L-Aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1) from human erythrocytes is purified for use as a reference material in clinical assays for the enzyme. The procedure involves heat treatment in the presence of a substrate (2-oxoglutarate), ammonium sulfate precipitation, and fractionation with n-butanol. The material is further purified by anion- and cation-exchange chromatography. Specific activity obtainable by a typical preparation is 110 μmol min⁻¹ mg⁻¹ at 30°C. Identical behavior is demonstrated between enzyme in human serum and purified erythrocyte enzyme when substrate concentrations, temperature, and pH are varied. A lyophilized preparation at 4°C is stable for longer than 400 days. Data regarding purity and behavior in currently used clinical assays is described.

Additional Keyphrases: cation- and ion-exchange chromatography • reference enzyme preparation • effect of protein • Arrhenius plots • intercomparison of assays by other methods • interlaboratory comparisons

Recently, there has been considerable activity in evaluation and standardization of methods for clinical enzyme assay (1–3). However, studies of this nature are hampered by the current vagaries of assay and reporting temperatures, incubation conditions, and units of activity. Control and comparison are further complicated by variances in calibration of colorimetric and other “one-point” methods. The standardization of single-point enzyme assays has long been a subject of controversy. The use of enzymes as reference materials in these assays has been marked with both disfavor and approbation (1, 3, 4). Much of the criticism has been directed against the use of serum-based enzyme “standards,” the deficiencies of which are well documented (3, 5–7). The use of enzymes as references would be more acceptable if adequate, well-defined materials were available. Enzyme reference materials are also preferable to the use of chemical standards. Since they are subjected to the assay in the exact manner of the unknown patient sera, systematic error is minimized during the substrate conversion steps. In addition, they offer a practicable means for comparison of methods. We think these materials should be developed and investigated, because they would facilitate interlaboratory comparisons as well as intralaboratory control and standardization of enzyme assays.

An evaluation program in New York state for testing the proficiency of clinical laboratories has demonstrated the feasibility and benefits of such an approach (2). In a mandated program, 315 laboratories participated in the L-aspartate: 2-oxoglutarate aminotransferase¹ determination. The coefficients of variation for the four most widely used assays² would have been three- to five-fold smaller if a uniformly standardized reference material had been used, minimizing interlaboratory bias. Moreover, the infrequent use of kinetic spectrophotometric assays by these laboratories (7.6%) underscores the need for such a reference material.

The concept of stable, purified enzyme reference materials for use in standardization and control of diagnostic enzyme assays is not new. Babson (10) in 1968 recognized the need for such materials, as have other workers (11). One goal of the National Committee for Clinical Laboratory Standards is to develop specifications for reference enzymes of high purity (12). Our efforts at this Division to prepare suitable enzyme reference materials began in 1967.

In developing a suitable L-aspartate: 2-oxoglutarate aminotransferase preparation, we considered the following criteria to be most important: (a) Optima. The material must have the same substrate and pH optima as human serum enzyme. Purified enzyme must exhibit the same temperature dependence as serum enzyme. Such conditions remove from consideration the use of most described materials (13–15), owing to their divergence

¹ EC 2.6.1.1; formerly known as glutamic-oxaloacetic transaminase (gat).
² Reitman–Frankel (9) [Sigma and Dade kits], SMA 12/60 [Technicon], and Babson et al. (9) [Warner–Chilcott kit] represented 80.6% of total methods used.
from human serum enzyme optima. (b) Stability. The long-term stability of the lyophilized preparation and the short-term stability of the reconstituted material should be as great as possible; the enzyme must be stable under conditions to which it would be exposed during transport to the laboratory. (c) Co-Factor. The transferase should be fully saturated with its co-factor, pyridoxal phosphate (16, 17). (d) Purity. Inhibitors and interfering enzymes, notably L-glutamate:NAD oxidoreductase (deaminating), must be absent. Purity should be high while stability and yield remain satisfactory. (e) Isolation. Easily reproducible procedures, compatible with processing large volumes, should be used in the preparation.

Although we used bovine heart enzyme in our original studies (2, 5), in 1969 the Enzyme Subcommittee of the National Committee of Clinical Laboratory Standards specified that enzyme reference materials be of human origin (12). We have found outdated human erythrocytes to be a moderately rich source of L-aspartate:2-oxoglutarate aminotransferase. Human red cells are readily available and legally dispensable, important considerations for commercial large-scale preparation. Furthermore, erythrocyte aminotransferase seems qualitatively appropriate for use as a clinical enzyme reference material. The enzyme from erythrocytes has the same K_m values (aspartate and 2-oxoglutarate) and immunochemical properties as does the enzyme from the cytoplasmic component of heart and liver tissue (18, 19). Both human serum and erythrocyte aspartate aminotransferase have been shown to be predominantly the cytoplasmic isoenzyme (18, 20).

