α-Ketoglutaric Semialdehyde Dehydrogenase of Pseudomonas

PROPERTIES OF THE PURIFIED ENZYME INDUCED BY HYDROXYPROLINE AND OF THE GLUCARATE-INDUCED AND CONSTITUTIVE ENZYMES*

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SUMMARY

Hydroxyproline-induced α-ketoglutaric semialdehyde dehydrogenase has been purified from a Pseudomonas strain and characterized with respect to substrate specificity and other properties. After a variety of growth conditions, Pseudomonas extracts also contain a constitutive dehydrogenase, which differs from the induced enzyme in sedimentation and electrophoretic behavior. Although the constitutive enzyme resembles the induced form in many kinetic respects, it is relatively more active with glutaric semialdehyde as a substrate and is tentatively assigned a role in lysine metabolism. Growth on D-glucarate induces a dehydrogenase which, in all kinetic and physical respects examined, appears identical with that induced by growth on hydroxyproline.

α-Ketoglutaric semialdehyde (2,5-dioxovalerate) is a recently discovered intermediate in several induced degradative pathways. Initially identified as a product of hydroxyproline metabolism (1–3), it has also been reported as an intermediate in the metabolism of L-arabonate (4) and of D-glucarate or D-galactarate (5) by Pseudomonas strains, and in the degradation of hexuronic acids by Agrobacterium tumefaciens (6). Other bacterial pathways in which it might hypothetically participate have been noted (3). Recent observations (7) have failed to support the consideration that it takes part in certain plausible reactions in rat tissues or in Escherichia coli.

We have briefly described (1, 2) the enzymatic oxidation of KGSA2 with triphosphopyridine nucleotide to yield α-ketoglutarate; KGSA dehydrogenase was purified slightly from extracts of hydroxyproline-grown cells of Pseudomonas striata (1, 2); this step completed the reaction sequence which converts hydroxyproline to α-ketoglutarate (8). Dagley and Trudgill (5) noted a similar reaction in crude extracts of Pseudomonas strains induced with any of several substrates, except that DPN appeared to be the preferred oxidant in extracts of galactarate-

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1 Y. F. Chang and D. S. Feingold, personal communication.

2 The abbreviations used are: KGSA, α-ketoglutaric semialdehyde; GSA, glutaric semialdehyde.

induced Pseudomonas "A." A similar oxidation of KGSA, reported by Stoolmiller and Ables (4), was catalyzed by an enzyme which had been partly purified from Pseudomonas oleovorans (9) after growth on hexane. Extracts of hexuronate-grown A. tumefaciens also catalyze KGSA oxidation to α-ketoglutarate(6).

A point of special interest concerning KGSA dehydrogenase in P. striata is its existence in at least two forms, one constitutive and the other induced by growth of cells on hydroxyproline or glucarate. We have already noted some comparative features of the constitutive and induced enzymes in bacterial extracts (10). The present paper describes the purification and certain properties of KGSA dehydrogenase from hydroxyproline-induced P. striata. The enzyme has been purified about 60-fold; while enzyme in the most purified fractions appears to represent at least 50% of the protein present, electrophoresis is capable of resolving several enzymatically active bands. Further details are also presented comparing the enzyme in extracts of cells grown on inducing and noninducing substrates.

EXPERIMENTAL PROCEDURE

Materials

Bacteria—The strain of Pseudomonas8 has been described earlier (8). Cells were maintained on stock slants, cultured, and harvested as described (12). During this work we observed that our cells yielded two distinct colonial forms on solid media; these resembled the translucent and more opaque colonies described by Stanier, Palleroni, and Doudoroff (11). No electrophoretic or activity differences were observed in either of the constitutive forms of KGSA dehydrogenase (growth on proline) or the induced form (growth on hydroxyproline) when either colony type was used as the cell source. Reference to a lyophilized culture of this strain prepared in 1962 suggested that the translucent colony type represented the original strain.

Substrates—α-Ketoglutaric semialdehyde was prepared chemically from 2,5-dimethoxystilbamides acid methyl ester and purified as described earlier (3). The precursor compound was furnished by Dr. N. Clauson-Kaas (13). After elution from Dowex 1, KGSA was kept at −15° in HCl (0.1 N to 1 N) at

8 Dr. R. Y. Stanier has examined our strain, previously designated P. striata (8), and assigns it to Pseudomonas putida biotype A (11).
concentrations of 0.05 to 0.5 m. No measurable loss was detected for at least several months by enzymatic assay with KGSA dehydrogenase (3). Although knowledge of the reactivity of KGSA with common components of biological assay systems is fragmentary, a rapid reaction with excess NH₃ has been noted (3). Observations of ours, in agreement with those of Dr. R. H. Abeles, also indicated that KGSA is unstable in Tris buffer. Incubation of 7 μmoles of KGSA in 0.1 m Tris, pH 8, for 6 hours at 25° led to almost complete loss of enzymatically assayable substrate. There was also significant loss, although less, under the same storage conditions at an acid pH produced by 0.3 m trichloracetic acid. In contrast, KGSA appeared quite stable in 0.1 m potassium phosphate, either at pH 8 or with added trichloracetic acid. Tris and other amine buffers were therefore avoided in subsequent assays.

An analogue of KGSA, presumptively 4-hydroxy-KGSA (3), was prepared by the condensation of glyoxal and oxalacetic acid, and purified by chromatography through Dowex 1-chloride. This material could be converted to hydroxyproline by treatment with NH₃ followed by reduction with NaBH₄, in a manner presumably analogous to the amination and reduction of KGSA to yield proline (3). Hydroxy-KGSA was estimated as hydroxyproline (14).

Glutaric semialdehyde was prepared by oxidation of α-amino adipate with chloramine-T, according to a procedure of Hendler and Anfinsen (18) for the oxidation of glutamate to succinic semialdehyde. Preparations beginning with 100 μmoles of α-amino adipate yielded approximately 50 μmoles of glutaric semialdehyde, assayed by the reduction of TPN with the use of partially purified KGSA dehydrogenase. For some purposes, glutaric semialdehyde was further purified by adsorption on Dowex 1-acetate and elution with 2 N acetic acid. The aldehyde was stable for at least several months on storage at 4° in acid solution, but large losses (enzymatic assay) were observed after lyophilization to dryness. Paper chromatography of the 2,4-dinitrophenylhydrazone gave Rf values that agreed with those reported by Ichihara, Ichihara, and Suda (16) for the solvents isopropyl alcohol-water-ammonia (120:20:10) (Rf 0.5) and tert-butyl alcohol-formic acid-water (40:10) (Rf 0.9). Reduction of the 2,4 dinitrophenylhydrazone in a Parr hydrogenator (suspension in water, PtO₂ catalyst, H₂ at 55 p.s.i., 2 hours, 25°) yielded a product which cochromatographed with reference δ-aminovalerate in three solvents (isopropyl alcohol-water-ammonia (120:20:10), Rf 0.14; tert-butyl alcohol-formic-acid-water (70:15:15), Rf 0.53; and n-butyl alcohol-water-ammonia (2:1:2), Rf 0.52).

