Presence of a Tightly Bound NAD\(^+\) in Urocanase of *Pseudomonas putida*

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The reduction of urocanase with NaB\(^-\)H\(_2\), plus NaB\(^+\)H, produced an inactive enzyme upon incorporation of 1 g atom of hydrogen/mol of enzyme. From acid hydrolysates of this reduced enzyme was isolated labeled nicotinic acid which was identified by chromatography, electrophoresis, and mass spectral analysis. The presence of a nicotinic acid-containing component was confirmed by incorporation of \(^{14}\)Cnicotinic acid into urocanase during growth of a nicotinic acid auxotroph of *Pseudomonas putida*. Treatment of this purified \(^{14}\)Curocanase with perchloric acid released a nicotinic acid containing cofactor which was subsequently determined to be NAD\(^-\) by electrophoresis and enzymatic analysis. Incorporation of tritium upon NaB\(^-\)H\(_2\), reduction of urocanase, microbiological analysis for nicotinic acid, and the specific radioactivity of \(^{'\circ}\)Hurocanase all supported the stoichiometry of 1 mol of NAD\(^+\) bound/mol of native enzyme. The necessity of using denaturing conditions to remove enzyme-bound NAD\(^+\) indicated that the NAD\(^+\) is very tightly bound. The principal criteria employed earlier for the identification of \(\alpha\)-ketobutyrate as a prosthetic group in urocanase (George, D. J., and Phillips, A. T. (1970) J. Biol. Chem. 245, 5255–537), namely similar chromatographic and electrophoretic properties for \(\alpha\)-hydroxy\[^{1}\]Hbutyrate and \[^{1}\]H material isolated from urocanase after NaB\(^-\)H\(_2\), reduction and acid hydrolysis, were shown to be unable to distinguish between these compounds and nicotinic acid, thereby accounting for the incorrect conclusion that \(\alpha\)-ketobutyrate was an essential cofactor. Present results indicate that NAD\(^+\) is a functional coenzyme in urocanase and thus raise the possibility that catalysis proceeds through an internal oxidation-reduction mechanism.

Urocanase \((4'\)-imidazolone-5'-propionate hydro-lyase, EC 4.2.1.49) was purified and crystallized from *Pseudomonas putida* earlier in this laboratory (1). Its inhibition by such common carbonyl-attacking reagents as hydroxylamine, borohydride, and phenylhydrazine led to the belief that pyridoxal 5'-phosphate would be a required cofactor, as had been reported for chicken liver urocanase by Gupta and Robinson (2), but no evidence could be gathered to support this expectation. Instead, based on chromatographic and electrophoretic properties of material isolated from acid-hydrolyzed, NaB\(^+\)H-reduced urocanase, \(\alpha\)-hydroxy\[^{1}\]Hbutyrate was identified as a reduction product, thereby suggesting that the parent material was a covalently bound \(\alpha\)-ketobutyrate (1). This finding was attractive because supplemental evidence indicated that the phenylhydrazine adduct of urocanase closely corresponded in spectral properties with a keto acid phenylhydrazone, stoichiometry of \(^3\)H incorporation upon NaB\(^-\)H\(_2\), reduction was approximately 1 g atom of hydrogen/mol of enzyme, the reduced enzyme was stable to dialysis but radioactivity could be removed by hydrolytic treatment with acid or proteases, and finally, several other enzymes had been found to contain the related cofactor, pyruvate, as an apparent substitute for pyridoxal phosphate (3–5).

In recent years, it has been difficult to reconcile the expected properties of a covalently bound \(\alpha\)-ketobutyrate moiety with an accumulation of data on the properties of enzyme modified with NaB\(^+\)H, and \(O\)^{14}\Cmethylhydroxylamine.† We herein report the finding that urocanase from *P. putida* contains a tightly bound NAD\(^+\) moiety, the properties of which account almost completely for the previous data leading to the conclusion that an \(\alpha\)-ketobutyrate group was present in urocanase.