We describe here the purification, properties, and characterization of erythrocyte aminotransferase.

Materials and Methods

Materials

Chemicals: L-Aspartic acid, 2-oxoglutaric acid, maleic acid, pyridoxal phosphate, glutaric acid, and β-nicotinamide adenine dinucleotide, reduced form [β-NADH (Grade III)], were obtained from Sigma Chemical Co., St. Louis, Mo. 63178; oxalacetic acid and reduced glutathione from Sigma and from Mann Research Laboratories, New York, N.Y. 10006; L-malate:NAD oxidoreductase* from Sigma and from Boehringer–Mannheim Corp., New York, N.Y. 10017; polyvinyl pyrrolidone (PVP; average mol wt, 40,000) from Calbiochem, San Diego, Calif. 92112; and polyethylene glycol (PEG; average mol wt, 6,000) from J. T. Baker Co., Phillipsburg, N.J. 08865. Human serum albumin, fraction V, was prepared by the procedures of Cohn et al. (21) and 4-thiazolidine carboxylic acid by the method of MacKenzie and Harris (22).

Maleate-EDTA buffer is prepared by dissolving 92.9 g of maleic acid and 30.4 g of disodium ethylenediaminetetraacetate dihydrate in 1500 ml of de-ionized water, increasing the pH to 6.0 with NaOH, and diluting to 2 liters.

Enzyme activities were measured by the method of Henry et al. (23) in a semiautomated spectrophotometric system ("Kintrac VII"; Beckman Instruments, Fullerton, Calif. 92634). The procedure was modified to include 1.8 U of L-malate:NAD oxidoreductase per assay. All units are expressed as μmol min^{-1} at 30°C. Aminotransferase activity was also determined by the methods of Reitman and Frankel (9) and Babson et al. (8) where noted. Protein concentrations were estimated by the method of Lowry et al. (24, 25), in which crystallized human serum albumin (Dade, Division American Hospital Supply Corp., Miami, Fla. 33152) was used as standard. Human red cells were obtained within three days after they became outdated. Specimens were lyophilized in a Repp shelf lyophilizer (Virtis Co., Gardiner, N.Y. 12525). Dialysis tubing (Union Carbide Corp., Chicago, Ill. 60638) was soaked in a solution of 1 g of Na_2EDTA per 100 ml and rinsed in distilled-de-ionized water.

Procedures

Polyacrylamide disc electrophoresis. The conditions and general method of Davis' alternative procedure (26) were used. The samples were dissolved in a sucrose solution (final concentration, 40 g/dl) for application and electrophoresed at a constant current of 3 mA/tube (Model 100 power supply; Canalco, Rockville, Md. 20852). The glass gel tubes were 75 mm long, 5 mm i.d. Electrophoresis was stopped when the bromphenol blue tracking dye had migrated 50 mm into the separation gel. Gels were stained for aminotransferase activity by incubating at room temperature for 5 min in 2-oxoglutarate (2.1 mmol/liter); L-aspartate (9 mmol/liter); phosphate buffer (pH 7.5, 80 mmol/liter); and 6-benzamido-4-methoxy-m-toluidine, diazo- nium chloride [Fast Violet B salt; grade I (Sigma)] (250 mg/dl); we used 5 ml of this mixture per gel. Incubation must immediately follow medium preparation. Five minutes or less is sufficient for visualization of 10 mU aminotransferase. If very low enzyme concentrations were applied, incubation medium was replaced after 5 min with freshly prepared medium for a further 5 min incubation. Protein was made visible by overnight staining with a solution of anazolene sodium (Coomassie Blue), 0.5 g/liter of a 25:10:65 (by vol) mixture of 2-propanol, acetic acid, and water (minimum, 170
ml/gel), with stirring. Gels were photographed through a G-15 Wratten deep-yellow filter. For disc electrophoresis in the presence of sodium lauryl sulfate we used the conditions of Fairbanks et al. (27). Chemicals used in electrophoresis were from: acrylamide and N,N'-methylenebisacrylamide (electrophoresis grade), Aldrich Chemical Co., Milwaukee, Wis. 53223; ammonium persulfate and N,N,N',N'-tetramethylethylenediamine (TEMED), Canaco; Coomassie Brilliant Blue R250, Bio-Rad Laboratories, Richmond, Calif. 94804; sodium lauryl sulfate (U.S.P.), Fisher, was recrystallized from ethanol. Molecular-weight markers for sodium lauryl sulfate gel calibration were: ATP:pyruvate phosphotransferase⁴ (EC 2.7.1.40), 2-phospho-D-glycerate hydro-lyase⁵ (EC 4.2.1.11), L-glycerol-3-phosphate: NAD-oxidoreductase⁶ (EC 1.1.1.8) (Boehringer); trypsin (EC 3.4.2.4) (Worthington Biochemical Corp., Freehold, N.J. 07728); pepsin (EC 3.4.1.1) (Difco Laboratories, Detroit, Mich. 48201); crystalline human serum albumin (Dade); and hemoglobin prepared from washed human erythrocytes (28).