Formaldehyde and acetaldehyde were dissolved as the crystals in small amounts of acid and acidified (5 N HCl) to liberate the free aldehydes. Malonic dialdehyde was obtained by HCl treatment of its dimethyl acetal, tetramethoxypropane. Succinic semialdehyde was obtained by refluxing diethyl formylsuccinate (a gift from Dr. S. Bessman) in 0.1 m HCl (17). Other aldehydes were of the highest purity obtainable commercially and were used without further purification. Uniformly labeled ²⁵C-L-lysine was a product of Schwarz BioResearch.

Calcium phosphate and alumina C₇ gels were purchased from Sigma; dry weight values cited are based on data furnished by Sigma. Polyethylene glycol (Carbowax 20,000) for dehydrating enzyme solutions was a product of Union Carbide. Sephadex gels were obtained from Pharmacia. Hydroxylapatite for column use was prepared according to the directions of Tiselius, Hjerten, and Levin (18). Other chemicals were obtained from standard commercial sources.

Methods

Preparation of Bacterial Extracts—Fresh or frozen cell pellets were homogenized with a motor-driven pestle in 5 volumes of 5 m potassium phosphate, pH 7.0, containing 5 mM GSH (fresh neutral solution) and 1 mM EDTA. Volumes of 30 to 50 ml were treated for 10 to 15 min in a 10-ko Raytheon sonic oscillator circulated with ice water. Sonic oscillation of small cell pellets was carried out for 1 to 2 min with similar suspensions of 5- to 10-ml volume in the microtip of a Branson ultrasonic oscillator with ice water cooling.

Cells were grown on hydroxy-L-proline as the major added carbon-nitrogen source in medium containing minerals and a small amount of yeast extract as noted earlier (8). Cultures were grown for 16 to 18 hours, and gave an absorbance at 650 μm (1-cm light path, Zeiss PMQ II spectrophotometer) of about 1.5. Medium composition for growth on substrates other than hydroxyproline is described below.

Assay of Enzyme—KGSA dehydrogenase activity was routinely assayed by following the rate of TPN reduction either in a Zeiss PMQ II spectrophotometer or in the Gilford recording attachment with a Beckman DU monochromator. The usual assay mixture was 1 ml in volume and contained 0.02 m pyrophosphate (pH 8.5), 1 mM TPN, and enzyme. KGSA was added last at about 0.5 μm, and the rate of change in absorbance at 340 μm was measured for several minutes. Incubation temperature was 25-28°. The reaction rate was linear with time and enzyme concentration over a convenient range with both crude and purified enzyme fractions. Crude extracts from constitutively grown cells required 50- to 200-fold dilution in the final volume for convenient assay, while crude enzyme from induced cells was generally diluted 10-fold further. Under these conditions, added α-ketoglutarate stimulated neither TPN reduction nor TPNH oxidation; the latter reaction was observed, however, on addition of ammonium salts, and undoubtedly reflects the TPN-specific glutamate dehydrogenase present in these extracts (8). One unit of enzyme was defined as the quantity catalyzing the reduction of 1 μmole of TPN per min under the conditions described.

For certain purposes a colorimetric assay was used in which TPNH was detected by the reduction of nitro blue tetrazolium chloride, mediated by phenazine methosulphate. Enzyme, KGSA, and TPN were incubated as described above for 5 min at 25°; 0.1 ml of a mixture of phenazine methosulphate (0.04%) and nitro blue tetrazolium chloride (0.2%) was then added with thorough mixing, and the mixture was shaken with 2 ml of ethyl acetate and centrifuged. Absorbance of the ethyl acetate layer was measured at 510 μm.

Gel Electrophoresis—The vertical method of Smithies (19) was used for starch. Gels were generally prepared in Tris or imidazole buffer, 0.025 m, pH 7. TPN at a final concentration of 0.1 to 0.2 μm in the gel (by addition to the hot gel suspension before pouring) greatly increased the stability of the enzyme during electrophoresis. Electrophoresis was carried out at 4° for 4 to 6 hours at about 4 volts per cm. After electrophoresis the starch slab was often cut horizontally into two parts. One
part was stained for protein in a solution of naphthol blue-black in 5% acetic acid and destained electrophoretically in 2% acetic acid. Enzyme activity was detected by flooding the freshly cut surface of the other portion of starch gel with a solution containing TPN, KGSA, and pyrophosphate buffer and, after several minutes, adding a few drops each of 0.2% phenazine methosulfate and 0.4% nitro blue tetrazolium to reveal purple bands in the position of enzyme. Direct elution of starch after a cycle of freezing and thawing indicated very extensive loss of enzyme units on electrophoresis, even when TPN was present in the starch block.

Polyacrylamide gel electrophoresis was carried out as described by Ornstein and Davis (20) with the Tris system of Jovin, of aliquots to remove GSH and EDTA. Sorbance at 280 nm was used to estimate protein. Occasionally of about 0.2 ml each were obtained by puncturing the bottom of approximately 10 cm, usually at 38,000 rpm for 16 to 18 hours. Fractions were layered with 0.2 ml of enzyme and centrifuged at approximately 5% to 20% in sucrose. The medium generally contained 5 mM described by Martin and Ames (22) through linear gradients from its omission reduced the effectiveness of subsequent steps.

Polyacrylamide gel electrophoresis was more discriminating than starch in revealing multiple enzyme-reactive bands in the most purified fraction, the starch method was used for most studies of extracts from variously grown cells because of the greater ease of detecting enzyme activity by direct staining.

**Sucrose Gradient Centrifugation**—This was carried out as described by Martin and Ames (22) through linear gradients from 5% to 20% in sucrose. The medium generally contained 5 mM phosphate (pH 7.0), 5 mM GSH, and 1 mM EDTA. Tubes were layered with 0.2 ml of enzyme and centrifuged at approximately 10 cm, usually at 38,000 rpm for 10 to 15 hours. Fractions of about 0.2 ml each were obtained by puncturing the bottom of the tube.

**Protein Determination**—For routine purposes, a turbidimetric method (23) was used. In monitoring column effluents, absorbance at 280 nm was used to estimate protein. Occasionally the method of Lowry et al. (24) was used, after thorough dialysis of aliquote to remove GSH and EDTA.

**RESULTS**

**Purification of Enzyme**

Our earlier efforts (2) to purify KGSA dehydrogenase failed to develop an effective method for the initial removal of nucleic acids and led to large losses of activity during fractionation. The earlier studies employed KGSA dehydrogenase primarily for the stoichiometry of KGSA formation and utilization, and dialysis against dilute buffers was always performed as an early step to remove possible interfering endogenous substrates. We have since learned that the enzyme becomes quite unstable on dialysis or storage, particularly in dilute salt solutions.