**EXPERIMENTAL PROCEDURES**

Organisms — *Pseudomonas putida* A3.12 (ATCC 12633) and *Lactobacillus plantarum* (ATCC 8014) were obtained from the American Type Culture Collection. *P. putida* PSP 73, a nicotinic acid auxotroph, was isolated following N-methyl-\(N\)'-nitro-\(N\)'-nitrosoguanidine mutagenesis (100 µg/ml, 30 min) of the A3.12 strain and selection by penicillin enrichment. The auxotroph was found to be blocked between quinolinic acid and nicotinic acid mononucleotide.

**Urocanase Purification and Assay** — Urocanase was purified essentially as described by George and Phillips (1), but scaled up to accommodate 2 kg of wet cell paste. For routine purifications, the final crystallization step was replaced by a DE52 column chromatography procedure. This consisted of elution of the enzyme from a column (2.2 × 45 cm) of DE52 with a linear gradient of Tria/HCl, pH 7.5, 0.05 to 0.3 M, in a total volume of 1.0 liter. Concentration of pooled enzyme fractions was achieved by ultrafiltration with Amicon PM-10 membranes. Enzyme thus purified had a specific activity of 2.2 µmol/min/mg of protein and was at least 98% pure as determined by polyacrylamide gel electrophoresis at pH 8.3 (6) and sodium dodecyl sulfate.

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gel electrophoresis (7), when 50 µg of protein was applied per gel and stained with Coomassie blue R250 to a detection limit of 0.3 µg.

Urocanase was assayed spectrophotometrically by following the decrease in absorbance of urocanate at 277 nm (1). One unit of activity is defined as that amount of enzyme decomposing 1 µmol of urocanate/min at 25°C.

Other Assays—Protein concentration was measured by the method of Groves et al. (8), and based on a standard curve prepared with bovine serum albumin.

All radioactivity measurements were conducted in a Beckman LS2000 liquid scintillation spectrometer with a scintillation fluid consisting of toluene plus Triton X-100 (2:1) containing 5 g of 2,5-diphenyloxazole/liter of mixture. Efficiencies were generally 90% for ¹⁴C and 30% for ³H.

Urocanase solution in 20-µl increments over a 30-min period at 0°C was added to varying portions of the neutralized extract were tested for the ability to reduce (NH₄)₂SO₄ and 1.6 µmol L-⁰⁻⁻carboxy-³⁴C-nicotinic acid (30.5 µCi/µmol). Cells were harvested by centrifugation (9) at 10,000 x g for 20 min and the urocanase purified as described earlier.

The L-⁰⁻⁻carboxy-³⁴C-nicotinic acid used in this experiment was found to be radiochemically pure by cellulose thin layer chromatography in 2-propanol:H₂O:HOAc (4:1:3). Two portions of urocanase (each 100 mg, 0.9 pmol, in 2.5 ml of 0.5 M Tris/HCl, pH 7.5) were treated (15) with 35% perchloric acid (10 µl) and incubated for 20 min at 0°C and centrifuged for 5 min at 6,000 x g. A portion of this supernatant was examined by electrophoresis in the N.-ethylmorpholine system to identify the L-³⁴C-labeled component. A second portion (140.000 cpm) of the same fraction was chromatographed on a column (1 x 25 cm) of DE52 ion exchange cellulose with a linear gradient (800 ml) from 0 to 0.2 M NH₄HCO₃.

Eighteen different patterns were obtained, each having at least 50% ninhydrin reactive material.

Incorporation of L-³⁴C-Nicotinic Acid into Urocanase—A nicotinic acid autoxroph of P. putida was grown at 30°C on 10 liters of medium containing 0.8% glucose, 20 mm L-histidine, salts A (14) with Na₂SO₄ replacing (NH₄)₂SO₄, and 1.6 µmol L-³⁴C-nicotinic acid (30.5 µCi/µmol). Also included with the sample prior to chromatography were 1- to 3-mg quantities of authentic NAD⁺, NADH, and NADP⁺.