Enzyme preparation. Maleate-EDTA buffer, 2.5 liters, is added to 6.0 liters of recently outdated, centrifugally packed human erythrocytes. The mixture is frozen at -50°C and lyophilized, producing about 2 kg of an easily stored stable powder.

Lyophilized powder, 310 g, is added to 3 liters of de-ionized water and mixed thoroughly. The mixture is poured into a 4-liter beaker, which is then placed in a water bath maintained at 85°C. The mixture is stirred constantly with a Plexiglas paddle operated by a rheostat-controlled 41-watt motor. When the temperature of the preparation reaches 55°C, 6.8 mmol of 2-oxoglutaric acid (Sigma) is added as a solution in maleate-EDTA buffer. When the hemolysate temperature reaches 75°C, denaturation is allowed to proceed at 75°C with slow constant stirring for 20 min. The beaker is then removed and cooled as rapidly as possible in a salt–ice bath to about 4°C. The mixture is centrifuged at 9,500 rpm [1.6 × 10⁴ g at Rmax (Model J-21 Centrifuge, Type JA-10 rotor, Beckman)] at 4°C for 15 to 20 min, and the dark-red precipitate discarded.

For each 100 ml of supernatant liquid, 31.0 g of ammonium sulfate is added with constant stirring. After addition is complete, the preparation is stirred gently for 30 min at 4°C. The suspension is centrifuged as described above for 30 min and the precipitate discarded. The enzyme is then precipitated by adding 12.1 g of ammonium sulfate for each 100 ml of supernatant liquid followed by stirring for 20 min at 4°C. The red precipitate is collected by the described centrifugation for 30 min and the supernatant liquid discarded.

The enzyme is taken up in 190 ml of a 10-fold dilution of maleate-EDTA buffer containing 2-oxoglutarate (1.7 mmol/liter) and pyridoxal phosphate (40 μmol/liter). The dissolved precipitate is transferred to a 500-ml Erlenmeyer flask and with constant rapid stirring, 75 ml of n-butanol is added slowly from a buret. When addition is complete the flask and contents are warmed to 37°C in a water bath and incubated for 5 min with occasional stirring. The mixture is poured into a separatory funnel and the phases allowed to separate at room temperature. The clear yellow to light-red aqueous layer is removed and dialyzed overnight against running distilled water.

A column (8.2 cm width; 9 to 18 cm bed height) of cm-Sephadex C-50 (Pharmacia Fine Chemicals Inc., Piscataway, N.J. 08854) is prepared and equilibrated with sodium acetate buffer (60 mmol/liter, pH 7.50 ± 0.03 with the Beckman glass electrode). Pooled dialyzed enzyme solution, 810 ml, is centrifuged at low speed to remove any precipitate forming during dialysis and the acetate concentration adjusted to 60 mmol/liter, pH 5.3 by adding 90 ml of sodium acetate buffer (0.60 mol/liter, pH 5.3). This solution is carefully poured onto the gel and allowed to run through the column. A red-brown band appears at the surface of the gel; the column is washed with 2.5 column volumes of buffer to ensure complete elution (Figure 1). A₅₅₀ is measured to facilitate localization of the eluted protein.

A column of equilibrated cyanide-Sephadex A-50 (Pharmacia) is poured exactly as described above and cm-Sephadex C-50 eluate applied to the top of this column. After the sample is added, the column is washed with 2.5 bed volumes of buffer to completely elute the enzyme (Figure 2).