Fractionation usually began with about 20 g of fresh or frozen cell pellets (wet weight). All procedures were carried out at 0-4°C. Sonic extracts were centrifuged at 25,000 × g for 30 min; the supernatant solution (Fraction I) was further centrifuged at 100,000 × g for 90 min, and the supernatant fluid was kept (Fraction II). Essentially all the units present in Fraction I were recovered in Fraction II. To Fraction II was added an equal volume of calcium phosphate gel (80 mg per ml), and the mixture was stirred for 10 min, followed by removal of the gel by centrifugation. To the supernatant solution (Fraction III) was added 0.2 volume of Cy gel (20 mg per ml); the mixture was stirred for 10 min, and the Cy gel was collected by centrifugation and eluted by stirring for 15 min with 0.02 M potassium phosphate, pH 7.0, containing 5 mM GSH and 1 mM EDTA, usually in a volume about half that of Fraction II. The Cy eluate (Fraction IV) was concentrated 2- to 3-fold, initially by dialysis against Carbowax dextric, and in later preparations by lyophilization. Carbowax concentration sometimes caused significant losses, while lyophilization did not. Concentrated Fraction IV was then passed through a column (2.5 × 60 cm) of Sephadex G-100 which had been equilibrated with buffer of the same composition used for elution of enzyme from Cy gel. The enzyme was washed off the column with the same buffer; fractions highest in enzyme were selected and pooled (Fraction V). Although little or no purification and some loss of total activity generally resulted from this step, it was retained because of the impression that its omission reduced the effectiveness of subsequent steps.

Fraction V was adjusted with a pH meter to pH 6.0 to 6.2 by adding 1% glacial acetic acid and was passed onto a hydroxylapatite column, 2.2 × 40 cm. The column was eluted with a linear gradient made with 120 ml each of 0.01 M and 0.07 M potassium phosphate, pH 7.0, containing 5 mM GSH and 1 mM EDTA. The enzyme was eluted rather sharply, beginning at about 0.03 M in phosphate (Fraction VI). In individual runs, fractions were pooled so as to obtain maximum purity with reasonable yield. The final step involved chromatography of Fraction VI through a DEAE-cellulose column, 1.1 × 25 cm. The enzyme was eluted with a linear gradient made with 70 ml of 0.02 M potassium phosphate, pH 7.0 (containing 5 mM GSH and 1 mM EDTA), and 70 ml of the same buffer mixture containing 0.4 M KCl. Enzyme was eluted in a sharp peak, generally beginning at about 0.06 M KCl (Fraction VII).

It was observed that Fraction V contained considerable activity for both Δ1-pyrroline-4-hydroxy-2-carboxylate deaminase (2) and hydroxyproline 2-epimerase (25). The deaminase, in particular, had been inadvertently purified 10- to 20-fold and was estimated to represent an appreciable protein contaminant of Fraction V. The subsequent two purification steps were introduced to remove these enzymes, based on previous knowledge of their fractionation behavior. The hydroxyxylapatite step removed deaminase activity largely or completely, while in the DEAE-cellulose step KGSA dehydrogenase activity was eluted before the epimerase.

The best preparations of Fraction VII contained enzyme purified 50- to 60-fold from the initial sonic extract. In later preparations, TPN was added to solutions used to elute the hydroxylapatite and DEAE-cellulose columns. This improved the recovery of enzyme in the two last steps to an over-all yield of about 25%, compared with yields averaging 10% from preparations without TPN addition. A specific preparation is summarized in Table I.

**Purity of Enzyme**—Starch electrophoresis of Fraction VII indicated a single major protein-staining band (Fig. 1), which agreed in position with the band detected by enzyme staining (Fig. 2). Both the band staining for enzyme activity and that staining for protein were quite broad, although comparably so. Electrophoresis on polyacrylamide gel led to resolution of several enzyme-staining bands, each corresponding to a band staining for protein. Under these conditions, too, it was judged that most of the stainable protein was enzymatically active in the KGSA-TPN assay described under "Methods." It is not clear whether the electrophoretically multiple enzyme bands of purified fractions are intrinsic or are produced during electrophoresis.
TABLE I

Purification of KGSA dehydrogenase

Assay, units, and procedure are described in the text.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>units/ml</td>
<td>mg/ml</td>
<td>units/mg</td>
<td>%</td>
</tr>
<tr>
<td>I. Sonic supernatant</td>
<td>90</td>
<td>29</td>
<td>33</td>
<td>0.88</td>
<td>100</td>
</tr>
<tr>
<td>II. &quot;Spinco&quot; supernatant</td>
<td>80</td>
<td>29</td>
<td>21</td>
<td>1.4</td>
<td>89</td>
</tr>
<tr>
<td>III. Calcium phosphate supernatant</td>
<td>135</td>
<td>17</td>
<td>3.5</td>
<td>4.9</td>
<td>88</td>
</tr>
<tr>
<td>IV. Cγ gel eluate</td>
<td>32</td>
<td>37</td>
<td>4.0</td>
<td>9.3</td>
<td>45</td>
</tr>
<tr>
<td>V. Sephadex effluent</td>
<td>19</td>
<td>37</td>
<td>3.2</td>
<td>12</td>
<td>27</td>
</tr>
<tr>
<td>VI. Hydroxylapatite eluate</td>
<td>42</td>
<td>16</td>
<td>0.43</td>
<td>37</td>
<td>26</td>
</tr>
<tr>
<td>VII. DEAE-cellulose eluate</td>
<td>23</td>
<td>27</td>
<td>0.51</td>
<td>53</td>
<td>24</td>
</tr>
</tbody>
</table>

Properties of Enzyme

Molecular Weight—A single estimation of molecular weight was made by the sucrose gradient method (22), with the use of crystalline catalase and crystalline yeast alcohol dehydrogenase as internal standards. Crude KGSA dehydrogenase (Fraction II was found repeatedly to give a sucrose gradient peak identical with that of Fraction VII (Fig. 8)) was centrifuged for 16 hours at 38,000 rpm with yeast alcohol dehydrogenase and bovine liver catalase as internal standards. Based on the midpoint of each sedimenting peak, the relative distances traveled from the meniscus were in the ratios 6.3, 7.6, and 10.8 for KGSA dehydrogenase, alcohol dehydrogenase, and catalase, respectively. From these data the molecular weight of KGSA dehydrogenase was estimated to be about 118,000. Approximate agreement with this estimate was also obtained from the emergence positions of the enzyme and several marker proteins on filtration through Sephadex G-200 (26), as described in a later section.

pH Optimum and Buffer Effects—Activity as a function of pH is shown in Fig. 3 with enzyme at the stage of Fraction VII. A point of interest is the higher activity at several pH values obtained with Tris buffer than with the other buffers used. Further investigation of this effect in a single pH region (8.0 to 8.5) showed that the initial rate of the reaction was about one-third higher with Tris (0.1 M) than with phosphate (0.1 M) but declined after 4 to 5 min to rates half that in phosphate; in contrast, the reaction in phosphate was maintained at a steady rate. Preincubation of KGSA with Tris for brief periods before addition of enzyme showed that after only 1 or 2 min of preincubation the initial rate was reduced about 30%, but did not decline further with longer preincubation. In view of the instability of KGSA in Tris (see “Methods”), it is plausible that a KGSA-Tris complex (or a complex with some impurity in Tris) forms and inhibits the enzyme. The estimated loss of KGSA alone, through a spontaneous reaction with Tris, is not sufficient to explain the declining rate.

