR transferase$^+$) and two types of mutant L cells (HGPR transferase$^-$ CRM$^-$, HGPR transferase$^-$ CRM$^+$), labeled with radioactive amino acids, were immunoprecipitated with highly specific HGPR transferase antiserum and the immunoprecipitates were analyzed on calibrated sodium dodecyl sulfate-urea gels. The HGPR transferase$^+$ and HGPR transferase$^-$ CRM$^+$ precipitates contained radioactive material which migrated with a sharp peak at 27,000, but no such material was derived from HGPR transferase$^-$ CRM$^-$ precipitates. Furthermore, this peak of radioactivity was reduced if purified mouse liver HGPR transferase was added prior to immunoprecipitation. From these results, we are confident that mouse HGPR transferase (liver and L cell) is composed of subunits with a molecular weight of 27,000.

Our results do not agree with the data reported for human HGPR transferase (15). We have pointed out that human and mouse HGPR transferase behave identically on Sephadex G-200. The disagreement arises over the subunit molecular weight of 34,000 reported by Arnold and Kelley (15). Because of this difference, we have immunoprecipitated HGPR transferase from human D98S cells and subjected the precipitate to electrophoresis on calibrated sodium dodecyl sulfate-urea gels. This experiment demonstrated that the subunit molecular weight of human HGPR transferase is also 27,000.

From our results, it appears unlikely that HGPR transferase is a dimer. The data are most consistent with the interpretation that the enzyme is a trimer. We cannot, however, completely rule out the possibility that the native state of the molecule is a higher multimer which has dissociated on sucrose gradients, polyacrylamide gel electrophoresis, and Sephadex gel filtration. Human and mouse HGPR transferase presumably have the same subunit composition due to their identical Stokes radii and subunit molecular weights.

REFERENCES

1. SZYBALSKI, W., SZYBALSKA, E. H., AND RAGNI, G. (1962) *Nat. Cancer Inst. Monogr.* **7**, 75–80
2. LITTLEFIELD, J. W. (1963) *Proc. Nat. Acad. Sci. U. S. A.* **50**, 568
3. HEDRICK, J. L., AND SMITH, A. J. (1968) *Arch. Biochem. Biophys.* **126**, 155
4. KAWATA, H., AND CHASE, M. W. (1968) *J. Chromatogr.* **35**, 565
5. SHARP, J. D., CAPECCHI, N. E., AND CAPECCHI, M. R. (1973) *Proc. Nat. Acad. Sci. U. S. A.* **70**, 3145
6. BEAUDET, A. L., ROUFA, D. J., AND CASKEY, C. T. (1973) *Proc. Nat. Acad. Sci. U. S. A.* **70**, 320
7. CUATRECASAS, P., AND ANFINSEN, C. B. (1971) *Methods Enzymol.* **22**, 343–378
8. GILHAM, P. T. (1971) *Methods Enzymol.* **21**, 191–197
9. FALVEY, A. K., AND STAEHELIN, T. (1970) *J. Mol. Biol.* **53**, 1
10. KAWAMURA, A. (1969) in *Fluorescent Antibody Techniques and Their Applications*, pp. 38–39, University of Tokyo Press, Tokyo
11. WARBURG, O., AND CHRISTIAN, W. (1941) *Biochem. Z.* **310**, 384
12. VALLEE, B., AND HOCH, F. L. (1955) *Proc. Nat. Acad. Sci. U. S. A.* **41**, 327
13. SCHACHMAN, H. K. (1963) *Cold Spring Harbor Symp. Quant. Biol.* **28**, 409
14. SIEGEL, L. M., AND MONTY, K. J. (1966) *Biochim. Biophys. Acta* **112**, 346
15. ARNOLD, W. J., AND KELLEY, W. N. (1971) *J. Biol. Chem.* **246**, 7398
16. RODBARD, D., AND CHRAMBACH, A. (1971) *Anal. Biochem.* **40**, 95
17. SEEGMILLER, J. E., ROSENBLOOM, F. M., AND KELLEY, W. N. (1967) *Science* **155**, 1682
18. WESTERVELD, A., VISSER, R. P. L. S., FREEKE, M. A., AND BOOTSMA, D. (1972) *Biochem. Genet.* **7**, 33
19. LYON, M. F. (1961) *Nature* **190**, 372