DEAE-Sephadex A-50 (Pharmacia) is prepared and equilibrated with 60 mmol/liter acetate-15 mmol/liter tris(hydroxymethyl)aminomethane buffer, pH 7.6, and the column is poured (2.5 cm width; 20 to 30 cm height). DEAE-Sephadex A-50 eluate is brought to the conditions of DEAE-Sephadex by adding 0.18 g of tris(hydroxymethyl)-aminomethane (Tris) to each 100 ml of solution; the pH should then be 7.6 ± 0.2. This enzyme solution is allowed to flow onto the DEAE-Sephadex column until completely applied—usually overnight at room temperature. The column is then washed with 100 to 200 ml of acetate–Tris buffer and the effluent discarded. The enzyme is now eluted with 0.12 mol/liter sodium acetate buffer, pH 4.75, and collected in 10-ml fractions. (Figure 3); A₅₅₀ is measured throughout. Fractions with A₅₅₀ greater than 0.03 are adjusted to pH 7.0 to 7.5.

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⁴ Trivial name, pyruvate kinase.
⁵ Formerly known as enolase.
⁶ Trivial name, glycerolphosphate dehydrogenase.
by addition of 1.0 molar phosphate buffer, pH 7.5, and dialyzed at 4°C for several hours against 2-oxoglutarate (1.0 mmol/liter) and pyridoxal phosphate (40 μmol/liter) in phosphate buffer (0.1 mol/liter, pH 7.5). Fractions are then dialyzed at 4°C against a 20-fold volume of the phosphate buffer, or of de-ionized water. Buffer or de-ionized water is changed four times in 32 h.

**Results**

**Preparation**

Average yields and purifications are shown in Table 1. Chromatographic eluates were collected in fractions (Figures 1–3) and those of arbitrary minimum specific activity used for further purification. Cut-off points chosen were: CM-Sephadex C-50 > 1.4 μmol min⁻¹mg⁻¹; QAE-Sephadex A-50 > 20 μmol min⁻¹mg⁻¹; DEAE-Sephadex A-50 > 98 μmol min⁻¹mg⁻¹. Yields are almost quantitative when a lower limit for minimum specific activity is chosen. The above limits allow high recovery without a great sacrifice in purity.

**Analytical Variables**

*Substrate optima.* Figures 4 and 5 show how aminotransferase activity varies with L-aspartate and 2-oxoglutarate concentration. Human serum activity represents the average percentage activity of three abnormal (>90 U/liter, Technicon SMA 12/60) and two normal (<50 U/liter, Technicon SMA 12/60) human serum pools. There is no significant difference between the substrate optima for the enzyme in human serum and for the purified erythrocyte enzyme. The data shown agree well with variations published by Henry et al.
related with L-aspartate concentration. (29) At 125 mmol of L-aspartate per liter, in phosphate buffer (93 mmol/liter), the purified enzyme from red cells exhibits a pH optimum plateau extending from pH 7.5 to 8.4 at 30°C. Activity is 78% ± 5% of maximum at pH 6.58 and 98% ± 2% of maximum at pH 8.75, in good agreement with other studies in which comparable L-aspartate concentrations and human serum enzyme were used (23, 17, 29).

Temperature variation. Aspartate aminotransferase activities of one normal and two elevated human serum pools, and of human erythrocyte preparation were determined at temperatures of 20.0°, 24.7°, 30.0°, 34.6°, 39.2°, and 45.0°C. Cuvet temperature was monitored with a 0°-50°C partial immersion thermometer that had been verified against a National Bureau of Standards certified total immersion thermometer. Each serum pool was assayed in triplicate, the purified erythrocyte specimen in quadruplicate.

Arrhenius plots of ln activity vs. reciprocal of absolute temperature, prepared from mean values by least squares regression, are shown in Figure 6. Data reported by earlier studies (23, 30) are converted to natural logarithms for ease in comparison. We find that human serum and purified erythrocyte aminotransferases follow identical Arrhenius relationships, which are in good agreement with previous studies (23, 30).

ln of activity vs. reciprocal temperature did not fall on the observed slope for 20°C (1/T = 2.41 X 10⁻¹ K⁻¹) or 45°C (1/T = 3.14 X 10⁻¹ K⁻¹); the latter is illustrated in Figure 6. Henry et al. (23) suggest that enzyme denaturation at 45°C is responsible for this deviation. On preincubating human serum in phosphate buffer,
92 mmol/liter, and L-aspartate, 150 mmol/liter, (assay preincubation concentrations) at 45°C, cooling, and carrying out the enzymatic reaction at 30°C, we concluded that sufficient activity was lost to give divergence from expected Arrhenius behavior.