Values of K_s for KGSA, TPN, and DPN—Fig. 4 presents reciprocal velocity-substrate plots for the principal substrates. In several sets of measurements with purified enzyme (Fraction VII), the value of K_s for KGSA ranged between 5 × 10^{-5} M and 1 × 10^{-3} M; inhibition at high substrate concentration is seen in Fig. 4.1. The value of K_s for TPN was approximately 1 × 10^{-1} M, and that for DPN was 1 × 10^{-3} to 3 × 10^{-2} M. The ratio of V_max for TPN:DPN was estimated as about 2.5.

It was clear from these data, as well as from a number of comparisons at single concentrations of each pyridine nucleotide, that the enzyme is selective for TPN, in contrast to the crude KGSA dehydrogenase of Pseudomonas A grown on α-galactarate, for which DPN was reported as the preferred oxidant (5). Excess substrate permitted complete reduction of either added DPN or TPN.

Substrate Specificity—Preparations of the enzyme at the stage of Fraction VII were tested with a number of aliphatic aldehydes.
FIG. 3. Activity as a function of pH for purified enzyme. Enzyme was Fraction VII (Table I) with a specific activity of 39 units per mg. A relative activity of 100 corresponded to 1.9 units per ml. Buffers were phosphate, 0.1 M (○); barbital, 0.05 M (●); carbonate, 0.1 M (▲); and Tris, 0.1 M (○). The pH in the cuvettes was determined following assay.

FIG. 4. Reciprocal plots of velocity as a function of concentration. Substrate concentrations varied were KGSA (A), TPN (B), and DPN (C). Two separate determinations are illustrated in A. The ordinates are in reciprocal units per ml of enzyme, and the abscissas are in reciprocal millimolar concentrations. For the assays of A, TPN was present at 1 mM; for the assays of B and C, KGSA was present at 0.26 mM. The enzyme corresponded to Fraction VII. Preparations of different activity were used for B and C.

Table II
Substrate specificity of KGSA dehydrogenase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Rate relative to KGSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>3.8</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>3.8</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>10-20</td>
<td>11*</td>
</tr>
<tr>
<td>Malonic aldehyde</td>
<td>2.5</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>3-Hydroxyglyceraldehyde</td>
<td>5</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>Pyruvic aldehyde</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Butyraldehyde</td>
<td>4-6</td>
<td>14*</td>
</tr>
<tr>
<td>Isobutyraldehyde</td>
<td>4-6</td>
<td>1.2*</td>
</tr>
<tr>
<td>2-Hydroxybutyraldehyde</td>
<td>6</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>Succinic semialdehyde</td>
<td>6</td>
<td>0.4*</td>
</tr>
<tr>
<td>Valeraldehyde</td>
<td>4</td>
<td>8*</td>
</tr>
<tr>
<td>2,3-Dimethylvaleraldehyde</td>
<td>4</td>
<td>0.5*</td>
</tr>
<tr>
<td>5-Hydroxyvaleraldehyde</td>
<td>4</td>
<td>2.8</td>
</tr>
<tr>
<td>Glutaric semialdehyde</td>
<td>2-20</td>
<td>10*</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>3-25</td>
<td>15*</td>
</tr>
<tr>
<td>4-Hydroxy-KGSA</td>
<td>6</td>
<td>0.8*</td>
</tr>
<tr>
<td>Hexylaldehyde</td>
<td>4</td>
<td>8*</td>
</tr>
<tr>
<td>Heptylaldehyde</td>
<td>4</td>
<td>15*</td>
</tr>
<tr>
<td>Oleylaldehyde</td>
<td>4</td>
<td>11*</td>
</tr>
<tr>
<td>Decylaldehyde</td>
<td>4</td>
<td>10*</td>
</tr>
</tbody>
</table>

* Average of two to four determinations.
$^b$ Value of Vmax.
$^c$ Competitive inhibitor; see the text.

(29) Certain modifications in the assay were desirable for the longer chain, partly water-insoluble aldehydes (C5 and above). These were assayed not only by the standard optical method but also by addition to the assay medium of the substrate in ethyl alcohol, together with the inclusion of ethyl alcohol (0.1 volume) in the incubation mixture. To correct for slight increasing absorbance due to emulsion formation, assays were carried out in a recording spectrophotometer with addition of enzyme last. TPNH was also measured by the formazan method (see "Methods"). Reasonable agreement was obtained for a number of the substrates assayed by different methods, and these averaged assay values appear in Table II.

It was clear that the enzyme was not highly specific, but acted on a number of aldehydes from C5 to C18 (the longest chain tested) at varying rates up to about one-fifth the rate with KGSA. Except for GSA, which had a Km of $1.6 \times 10^{-5}$ M, all other substrates besides KGSA had low affinity for the enzyme. The Km for glutaraldehyde, for example, was about 0.05 M. In most instances little or no inhibition of KGSA oxidation (usually at 0.4 mM) was observed in the presence of a 10-fold or greater concentration of the less active substrate, signifying relatively low Km values for the latter. Low rates of reaction were not due to traces of a contaminating active substrate, since glutaraldehyde, propionaldehyde, valeraldehyde, and heptylaldehyde were oxidized stoichiometrically by the enzyme. The reaction with glutaraldehyde resulted in the uninterrupted production of 2 moles of TPNH per mole of substrate, indicating the oxidation of both aldehyde groups. At low concentrations of glutaraldehyde, the rate-limiting step is that of the first oxidation, since the dialdehyde is bound about three orders of magnitude more weakly than the semialdehyde.

Inhibitors—No significant inhibitor activity was observed in the compounds tested, with the single exception of presumptive 4-hydroxy-KGSA. This compound behaved like a competitive inhibitor of the oxidation of KGSA (Fig. 5); the values of KI calculated from the two concentrations of inhibitor shown in Fig. 5 were $3 \times 10^{-3}$ M and $4 \times 10^{-3}$ M. The high affinity of this compound for the enzyme (matched only by KGSA and GSA) is a further point in favor of the structure considered for the inhibitory compound.

Stability and Protection by TPN—The heat lability of KGSA...
dehydrogenase was noted earlier (2) and was then utilized to remove this activity from preparations of \( \Delta \)-pyrroline-4-hydroxy-2-carboxylate deaminase. Crude preparations of the dehydrogenase, although always stored with GSH and EDTA, irregularly lost activity on storage at \(-15\)° or \(4\)° and on dialysis at \(4\)°. Storage or dialysis was also found to render the enzyme more heat-sensitive, even when no loss of activity was directly demonstrable. Any of a number of additions markedly stabilized both crude and purified preparations tested by inactivation rates at temperatures of 40-50°. Stabilizing additions included inorganic salts, KGSA, thiols, and TPN or DPN. For comparable protection, DPN was required at about 10 times the concentration of TPN, in agreement with their respective \( K_m \) values. TPN stabilized the enzyme during dialysis and column fractionation procedures as well as during heat treatment. Fig. 6 illustrates the stabilizing effect of TPN on purified enzyme \((A)\), together with the dependence of enzyme stability on ionic strength \((B\) and \(C)\). Less stable preparations of enzyme, e.g. samples of Fraction II in relatively low salt, frequently showed two-slope heat-decay curves, similar to that seen in Fig. 6B for dialyzed Fraction VII.