For analysis by combined gas chromatography-mass spectrometry two portions of urocanase (each 100 mg, 0.9 pmol, in 2.5 ml of 0.5 M Tris/HCl, pH 7.5) were reduced with 100 µl of NaBH₄ in 50 mM sodium borate, pH 8.0. The solution was not treated with (NH₄)₂SO₄ and 1.6 µmol L-³⁴C-nicotinic acid (30.5 µCi/µmol).

The migration of the tritiated material obtained from urocanase after reduction was followed by autoradiography and by radiochemical purity by cellulose thin layer chromatography in 2-propanol:H₂O:HOAc (4:1:3). For each 100 µg of sample, 200 µl of bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and 50 µl of 2.5 M HCl in all experiments. This tritiated material was collected, dried in vacuo over NaOH pellets; the residue was taken up in water and applied to columns of AG 50-X4 (1 x 1 cm) and a 1 M sodium hydroxide eluate was monitored by turbidity at 600 nm. Standard curves based on NAD⁺ were identical with those based on nicotinic acid. Unknowns were assayed in triplicate at eight levels of urocanase ranging from 0.1 to 7.0 nmol of enzyme.

Acid was removed by drying in vacuo over NaOH. The residue was taken up in water and applied to columns of AG 50-X4 (1 x 1 cm). Electrophoresis was carried out for 60 min at 2500 V in 0.3 M N-ethylmorpholine acetate, pH 8.0, containing 2.5 mm diithiothreitol.

Stands were visualized with ultraviolet light at 254 nm.

Like the other ³⁴C-labeled material bound to urocanase, ¹⁴C]urocanase (6.4 nmol, 0.71 ng) in 100 µl of 0.3 M Tris/HCl, pH 7.5, was treated (15) with 35% perchloric acid (10 µl) and incubated for 20 min at 0°C. The solution was neutralized with 0.01 M K ROH and centrifuged for 5 min at 6,000 x g. A portion (23,000 cpm) of the supernatant was examined by electrophoresis in the N.-ethylmorpholine system to identify the ³⁴C-labeled component. A second portion (140,000 cpm) of the same fraction was chromatographed on a column (1 x 25 cm) of DE52 ion exchange cellulose with a linear gradient (800 ml) from 0 to 0.2 M NH₄HCO₃. Also included with the sample prior to chromatography were 1- to 3-mg quantities of authentic NAD⁺, NADH, and NADP⁺.

Results of Mass Spectrometry

Identification of Material Isolated from NaBH₄-reduced Urocanase—Earlier work from this laboratory (1) revealed that the tritiated compound isolated from urocanase after reduction with NaBH₄ and acid hydrolysis migrated with a hydroxybutyrate in a number of chromatographic and electrophoretic systems. To substantiate the identity of this material, 100 mg of urocanase (0.9 µmol) was reduced with NaBH₄ and a further 100 mg with 1:1 mixture of Na₂SO₄ and NaBH₄, based on the specific radioactivity of NaBH₄ used, stoichiometrically.

L. M. Henderson, personal communication.

RESULTS

Identification of Material Isolated from NaBH₄-reduced Urocanase—Earlier work from this laboratory (1) revealed that the tritiated compound isolated from urocanase after reduction with NaBH₄ and acid hydrolysis migrated with a hydroxybutyrate in a number of chromatographic and electrophoretic systems. To substantiate the identity of this material, 100 mg of urocanase (0.9 µmol) was reduced with NaBH₄ and a further 100 mg with 1:1 mixture of Na₂SO₄ and NaBH₄, based on the specific radioactivity of NaBH₄ used, stoichiometrically.

[Further text would be here, but it is not included in this transcription.]
etry determinations on each sample revealed that 1.0 ± 0.1 g atom of hydrogen was incorporated per mol (110,000 g) of enzyme, in agreement with earlier results (1).