**Effect of aspartate.** Scardi et al. (31) used phosphate and aspartate under different conditions to convert the enzyme to the less stable pyridoxamine form. We speculated that the pyridoxamine form may be partially denatured or apoenzyme and coenzyme may be separated during the preincubation in the assay of the Henry et al. (25). We incubated human serum and purified erythrocyte specimens, with and without aspartate, in phosphate buffer (assay preincubation concentrations). Human serum and purified red cell material retain full enzymatic activity when incubated at 45°C for 2 h in the absence of aspartate, but in the presence of aspartate activity continuously decreases (Figure 7). Human serum aspartate aminotransferase violates Arrhenius behavior above 40°C (25), at least in part because of an artifactual lowering of enzyme activity by incubation with aspartate as called for by the assay of Henry et al. (25).

**Stability**

The enzyme (9600 U/liter) retains 100% activity at 4°C for one month in distilled water (containing 1 g of sodium azide per liter). When diluted to concentrations that are about the same as those in serum (50 U/liter) and lyophilized in Tris buffer (50 mmol/liter, pH 7.5), the transferase is 50% inactivated in 135 days at 4°C. Less activity was lost if any of the following were included (per liter) during lyophilization: 1 g of human serum albumin (fraction V or crystalline); 0.13 mmol of glutathione (reduced); 0.03 mmol of pyridoxal phosphate; 0.5 mmol of 2-oxoglutarate; 0.25 mol of sorbitol; 7.5 mmol of 4-thiazolidine-carboxylate; 10 g of polyvinyl pyrrolidone; or 20 g of polyethylene glycol (mol wt, 6000). Although some activity was lost with use of each and several combinations of the above, the inactivation rate progressively decreased to a stable minimal activity.

Aspartate aminotransferase was diluted with Tris buffer (50 mmol/liter pH 7.5), human serum albumin, fraction V (1 g/liter), was added and the mixture was lyophilized. Initial reconstituted activity was 50 U/liter; after 395 days at 4°C it was 35 U/liter. The data, by least-squares regression, yielded a linear equation of

\[
A = 42.9 - 0.05d
\]

where A is aminotransferase activity in U/liter on any day, d, after lyophilization. These data represent 29 degrees of freedom over 395 days with a 3.8 U/liter standard error of estimate. The standard error of estimate (s_e) is given by

\[
s_e = \sqrt{\frac{\sum{(A_i - (b_0 + b_1d_i + b_2d_i^2))^2}}{n - 2}}
\]

where \( b_j \) (\( j = 0, 1, 2 \)) are the least-squares coefficients from the fitted estimate and \( A_i \) the experimental value of transferase activity on day \( d \), after lyophilization (\( i = 1, 2, \ldots, n \)). Dependence of enzymatic activity on time is better described by the quadratic

\[
A = 50.2 - 0.125d + 0.0002d^2
\]

representing the above data with \( s_e \) of 2.3 U/liter. When activity measurements before day 120 are omitted, the data are described by

\[
A = 38.2 - 0.007d
\]

with 19 degrees of freedom (\( s_e = 1.7 \) U/liter); this is 0.2 the overall daily activity loss. This "aging" phenomenon was seen in most lots prepared (Figure 8). However, one mixture stabilized enzyme activity particularly well when observed for 486 days. For this bottling, enzyme solution containing (per liter) 1 g of human serum albumin (fraction V), 1.1 mmol of 2-oxoglutarate, 30 \( \mu \)mol of pyridoxal phosphate, 130 \( \mu \)mol of glutathione (reduced), and 50 mmol of Tris buffer (pH 7.5) was lyophilized in 5-ml serum vials and stoppered under reduced (5–6 mm Hg) pressure [Preparation A]. At that time the enzyme activity was 55 U/liter. Over the 486 days, the mean value for Preparation A was 55 ± 2.9 U/liter (1 sd). The least-squares regression linear equation for 394 days is

**Fig. 7. Effect of L-aspartate on preincubation**

Three human serum pools [HSP (1, 2, 3)] and purified erythrocyte aminotransferase, [EGOT (O)], were incubated in phosphate (--) and phosphate + L-aspartate (-----) at 45°C. After incubation, samples were immediately cooled and assayed for aspartate aminotransferase at 30°C at times shown. Activity in U/liter