Other Forms of KGSA Dehydrogenase

Enzymes capable of oxidizing KGSA have been detected in several strains of \textit{Pseudomonas} and in \textit{Agrobacterium} \((6)\), and during growth on any of several substrates \((2, 4, 5, 9)\). It seemed of interest to determine in a single strain whether separate inducers of KGSA dehydrogenase led to the synthesis of demonstrably different or similar proteins, and if comparable enzyme activity was present in extracts of uninduced cells.

In surveying growth substrates other than hydroxyproline that might induce KGSA dehydrogenase in our strain, \( L \)-arabinose, \( D \)-arabinose, \( D \)-gluconate, and \( 2 \)-furoate were tested as carbon sources, each at 0.2\% and with the addition of 0.1\% \((\text{NH}_4\text{})_2\text{SO}_4\) as a nitrogen source; other features of the medium and growth conditions were those used for growth on hydroxyproline. Hexane as a carbon source was also tested under conditions similar to those described by Baptist, Gholson, and Coon \((9)\). Cells failed to grow on either isomer of arabinose, on 2-furoate, or on hexane. Growth was good on \( D \)-gluconate as carbon source and, as reported for another \textit{Pseudomonas} strain \((6)\), led to relatively high levels of KGSA dehydrogenase in extracts.

To obtain so-called constitutive enzyme, cells were grown in the same mineral media under the same conditions, except that hydroxyproline was replaced by \( L \)-proline, \( L \)-glutamate, \( L \)-lysine, or yeast extract (Difco), each at 0.2\%. Alternatively, in place of hydroxyproline, the growth medium contained 0.2\% glucose (autoclaved separately) plus 0.1\% \((\text{NH}_4\text{})_2\text{SO}_4\). To rule out possible dependence of enzyme formation on yeast extract (the only undefined supplement present in the medium), the latter was occasionally omitted from media containing proline, glucone, or lysine without effect on enzyme levels. All the non-inducing media tested yielded cells the extracts of which contained low levels of KGSA dehydrogenase, ranging from 7\% (glucose growth) to about 30\% (lysine growth) of the specific activity found in extracts of hydroxyproline-grown or gluconate-grown cells. These data were reported in more detail elsewhere \((10)\) and are summarized in Table III for only the proline-, hydroxyproline-, or gluconate-derived enzyme.

Comparison of Induced and Constitutive Enzymes

Kinetic Features—The finding of activity for TPN-linked KGSA oxidation in cells grown on a variety of substrates not known to involve KGSA as a metabolic intermediate suggested that the so-called constitutive enzyme might be an aldehyde dehydrogenase unrelated to induced KGSA dehydrogenase, but with an overlapping range of substrates. A number of kinetic comparisons were therefore made between the enzyme in extracts of proline-grown cells, as an example of the constitutive group, and the enzyme in similar extracts of gluconate-grown cells and of hydroxyproline-grown cells. Fig. 7 shows the pH-activity curves for these three types of extracts. Crude and

![Fig. 5. Inhibition by 4-hydroxy-\( \alpha \)-ketoglutaric semialdehyde. Enzyme at the stage of Fraction VII was assayed without the inhibitor and with 0.022 \text{mM} and 0.006 \text{mM} concentrations of inhibitor. The \( \text{abcissa} \) is reciprocal millimolar concentrations of KGSA, and the \( \text{ordinate} \) is reciprocal units per ml of enzyme. \( \text{Numbers at each time} \) refer to the millimolar concentration of inhibitor. \( \text{Lines were drawn by the method of least squares, omitting the two highest (inhibitory) substrate concentrations.} \)

![Fig. 6. Stabilization of purified enzyme by TPN or salt. \( A \), heat inactivation of Fraction VII in about 0.1 \text{m KCl}, as eluted from DEAE-cellulose, without added TPN \((\bullet)\); \( B \), heat inactivation of the same enzyme as in \( A \), after dialysis to remove KCl (6 hours at \(4\)° against 250 volumes of 5 \text{mM phosphate (pH 7)} , 5 \text{mM GSH, and 1 \text{mM EDTA)}} ; \( C \), heat inactivation of the same enzyme as in \( B \) after addition of KCl to 0.2 \text{m}.)](http://www.jbc.org/content/jbc/242/9/1807)
**TABLE III**  
Kinetic comparisons of KGSA dehydrogenase from cells grown on different substrates

All values were obtained with fractions corresponding to Fraction II, Table I. Ranges of values given for $K_m$ determinations represent two or three separate determinations with different preparations. Specific activity ranges and averages are based on 18 separate cultures for hydroxyproline, 7 for proline, and 3 for glucarate.

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Specific activity $^{a}$</th>
<th>$K_m$</th>
<th>Rate relative to KGSA with the following substrates:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proline</td>
<td>0.13–0.19 (0.14)</td>
<td>$4 \times 10^{-5}$</td>
<td>Acetaldehyde</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0.51–1.4 (0.68)</td>
<td>$2 \times 10^{-5}$</td>
<td>Propanaldehyde</td>
</tr>
<tr>
<td>Glucarate</td>
<td>0.40–1.7 (0.91)</td>
<td>$3 \times 10^{-5}$</td>
<td>Butyraldehyde</td>
</tr>
</tbody>
</table>

$^{a}$ The range of values is shown, with mean values in parentheses.

Purified preparations of the hydroxyproline-induced enzyme gave almost superimposable curves (Fig. 7), suggesting that the comparison of crude enzyme preparations is valid. Although the glucarate-induced enzyme and the enzyme from proline-grown cells both showed a minor shift of peak $pH$ to slightly higher $pH$ values than the hydroxyproline-induced enzyme, no distinction between the enzymes could be based on these curves.

For the three types of enzyme, a comparison of $K_m$ values for each of the three substrates, KGSA, TPN, and DPN, was also made (Table III). Because of the variation in replicate determinations with the same type of enzyme, certain questionable differences among values for the separate enzymes (e.g. the $K_m$ for DPN of glucarate- and hydroxyproline-induced enzyme) could not be used to establish kinetic differences among the three types of enzyme. As with the $pH$-activity curves, $K_m$ values obtained with Fraction VII of the hydroxyproline-induced enzyme (see text above) were similar to those obtained with Fraction II.

A further comparison was made with the use of several substrates, all at concentrations believed to give maximal rates. It was of interest that extracts of proline- or hydroxyproline-grown cells contained no acetaldehyde-oxidizing activity. Ratios of activity for KGSA and for other substrates tested were similar for enzyme derived from the third growth conditions and agreed fairly well with the substrate specificity pattern of the purified hydroxyproline-induced enzyme. An exception to the kinetic similarities so far noted, however, was glutaric semialdehyde. While both glucarate- and hydroxyproline-induced crude enzymes oxidized GSA at only one fifth to one fourth the rate of KGSA (at saturating concentrations of either substrate), extracts of proline-grown cells oxidized both substrates at approximately equal rates. As previously reported (10), the ratio of KGSA:GSA rates appeared to represent the single reliable kinetic feature distinguishing induced from constitutive enzyme; growth on all substrates tested other than hydroxyproline or glucarate yielded crude enzyme having approximately equal activity for KGSA and GSA. Although $K_m$ for GSA was not determined systematically for extracts grown on the noninducing substrates tested, occasional assays at low GSA concentration suggested that its $K_m$ was comparable with that of the hydroxyproline-induced enzyme.