The procedure previously employed by George and Phillips (1) for isolation of a-hydroxy[1H]butyrate involved a proteolytic digestion and HCl hydrolysis of 1H-labeled enzyme, with subsequent identification based largely on the chromatographic properties of tritiated material remaining after removal of HCl. No accurate accounting of radioactivity recovered at this stage was conducted, nor was further purification attempted. We now report (Table I) that careful monitoring of protein from each sample revealed that significant amounts of tritium were lost (presumably to water) at all steps subsequent to the initial separation of protein from unbound radioactivity. Standard a-hydroxy[1H]butyrate made by NaBH₄ reduction of a ketobutyrate does not lose 1H under similar conditions. Moreover, the bulk of 1H-labeled material remaining after acid hydrolysis bound to a cation exchanger at pH 2 (dilute HCl solution) while standard a-hydroxybutyrate does not. That portion of radioactivity which did not bind, roughly 35% of the amount applied, was not a-hydroxybutyrate since it was completely volatile after neutralization and drying; we believe this material to be tritiated water. Thus, the properties of the nonvolatile tritiated material studied by George and Phillips (1) are not compatible with its identification as a-hydroxybutyrate.

A more thorough structural analysis of the ion exchange-purified material was undertaken by combined gas chromatography-mass spectrometry of the trimethylsilyl derivative. One major component, with a retention time of 11.2 min, was detected. The mass spectrum of this material derived from urocanase which had been reduced with NaBH₄, (the mass contribution of 1H was negligible) is shown in Fig. 1 (middle panel). A weak parent ion of m/e 195 is present, with greater intensity fragments seen at m/e 180, 136, and 106, plus the expected trimethylsilyl fragments at 73 and 75 (not shown).

The deuterated sample, initially prepared by reduction of urocanase with NaBD₄ containing 50% 2H, was likewise ex-

Table I

| Treatment                             | Total µCl recovered | Per cent recovery |
|----------------------------------------|---------------------|-------------------|
| Sephadex G-25                          | 82.0                | 100*              |
| Heat denaturation                      | 33.0                | 40                |
| Acid hydrolysis                        | 16.0                | 19                |
| AG 50-1H⁺ chromatography               | 10.0                | 12                |

* The Sephadex G-25 step, which separated unbound radioactivity from the enzyme, was assumed to be 100% recovery.

![Fig. 1. Mass spectra of standard nicotinic acid and the unknown derived from the borohydride reduction of urocanase. Mass spectra were taken from the injection of 25 nmol of sample as the trimethylsilyl (TMS) derivative. Top, NaBH₄-reduced urocanase; middle, NaBD₄-reduced urocanase; bottom, standard [1H]nicotinic acid.](http://www.jbc.org/)

A search for compounds exhibiting the chromatographic and mass spectral properties of the unknown revealed only one compound which fit all characteristics. The monooctytrimethylsilyle derivative of nicotinic acid chromatographed in a fashion identical with the unknown, and, as seen in Fig. 1 (bottom panel), yielded an essentially similar mass spectrum. The parent ion at m/e 195) fragments into ions at m/e 180 (–CH₃), m/e 136 (–CH₂–CO₂⁻), and m/e 106 (–O-trimethylsilyl).
Presence of NAD⁺ in Urocanase