CLINICAL CHEMISTRY, Vol. 18, No. 4, 1972
Activity under temperature.

\[
A = 54.5 + 0.003 \, d
\]

with 30 degrees of freedom, and a standard error of estimate of 3.1 U/liter (Figure 8). Bottling under decreased pressure appears to be necessary: enzyme prepared from the same mixture, but lyophilized and bottled under air [Preparation A1], showed an activity of 55 U/liter at that time but a 366-day average of 49.0 U/liter and the linear regression equation was

\[
A = 53.5 - 0.017 \, d
\]

with 26 degrees of freedom over 366 days; \( s_a = 3.1 \) U/liter. The activity loss observed also showed an “aging” effect, with no significant loss in activity between days 140 and 395 (Figure 8).

Preliminary studies indicate that the following mixture (per liter) is also an effective stabilizing preparation: 80 µmol of 2-oxoglutarate, 80 µmol of glutathione (reduced), 16 µmol of pyridoxal phosphate, 50 mmol of Tris, and 10 g of polyvinyl pyrrolidone [Preparation B]. Three lots with enzyme activities of 48, 93, and 18 U/liter have retained 100% activity for nine months (to date).

After reconstitution, preparations A, A1, and B are essentially stable for 30 days. Vials with their contents reconstituted were stored at 4°C for this period with a total of 10-15 h at room temperature. Activity was 10% less 41 days after reconstitution.

Purity

Aspartate aminotransferase with a specific activity of 98-116 µmol min\(^{-1}\)mg\(^{-1}\) can be obtained as described. Specific activity of 176 µmol min\(^{-1}\)mg\(^{-1}\) at 37°C (106 µmol min\(^{-1}\)mg\(^{-1}\) at 30°C) is reported for the enzyme from porcine heart (32). Bonavita (33) was able to prepare the enzyme from human brain; the preparation had a specific activity of 20.7 µmol min\(^{-1}\)mg\(^{-1}\) at 37°C (13.4 µmol min\(^{-1}\)mg\(^{-1}\) at 30°C).

Disc gel electrophoresis demonstrated one contaminant band when 50 µg of protein was applied (Figure 9). In a sodium lauryl sulfate disc gel electrophoresis system (37) the purified erythrocyte aminotransferase has an apparent monomer molecular weight of 44,500 daltons when calculated by the method of Weber and Osborn (34). An identical monomer molecular weight was determined for porcine heart aminotransferase (Sigma), dimer molecular weight 90,000 (32, 35). In this system a 60-µg sample showed two contaminant bands with apparent monomer molecular weights of 55,000 and 24,500.

In a relatively crude stage in purification, after butanol fractionation and dialysis, using 900 mU aminotransferase per assay, we could detect no activity of the following enzymes: L-glutamate:NAD oxidoreductase (deaminating) [Strecker (36)], L-alanine:2-oxoglutarate aminotransferase\(^8\) (EC 2.6.1.2) [Bergmeyer and Bernt (37)], or L-lactate:NAD oxidoreductase\(^10\) (EC 1.1.1.27) [Amador and Wacker (38)]. L-malate:NAD oxidoreductase was 5 mU by the method of Henry (39); after the first ion-exchange chromatography (cm-Sephadex C-50) no such enzyme activity could be detected.

Cofactor Saturation

Pyridoxal phosphate (30 µmol/liter) is used as a preservative for the prepared enzyme. This concentration is sufficient for saturation (16) and the enzyme can be diluted extensively while maintaining activity: dilution linearity. In addition, when isolated with use of maleate buffer and 2-oxoglutarate, the enzyme is in the pyridoxal form. Resolution of the coenzyme moiety occurs much more easily with the enzyme in the pyridoxamine form (31).

Assay of the Purified Enzyme by Different Methods

We evaluated the compatibility of purified enzyme (Preparation B) with the assays of Babson et al. (8), Reitman–Frankel (9), Technicon SMA 12/60, and Henry et al. (23). All values were converted to U at 30°C under optimized conditions (23). Conversion factors, 0.88 for the assay of Babson and 0.80 for that of Reitman–Frankel were calculated from published data (8, 9, 23, 40) and verified experimentally in our laboratory with serum from patients. These factors have also been used routinely with success for conversion of inter-laboratory proficiency test data.

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\(^8\) Human enzyme conversion values used for ease in comparison.