**Sedimentation and Electrophoresis**—In addition to the single
kinetic difference noted, a constant difference in sucrose gradient sedimentation and in electrophoretic mobility was repeatedly observed, and distinguished the glucarate- or hydroxyproline-induced enzyme from enzyme obtained after growth on all other substrates.

The sedimentation difference is illustrated in Fig. 8 for enzyme from hydroxyproline-grown or proline-grown cells. The molecular weight of the constitutive species was estimated to be about 50% greater than that of the inducible enzyme, or approximately 170,000; a similar value was obtained from the emergence position of the constitutive enzyme on filtration through Sephadex G-200 (26) columns, described below. As noted earlier (10), enzyme from glucarate-grown cells sedimented identically with that from hydroxyproline-grown cells, while enzyme from cells grown under all constitutive conditions tested sedimented like that illustrated from proline-grown cells. Mixing experiments (Fig. 8D) indicated that these sedimentation differences were maintained in a common environment. Addition of KGSA or glucarate to the sucrose solution before sedimentation did not influence the position or height of the enzyme peak from constitutively grown cells.

As illustrated schematically (10) and in photographs (Fig. 9), the enzyme from cells grown on noninducing substrates could also be distinguished electrophoretically from the enzyme extracted from hydroxyproline- or glucarate-grown cells. After all growth conditions, extracts showed an enzyme-staining band of relatively slow migration. After growth on hydroxyproline or glucarate, this band was similarly visible but was accompanied by a more intensely staining enzyme band, which moved more rapidly toward the positive pole. Under some conditions the induced enzyme species was a rather sharply defined electrophoretic band (Fig. 9A), while under others it was quite diffuse, as described for the purified enzyme (Fig. 9B). When TPN was routinely added to starch slabs, a faint band in the position of the induced enzyme could be discerned in all of the constitutive extracts examined (Fig. 9, B and C). The electrophoretic distinction between the constitutive and inducible types of enzyme was maintained at several pH values between 6.8 and 7.5, and persisted in mixed extracts (Fig. 9A). That these two species of enzyme alone accounted for the oxidation of both GSA and KGSA in crude extracts was also demonstrated electrophoretically (Fig. 9C). No electrophoretic difference could be detected between the major bands induced by growth on hydroxyproline or on glucarate.

Heat Stability A further apparent difference between the induced type of enzyme and that in extracts of constitutively-grown cells was the relatively greater heat stability of the latter type, illustrated in Fig. 10 for enzyme from proline-grown cells (Curve A) compared with that from glucarate-grown cells (Curve B). The difference was consistent in many trials but appeared to depend on the environment of the enzyme, rather than on its intrinsic properties. Thus, the unstable induced form of the enzyme was stabilized when added to extracts of constitutively-grown cells (Fig. 10). Stabilization could also be accomplished by addition to heat-inactivated (15 min, 65°C) extracts of cells grown under either induced or constitutive conditions. Aged extracts, stored for some months at -15°C, however, had lost the capacity to protect the inducible enzyme against rapid heat inactivation. The relatively stable constitutive enzyme could sometimes be made as labile as the induced enzyme by brief treatment with charcoal (Fig. 11), although this was not a consistently reproducible finding.

Although the significance of the two-slope decay curves for unstable preparations of KGSA dehydrogenase is unexplained, this type of inactivation curve is not unprecedented. Examples of such two-slope inactivation curves can be found illustrated for purified homoserine dehydrogenase (27) and for purified alkaline phosphatase (28). In general, a satisfactory explanation is lacking for the complex nature of such inactivation curves. In the present case, the possible interpretation that each slope represents a separate enzyme species, perhaps corresponding to the constitutive and inducible forms, was not supported. If this were the case, the hypothetical separate species defined by heat inactivation should be present in approximately a 1:10 proportion instead of the more nearly equal quantities estimated from heat inactivation curves. In addition, samples of the "constitutive" shoulder from sucrose sedimentation profiles of induced enzyme gave a two-slope heat inactivation curve much like that of samples taken from the peak tubes of the sedimentation pattern (Fig. 12). This finding also supports the belief that the relative heat stability of various forms of the enzyme was not intrinsic, but was determined by the environment.

The possibility that the inducible enzyme and constitutive enzyme are interconvertible forms dependent on the environment of the enzyme is suggested by the observations that the inducible enzyme can be stabilized by admixture with fresh crude prepara-
Fig. 9. Electrophoretic properties of constitutive and inducible enzyme. A, enzyme-staining bands from proline-grown cells (first channel), hydroxyproline-grown cells (center channel), and an equal mixture of these two extracts (right channel); B, enzyme-staining bands from cells grown on (from left to right) proline, lysine, hydroxyproline, yeast extract, glucose, and glutamate; C, enzyme-staining bands from lysine-grown cells. The two mirror image halves result from a horizontal cut and are stained, respectively, with GSA as substrate (left) and KGSA as substrate (right). All extracts shown correspond to Fraction II, Table I.

Fig. 10. Heat inactivation of induced and constitutive enzymes and their mixture. A, crude enzyme (Fraction II, Table I) from proline-grown cells; B, similar fraction from glucarate-grown cells; A + B, mixture of A and B in a ratio of 9:1 to provide approximately equal activity from each source; C, theoretical decay curve for A + B if each component behaved independently. Each extract and the mixture, all containing approximately equal protein concentration, were heated without dilution. Units given are for small samples (taken in the proportion of 10:5:1 for A:(A + B):B, chosen to yield approximately equal zero time activity.

Fig. 11. Labilization of constitutive enzyme by charcoal treatment. One milliliter of extract (Fraction II, Table I) from proline-grown cells was treated for 3 min at 0° with 50 mg of acid-washed charcoal (Norit A), then centrifuged and filtered (B). A control sample (A) was treated similarly except for omission of charcoal. Both were then tested simultaneously for inactivation at 43°.

and sedimentation properties expected from the combined individual components. More explicit experiments were done in which the inducible enzyme (an extract of glucarate-grown cells) was stabilized to heat denaturation by adding to it several volumes of a freshly prepared extract of lysine-grown cells. It was possible also to show marked stabilization of the glucarate-derived enzyme by adding an equal volume of heat-inactivated lysine-derived extract. This mixture, in which the induced enzyme
was the only KGSA dehydrogenase activity demonstrable, behaved in a manner typical for the induced enzyme with respect to the KGSA:GSA $V_{\text{max}}$ ratio, electrophoretic mobility in starch gel, and filtration through Sephadex G-200 (see below). An extract derived from lysine-grown cells was treated with charcoal and then exhibited greater heat lability, much as illustrated in Fig. 11. The charcoal-treated enzyme, although much more heat-labile, gave the typical KGSA:GSA $V_{\text{max}}$ ratio of about 1, both immediately and after heating samples so as to reduce enzyme activity to about one-third the unheated level. Other samples of lysine-derived enzyme, similarly treated with charcoal, showed no alteration of the electrophoretic or Sephadex filtration behavior characteristic of this form of the enzyme.