Hydroxybutyrate and Nicotinic Acid – The obvious dissimilarity between the structures of α-hydroxybutyrate and nicotinic acid raises the question of whether these two compounds could have been distinguished from one another by the chromatographic systems originally used for the identification of α-hydroxybutyrate derived from urocanase. In Table II, the migration of authentic nicotinic acid, the [3H]nicotinic acid isolated from urocanase and α-hydroxybutyrate are compared in the chromatographic systems described by George and Phillips (1). In all systems, the Rf values of α-hydroxybutyrate, nicotinic acid, and the treated material were indistinguishable. Electrophoresis at pH 8.9 likewise failed to separate these compounds. Of the original systems, only electrophoresis at pH 3.3 gave evidence that separation could be achieved. Because nicotinic acid is isoelectric at pH 3.4 (17) and the pKₐ of α-hydroxybutyrate is 3.65 (18), a slight variation of pH in this region could significantly affect the migration of both compounds and thus might explain the identical migration at pH 3.3 of the [3H]material and α-hydroxybutyrate seen by George and Phillips (1). Electrophoresis at pH 2.2, a condition not originally studied, was particularly effective in separating these compounds (Table II) and clearly indicated that the [3H]unknown derived from urocanase was not α-hydroxybutyrate.

Microbiological Assay for Nicotinic Acid – To confirm the presence of nicotinic acid in urocanase and to determine its stoichiometry with respect to the enzyme, the microbiological assay developed by Snell (9) was performed on native urocanase. Bovine serum albumin, molecular weight 66,000 (19), was used as a control and was found to contain no nicotinic acid when assayed at the same molar concentrations as urocanase. Based on a molecular weight of 110,000 (1), native urocanase contained 1.3 ± 0.1 mol of "nicotinic acid"/mol of enzyme.

Difference Spectra between Borohydride-reduced and NaBH₄-reduced Urocanase – A portion (3.2 mg) of the urocanase which had been treated with NaBH₄, was taken after the gel filtration step (see Table I) and its ultraviolet absorption spectrum compared to that of native urocanase at the same concentration (Fig. 2). A peak was observed at 395 nm having an absorbance difference of 0.08 cm⁻¹. The wavelength maximum of enzyme-bound NADH is commonly offset from that of unbound NADH (20). Based on an extinction coefficient of 6.2 × 10⁵ cm⁻¹·mol⁻¹ at 339 nm for free NADH, 0.46 mol of NADH was present/mol of enzyme. This is significantly lower than the stoichiometry of 1.0 g atom of hydrogren-bound/mol of enzyme calculated from [3H]incorporation upon NaBH₄ reduction; however, Chaykin and Meissner (21) demonstrated that borohydride reduction of NAD⁺ yields not only 1,4-NADH but also 1,2- and 1,6-NADH, the latter of which have absorption maxima at 395 and 345 nm, respectively. Values for extinction coefficients of 1.2 and 1,6-NADH were not presented by Chaykin and Meissner (21), but their data permit the calculation that roughly 60% (± 10%) of the total NADH (mixed isomers) formed would be accounted for by absorbance measurements made at 340 nm, using 6.2 × 10⁵ cm⁻¹·mol⁻¹ as extinction coefficient. This follows from the fact that 1,4-NADH comprises only 29% of the recoverable NADH and 34% was 1,6-NADH, the latter exhibiting a ratio of 0.9 for absorbance at 340 nm/A at 345 nm. While the proportion of these forms of NADH resulting from borohydride reduction of urocanase is not known, it is probable that this mixture of reduction products accounts for the discrepancy in stoichiometries.5

Electrophoresis of NaBH₄-reduced Material from Urocanase Prior to Hydrolisis – A portion of NaBH₄-reduced urocanase was treated with NaOH by the procedure described by Burch et al. (13) to remove NAD⁺ and NADH from tissues. Electrophoreses of this material and of labeled (reduced) NAD which had been reduced and treated under the same conditions as shown in Fig. 3. The compound isolated from the enzyme and authentic reduced NAD showed similar patterns. In both samples, the largest proportion of the tritium label migrated with NADH and the remainder with nicotinamide and NAD⁺. The presence of the latter compound is expected because NADH is known to be reoxidized in very dilute solutions (16). In addition, the decomposition of both NAD⁺ and NADH is accelerated in bicarbonate buffer, the buffer used during chromatography to purify both the reduced urocanase and reduced NAD prior to NaOH treatment.