\(^10\) Trivial name, lactate dehydrogenase (LD).
Fig. 9. Polyacrylamide electrophoretic patterns of erythrocyte aspartate aminotransferase preparations
Preparation stained for enzyme activity, column 1. Preparations stained for protein: column 2 (9 µg protein); column 3 (19 µg protein); column 4 (50 µg protein). Preparations after butanol fractionation and dialysis are shown stained for protein in column 5 and stained for both enzyme and protein (350 µg protein each) in column 6. Gels were stained as described in Materials and Methods in text.

Results obtained with purified enzyme after data conversion are shown in Figure 10. These show a linear, one to one, agreement for assays by the Reitman–Frankel and Henry methods. Although the correlation between the results of assays by the methods of Babson and Henry is also linear, values obtained by Babson's assay were 40% higher than expected. We studied the enzymatic and colorimetric portions of the assay and found that serum protein inhibits color formation. These results prompted further investigation of the Babson assay.

Initial studies showed nonadditive recovery when lyophilized purified enzyme samples were reconstituted with human serum (Table 2). In addition, these recovered activities were about equal to those obtained in the Henry and Reitman–Frankel assays. Further work indicated that this decreased recovery is caused by inhibition of oxalacetate–diazonium salt color development by human serum. Human serum that had been deproteinized by boiling had no effect on oxalacetate–diazonium salt color development, while human serum [Cohn et al. (21)] fractions I, II, III, IV-1, IV-2, and V all decreased oxalacetate–diazonium salt reactivity. In each case increasing protein concentrations (0–100 g/liter) produced decreasing color (e.g., by 30% when concentration of Fraction V was 100 g/liter). Protein exerted its effect both with and without Lipal11 diluent.

Therefore, the observed lack of compatibility in the Babson assay is due to the negligible protein content of our specimens. This does not vitiate use of this material for standardization of the

11 "Lipal" is ethoxylated tridecyl alcohol (obtained from Warner-Chilcott Laboratories, Morris Plains, N.J.).

Fig. 10. Aspartate aminotransferase activity by two colorimetric assays compared with kinetic determinations
Kinetic values (Δ) were determined by the method of Henry et al. (80) in five laboratories; 27 determinations on each sample. Reitman–Frankel (2) data (○) from four laboratories; n = 19. Babson et al. (8) data (■) from two laboratories; n = 25. All values have been converted to correspond with values by the Henry assay (see Results). For the Reitman–Frankel assay: Y = 3.31 + 0.974 X. For the Babson assay: Y = 6.66 + 1.363 X

Table 2. Effect of Human Serum on Aspartate Aminotransferase Values by the Assay of Babson et al. (8)†

<table>
<thead>
<tr>
<th>Enzyme activity, U/liter</th>
<th>Purified enzyme</th>
<th>Serum enzyme</th>
<th>Purified enzyme reconstituted with human serum</th>
<th>Recovery of purified enzyme (3 – 2)</th>
<th>Kinetic reference activity of purified enzyme</th>
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† All values obtained by the assay of Babson et al. (8) (except column 5) and converted as described in text.

Babson assay, because reconstitution with protein solution reconstitutes the color inhibition. With Fast Ponceau L15 dye of the Sax–Moore (41) assay, oxalacetate recoveries were lowered by only 4%, even if the concentration of protein in the sample was 100 g/liter.

High results were also measured in Technicon SMA 12/60 colorimetric and ultraviolet systems. Since oxalacetate–diazonium chromogen is measured in the Technicon colorimetric system, it is likely that there is a similar protein effect. A more pronounced effect was observed in the Technicon ultraviolet assay. This method showed an oxalacetate recovery of only 24% from 100 g/liter human albumin (Fraction V), as compared to saline. This may be due to decreased dialysis efficiency of oxalacetate because of the binding action of albu-

15 Fast Ponceau L Salt (Fast Red PDC) is diazoate-N'-butyl-4-methoxymethylanilide (Sigma).
min. We plan further study of these two reactions.

Blank values for the various assays were: Henry, 1 ± 1 U/liter; Reitman–Frankel, 3 ± 2 U/liter; Babson, 8 ± 5 U/liter; SMA 12/60 (colorimetric and ultraviolet), 1 ± 1 U/liter. In all cases these blank values are nonenzymatic, nonprotein in origin, and attributable solely to preservative(s). Because they are present in equal concentrations for any amount of enzyme, a preservative blank may serve as a zero standard.