**GSA as Substrate**—Unlike heat stability differences, the ratio of activity with KGSA and GSA as substrates did appear to distinguish the constitutive and inducible components even when these were present in the same extract. Thus filtration of hydroxyproline-induced enzyme through Sephadex G-200 yielded the same type of asymmetrical peak observed with sedimentation in sucrose gradients, with an earlier emergent shoulder of activity corresponding to the constitutive enzyme (Fig. 13). Enzyme in the earliest tubes of such gel fractionations of induced enzyme gave KGSA:GSA ratios characteristic of the constitutive enzyme, while that in successively later tubes gave increasingly higher KGSA:GSA activity ratios. Similarly, enzyme in the last fractions from gel-filtered constitutive enzyme gave KGSA:GSA ratios ranging up to 3; the very small quantities of the inducible form present in constitutive enzyme preparations made this demonstration less clear than for the larger quantities of the constitutive enzyme present in extracts from induced cells. Qualitatively similar observations were also made from appropriate regions of the sucrose gradients in which constitutive or induced enzyme had been sedimented.

Efforts to obtain clear-cut separation of the constitutive and inducible components from crude extracts (after both constitutive and inducing growth conditions) were made by fractionation with DEAE-cellulose, hydroxylapatite, by preparative electrophoresis on starch gel or acrylamide, and by recycling pooled fractions from Sephadex gel filtrations similar to those shown in Fig. 13. These were unsuccessful; although electrophoresis was the most promising method in principle, the large losses of enzyme sustained on eluting gels precluded useful preparative separation under the conditions used.

**GSA as Possible Intermediate in Lysine Degradation**—The consideration that the constitutive form of the dehydrogenase might be related to lysine metabolism initially prompted our efforts to prepare and test GSA as a substrate, since an inducible pathway for lysine described in a Pseudomonas strain involves the formation (16) of GSA via transamination of $\alpha$-aminovalerate.

A recent communication by Takeda and Hayaishi (29) reports that the product of crystalline lysine oxygenase, considered to be the first enzyme of this pathway, is not $\alpha$-aminovalerate, but its amide, $\alpha$-aminovaleric amide. The amide can evidently be readily deamidated in less purified preparations to yield $\alpha$-aminovalerate, earlier isolated as a metabolic product of lysine (30).
and oxidation of GSA to α-ketoglutarate (31). It was therefore of interest to determine whether this pathway is operative in our strain, particularly after growth on lysine.

That δ-aminovalerate (or a closely related precursor) is derived from lysine in our extracts was shown by incubations of crude bacterial extracts with 14C-l-lysine and unlabeled δ-aminovalerate. Following a 2-hour incubation at 25° of 10 μmoles of l-lysine (2.6 × 10^8 cpm) with 10 μmoles of δ-aminovalerate and 0.2 ml of extract (Fraction II) of lysine-grown cells (Tris buffer, pH 8), approximately half the initial counts were associated with δ-aminovalerate, separated chromatographically on paper (n-butyl alcohol-acetic acid-water, 4:1:1) from lysine and α-aminoadipate. Under the same conditions, an extract of hydroxyproline-grown cells catalyzed the transfer of only about one-eighth the initial counts in lysine to the δ-aminovalerate region. With either extract, essentially all of the initial counts were accounted for (by elution from paper chromatograms) as associated with either lysine or δ-aminovalerate.

Transaminase activity for δ-aminovalerate was demonstrated by coupling this reaction to GSA oxidation. The latter reaction, observed in the usual way by TPN reduction, was dependent on the addition to crude extracts of both α-ketoglutarate and δ-aminovalerate or lysine. Extracts of hydroxyproline-grown or proline-grown cells were as active in catalyzing the presumptive transamination reaction as were extracts from lysine grown cells, in agreement with the findings of Suda, Kamahara, and Hagiwa (32) that the transaminase is not inducible, or only slightly so, by growth on lysine.

**DISCUSSION**

Enzymes catalyzing pyridine nucleotide-linked oxidation of α-ketoglutaric semialdehyde have recently been recognized in bacterial extracts following growth on hydroxyproline (2), glucarate or galactarate (5), arabinose (4), or hexuronates (6). The studies described here present some properties of such an enzyme purified extensively from a *Pseudomonas* strain grown on hydroxyproline. Since growth on any of these substrates leads both to the formation of α-ketoglutaric semialdehyde and to the appearance of increased levels of the dehydrogenase, it would be expected that the enzyme is more or less specifically adapted to this substrate. This expectation was in general borne out by the substrate specificity studies presented above: apart from glutaric semialdehyde, α-ketoglutaric semialdehyde was the only substrate tested that had high affinity for the enzyme. Specificity was not limited strictly to these 5-carbon semialdehydes, however, in that a number of aliphatic aldehydes with chain length of 3 or greater could be oxidized at appreciable rates, although with much higher K_m values. The similar ratios for several substrates exhibited by both crude and purified hydroxyproline-induced enzyme (Tables II and III) make it unlikely that several aldehyde dehydrogenases of different specificity are represented by the purified fraction; it appears also that no significant activity exists for the oxidation of acetaldehyde in crude extracts of cells grown on either proline or hydroxyproline (Table III).

A few structural generalizations emerge from the specificity data of Table II. Only the three 5-carbon semialdehydes, glutaric semialdehyde, α-ketoglutaric semialdehyde, and 4-hydroxy-α-ketoglutaric semialdehyde, appeared to be tightly bound to the purified enzyme, the first two as substrates and the last as a competitive inhibitor. Although unsubstituted aliphatic aldehydes of chain length 3 and above were substrates at high concentration, substitution by methyl or hydroxy groups greatly reduced or eliminated activity; as examples, glyceraldehyde may be compared with propionaldehyde; isobutyraldehyde and 2-hydroxybutyraldehyde, with butyraldehyde; methyl- or hydroxyl-substituted valeraldehyde, with unsubstituted valeraldehyde; and hydroxy-KGSA, with KGSA. It is notable that a semialdehyde of 4 carbons (succinic semialdehyde) was a much poorer substrate than the corresponding butyraldehyde, while a ketoaldehyde of 3 carbons (pyruvic aldehyde) was a poorer substrate than the corresponding propionaldehyde. Functional groups which appear to promote activity in 5-carbon aldehydes thus reduce activity in shorter chains.

The low level of substrate activity for aliphatic aldehydes from 3 to 10 carbons (and perhaps higher) may be relevant to the use of an aldehyde dehydrogenase from hexane-grown cells ("octaldehyde dehydrogenase" (9)) to oxidize KGSA to α-ketoglutarate (4). As yet, however, there is little information on the substrate specificity of the aldehyde dehydrogenase from hexane-grown *P. oleovorans*, nor is it clear whether this enzyme was induced by growth on hydroxycarbons or was constitutive, perhaps resembling the constitutive dehydrogenase described in the present work.