Evidence for the instability of NADH at neutral pH (16) plus the above observations on decomposition of NADH to NAD⁺ and nicotinamide can account for the appearance of [3H]nicotinic acid in acid hydrolysates of NaBH₄-reduced urocanase; these facts also provide an explanation for the loss of tritium during the isolation of [3H]nicotinic acid (Table I) and for the low [3H]content of nicotinamide obtained from the reduction of urocanase with NaBH₄. It is probable that the 5-min boiling treatment of reduced enzyme in bicarbonate buffer at pH 8.0 (after the Sephadex G-25 step in Table I) resulted in extensive oxidation of NADH to NAD⁺, with the accompanying release of one hydrogen (or tritium) atom nonstereospecifically.

5 In response to reviewer comments requesting quantification of the relative amounts of 1,4-, 1,2-, and 1,6-NADH resulting from borohydride reduction of NAD⁺, we offer the following data. Reduction of 10 μmol of NAD⁺ with NaBH₄ at pH 7.6, followed by chromatography on Sephadex G-10, resulted in the isolation of a peak fraction containing 5.5 μmol of [3H]NADH as estimated from absorbance measurements at 340 nm. This material contained an amount equivalent to 0.95 g atom of hydrogen incorporated/mol of NADH formed. Subsequent analysis of these mixed NADH isomers at a concentration of 0.07 μmol/ml (estimated by A₂₆₀) revealed an absorbance of 0.24 cm⁻¹ at 340 nm, indicating a concentration of 0.039 μmol/ml of 1,4- plus 1,6-NADH. Therefore, concentrations of NADH bound in the absorbance measurements at 340 nm are only 33% of the total concentration when NADH produced by borohydride reduction of NAD⁺ is being analyzed.

TABLE II

Migration on paper chromatography and high voltage electrophoresis of material derived from NaBH₄-reduced urocanase compared to standard nicotinic acid and α-hydroxybutyrate

| System | Migration |
|--------|-----------|
| | Authentic nicotinic acid | Authentic α-hydroxybutyrate | [3H]Nicotinic acid from urocanase |
| A. Chromatography | | |
| I. 1-Propanol:conc. NH₄OH (3:2) | 0.72 | 0.73 | 0.73 |
| II. Ethanol:conc. NH₄OH: H₂O (80:5:15) | 0.74 | 0.73 | 0.73 |
| III. 0.5 M ammonium acetate:acetone (1:3) | 0.45 | 0.46 | 0.46 |
| IV. Chloroform:methanol:formic acid (50:50:1) | 0.89 | 0.89 | 0.89 |
| B. Electrophoresis | | |
| V. 1% (NH₄)₂CO₃, pH 8.9 | +10.8 | +10.8 | +10.8 |
| VI. 50 mM ammonium formate, pH 3.3 | −1.1 | +1.5 | −1.1 |
| VII. 1 M Formic acid, 1.5 M acetic acid (1:1), pH 2.2 | −15.5 | −3.5 | −15.5 |
Presence of NAD\(^+\) in Urocanase

**Table III**

| Purification step | Total protein (mg) | Total units | Specific activity (μCi/mg) | Total radioactivity (μCi) | Protein (μg) |
|------------------|-------------------|-------------|----------------------------|--------------------------|-------------|
| 1. Crude extract  | 2953              | 148         | 0.05                        | 332.5                    | 0.11        |
| 2. Protamine sulfate | 2240             | 153         | 0.07                        | 282.0                    | 0.12        |
| 3. 50 to 75% (NH\(_4\))SO\(_4\) | 790              | 110         | 0.14                        | 54.0                     | 0.07        |
| 4. DEAE-cellulose | 98.4              | 58.5        | 0.59                        | 11.3                     | 0.11        |
| 5. Heat, 60°C    | 42.0              | 42.4        | 1.01                        | 8.5                      | 0.20        |
| 6. Sephadex G-200 | 25.0              | 35.4        | 1.41                        | 6.5                      | 0.26        |
| 7. DES52         | 9.9               | 21.6        | 2.16                        | 3.2                      | 0.32        |