**Discussion**

The first stages of the described purification are modifications of those reported by Jenkins et al. (13) for porcine enzyme and used by other workers (18, 55, 42). Variations in heating times and ammonium sulfate concentrations indicate it is practicable to use the same conditions for the initial fractionation of human enzyme. Polyethylene glycol (mol wt, 6000) can be substituted for ammonium sulfate in the fractionation, and we found the enzyme is largely precipitated by 140 to 175 g of polyethylene glycol per liter at room temperature. Neither yields nor purification are increased by this substitution. Packed red cells are lyophilized for convenient storage and to ensure hemolysis; however, results are identical when nonlyophilized hemolysate is substituted. In our hands, increasing hemolysate-buffer ratio during heat fractionation decreases yields because of occlusion of the enzyme. Polyanovskii and Telegdi (43), who used glutarate buffer (pH 6.0) in their preparations, reported partial derivatization of porcine enzyme sulfhydryl groups if maleate buffer is used. However, glutarate buffer (pH 6.0) does not protect human enzyme from heat denaturation, and no activity can be recovered by incubation with pyridoxal phosphate and (or) reduced glutathione. We did all our chromatography at room temperature because chromatography at 4°C did not improve yield.

In acetate buffer, pH 5.3, we find human erythrocyte aspartate aminotransferase does not bind appreciably to either cation- (CM-Sephadex C-50) or anion- (QAE-Sephadex A-50) exchange gels. We exploit this property by allowing the crude enzyme preparation to flow through each column—contaminating protein binds to surfaces of either gel. Use of the high-flow-rate columns described is as rapid as batch processing but provides greater yield and purity. Concentration is unnecessary before the sample is applied to these columns; an entire CM-Sephadex eluate and the following wash passes through a QAE-Sephadex column in less than 2 h.

In our routine procedure, the first 100 ml of eluate after sample application is discarded and the gels are allowed to run dry after the prescribed wash (see Materials and Methods). Adsorption of the enzyme onto DEAE-Sephadex is the slowest step in the current procedure. However, once started, the sample can be unattended overnight while it goes through the column. Yields of highly purified enzyme (specific activity >98 \( \mu \text{mol min}^{-1}\text{mg}^{-1} \)) can be increased by gradient or stepwise elution from the DEAE-Sephadex column. Because these steps are time consuming and necessitate rigorous control of buffer conditions, we omitted them from the routine preparation procedure. With the simplified technique, significant amounts of highly purified material can be prepared. We used the described method to obtain sufficient enzyme (93 \( \mu \text{mol min}^{-1}\text{mg}^{-1} \)) from 1.1 liters of red cells, for multiple assay by 450 clinical laboratories in New York State. For use in this evaluation, we prepared three 600-bottle lots (5 ml per bottle) with activities of 18, 48, and 93 U/liter.

We think that the preparation herein described meets the five criteria for a reference enzyme set forth in our introduction: human serum enzyme optima, maximum stability, coenzyme saturation, purity, and ease in isolation. With regard to purity, some additional considerations are pertinent. High-purity enzyme has been shown to be unstable at serum concentrations without the addition of albumin or polyvinylpyrrolidone. Certain conditions of purity, however, must be met: absence of glutamate dehydrogenase (an enzyme that interferes with the kinetic assay: \( L\text{-glutamate} + H_2O + \text{NAD} \rightarrow \text{2-oxoglutarate} + \text{NH}_3 + \text{NADH} \)), absence of malate dehydrogenase (high activities in a reference material would prevent detection of a deficiency of this coupling enzyme (6)), and absence of blank in colorimetric assays. Although absolute homogeneity can be justifiably demanded, we think the above specifications are sufficient. In fact, we find that the enzyme in any stage of purification after butanol fractionation adequately fulfills these conditions of purity, and material prepared as described has been successfully used in interlaboratory surveys.

The need for a uniformly standardized reference material is particularly well illustrated in the findings of Helman et al. (44). The described aspartate aminotransferase reference material provides a practical solution to this dilemma. Furthermore, the practical advantages of a purified enzyme reference material are demonstrated by our finding that the protein concentration of the sample markedly influences the results of certain colorimetric procedures, as is also true for a similar human lactate dehydrogenase reference material [Copeland, W. H., Graffunder, B.Y., and Vanderlinde, R.E., Preparation stability and characterization of human erythrocyte LDH I as an enzyme standard. Manuscript in preparation]. We anticipate that other methods will be subject to criti-
cism as enzyme reference materials become more widely used.

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