The observation of multiple enzyme-reactive bands demonstrable by polyaniumamide gel electrophoresis of the purified enzyme (and the suggestion of similar heterogeneity on starch gel electrophoresis) raises the question whether these are intrinsically multiple forms of the purified enzyme or an artifact of the isolation or the electrophoretic technique. It should be noted that in many trials of starch gel electrophoresis the induced enzyme in crude extracts migrated as a rather sharp band made visible by enzyme staining (Fig. 9A); more diffuse enzyme-reactive bands have been the rule in a number of recent electrophoresis trials (Fig. 9B), suggesting that some variable of technique may influence band width and multiplicity. A number of possible factors have been tested (including the manner and time of sonic extraction, the buffers and other components of the starch gel, the purity of substrates used for staining enzyme on starch surfaces) that do not explain this variation in band appearance.

Apart from possible electrophoretic heterogeneity of the hydroxyproline- or glucarate-induced enzyme, it has been noted that extracts of uninduced cells grown on any of a variety of substrates uniformly contain lower levels of a dehydrogenase with catalytic properties that resemble the purified hydroxyproline-induced enzyme (except for the relatively lower activity of the latter with GSA), but with distinctive electrophoretic and sedimentation properties. In addition to this enzyme species, a trace of enzyme activity is demonstrable which has the same electrophoretic mobility as that of the species selectively increased by growth on glucarate or hydroxyproline. Although the latter component of constitutively grown cells is present in low quantity, partial separation by gel filtration and sedimentation suggests that it also shares with the induced form the lower molecular weight and the greater activity with KGSA than GSA.

It thus qualifies as the true basal enzyme, the synthesis of which may be specifically stimulated by growth on those substrates, glucarate or hydroxyproline, in the metabolism of which KGSA is involved as an intermediate. Its low level, estimated not to exceed a small percentage of the induced level, is consistent with the level of basal activity for other enzymes in the hydroxyproline-induced pathway (2, 12, 25).

The metabolic significance of the constitutive form of the enzyme and its relationship to the inducible form are unsettled.
One possibility is that the constitutive isozyme is related to lysine metabolism; findings consistent with this hypothesis are the relatively greater activity of this form of the enzyme with GSA than KGSA, the demonstration that the lysine pathway yielding 5-aminovalerate and GSA exists in this strain, and the finding that growth on lysine leads to levels of this form of the enzyme several times greater than obtained with growth on other "constitutive" substrates (10). That growth on lysine stimulates only a modest increase in the constitutive form further suggests that the presence of this enzyme species under a variety of growth conditions may represent endogenous induction by the normal lysine pool. It will be of interest to test these possibilities with the use of lysine auxotrophs, in which the lysine pool might be subject to external control, in an effort to influence the level of the constitutive form of the enzyme.

Since the so-called constitutive form of the enzyme appears to be little influenced by growth on inducing substrates, while the inducible form (represented by a minute quantity in uninduced cells) is selectively increased by growth on glucarate or hydroxyproline, it appears likely that these two forms of the enzyme are distinct in primary structure. The recent literature contains many examples of multiple forms of enzymes similar enough in catalytic properties to be designated isozymes, but for which evidence exists indicating separate metabolic functions and separate regulation; in some cases, genetic distinctness of individual isozymes has also been demonstrated by selective loss in mutants. Well known instances of bacterial isozymes which are separately repressible or inducible include the earlier aspartokinases (34, 35) of E. coli. As more recent examples may be cited the recognition of multiple enzymes catalyzing the synthesis of 7-phospho-3-deoxy-d-arabinoheptulosonate (36, 37) in E. coli, and the two A^-pyruvyl dehydrogenases of Bacillus subtilis, separately inducible by arginine or proline (38). As an example from mammalian systems, the constitutive and drug-induced succinyl-CoA synthetases of mouse liver were reported to differ in electrophoretic, pH-activity, and heat stability properties (39).

The catalytic similarities between the constitutive and induced forms of KGSA dehydrogenase raise the possibility that the latter may represent activation of the former rather than a genetically distinct protein. As reported, however, direct treatment of the constitutive enzyme with KGSA or glucarate (a single experiment with hydroxyproline was technically unsatisfactory) did not alter the typical sedimentation behavior expected for this form of the enzyme. If these two species of enzyme are indeed genetically distinct proteins with different metabolic functions, their close catalytic similarity might seem surprising. However, structural and serological similarities of several distinctly different dehydrogenases of B. subtilis have been described and used to support the suggestion of an evolutionary relationship (40). In this light, the induced form of KGSA dehydrogenase may represent evolutionary modification (with an appropriate increase in activity for KGSA) of the constitutive dehydrogenase. The constitutive enzyme should perhaps be designated GSA dehydrogenase to indicate that GSA is as good a substrate as KGSA for this enzyme species. An additional consideration is the extent to which adaptation to two similar substrates dictates more extensive kinetic similarity in two distinct enzymes; thus the similarities noted between the constitutive and inducible enzymes may merely reflect the constraints on structural requirements for a protein catalyzing the dehydrogenation of 5-carbon semialdehydes.

Apart from speculation, the present example joins others in indicating that a constitutive level of an enzyme, even while catalytically similar to its induced counterpart, may represent a different protein rather than a basal level of the induced enzyme. Relevant to technical aspects of distinguishing separate enzymes, it should be noted that in our case an apparent marked difference in heat stability between the constitutive and induced dehydrogenases did not prove to be a reliable index of protein difference, since provision of a common environment for both enzymes abolished the stability difference. In our case, the stability difference probably results from differences in stabilizing components contained in extracts of cells grown under different conditions.

A further question concerns the identity or nonidentity of the enzymes induced by growth on glucarate or on hydroxyproline. No consistent or reliable difference distinguished this form of the enzyme in either type of extract, whether by kinetic or by physical criteria. The tentative conclusion now warranted, therefore, is that the glucarate-induced and the hydroxyproline-induced enzymes may be identical and represent the same structural gene. If this is so, it is simplest to postulate a single inducer common to both pathways that can stimulate the synthesis of enzyme during growth on glucarate or hydroxyproline; KGSA is the obvious candidate for such a role. However, in a direct test of this possibility, KGSA did not induce the enzyme in cell suspensions, although separate data suggested that it could be taken up and metabolized by whole cells. Furthermore, current studies with a mutant apparently lacking hydroxyproline epimerase indicate that hydroxy-L-proline can itself induce KGSA dehydrogenase. These observations raise the possibility that two compounds of quite different structure (hydroxy-L-proline and D-glucarate or a metabolic product of the latter) can induce synthesis of the same enzyme. Alternatively, the glucarate-induced and the hydroxyproline-induced enzymes may be coded by distinct structural genes separately regulated by inducers of the hydroxyproline and glucarate pathways, but the enzymes may be too similar for differentiation by the criteria applied so far. We hope to investigate these alternatives by the use of appropriate mutants and by purification of the glucarate-induced enzyme for a more complete study of its properties.

REFERENCES


R M Gryder and E Adams, unpublished observations.
α-Ketoglutaric Semialdehyde Dehydrogenase of *Pseudomonas*: PROPERTIES OF THE PURIFIED ENZYME INDUCED BY HYDROXYPROLINE AND OF THE GLUCARATE-INDUCED AND CONSTITUTIVE ENZYMES
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