*One mole of \([^{14}C]\) nicotinic acid/m mole of enzyme would be equivalent to 0.28 μCi/mg of enzyme based on a value of 110,000 for the molecular weight of urocanase.*

To confirm that the coenzyme of urocanase was NAD\(^+\), the \([^{14}C]\)-labeled material was removed from the enzyme by acid precipitation with 3.2% perchloric acid (final concentration). Treatment of the enzyme with perchloric acid released 70% of the \([^{14}C]\) originally bound to urocanase into the supernatant fraction. Electrophoresis at pH 8.0 of this material after neutralization along with standard nicotinic acid-containing compounds revealed that 88% of the counts migrated with NAD\(^+\) (Fig. 5). The other 12% of the \([^{14}C]\) migrated in the region coinciding with NADH (roughly 6%) or trailed the main peak toward the origin (6%). The presence of NADH in the sample is an artifact due to nonenzymatic reduction of NAD\(^+\) by dithiothreitol present in the electrophoresis buffer.

Further analysis of the \([^{14}C]\)-labeled compound isolated from \([^{14}C]\)urocanase was accomplished by DEAE-cellulose column chromatography with an elution gradient of NH\(_4\)HCO\(_3\). This system (22) cleanly separates NAD\(^+\), NADH, and NADP\(^+\) from one another, as shown in Fig. 6. Approximately 92% of the radioactivity applied to the column co-eluted with NAD\(^+\).

**Enzymatic Analysis of Coenzyme from Urocanase** — To demonstrate that the coenzyme isolated from urocanase was NAD\(^+\), 160 nmol of urocanase containing 68,000 cpm of \([^{14}C]\)urocanase was treated with perchloric acid and the isolated neutralized coenzyme (73% recovery, 49,200 cpm) tested for its extent of reduction by yeast alcohol dehydrogenase and ethanol. Portions of this extract equivalent tobetween 10 and 40 nmol of urocanase were examined and reduction was complete after 10 min. Under these conditions, reduction of similar amounts of NAD\(^+\) was less than 4% complete, thereby indicating that the reducible material was not NADP\(^+\). The assays showed that 107 ± 3 (S.D., n = 4) nmol of NAD\(^+\) were recovered from acid-treated enzyme or after correction for the 73% recovery of label, 146 nmol of NAD\(^+\) were originally present in the 160 nmol of urocanase. The results confirm that the cofactor of urocanase is indeed NAD\(^+\) and that roughly 1 mol of NAD\(^+\) is bound per mol of enzyme.

A second enzymatic test was employed for the identification of NAD\(^+\) in urocanase. In this test, 192 nmol of native urocanase was treated with HClO\(_4\), as described above and neutralized with KOH. The supernatant fraction was lyophilized and reconstituted in 0.8 ml of 0.12 M 2-amino-2-methyl-1-propanol, pH 9.9. One-half of this solution was then treated with 2 μmol of DL-lactate, 50 μmol of L-glutamate, and a mixture of bovine...
The results described clearly indicate the presence of 1 mol of NAD\(^+\) firmly bound to each mole of urocanase and illustrate how nicotinic acid produced from the degradation of NAD\(^+\) was mistakenly identified as \(\alpha\)-hydroxybutyrate in our earlier investigations. The almost complete reliance upon chromatographic and electrophoretic similarities between \(\alpha\)-hydroxybutyrate and the \(^3\)H unknown obtained from reduced and hydrolyzed urocanase can now be seen to have been inadequate for purposes of structural analysis of the urocanase coenzyme, in that the criteria did not differentiate between \(\alpha\)-hydroxybutyrate and nicotinate. Two other chemical properties of the urocanase coenzyme or its reduction product were examined in the original study but have not been dealt with here. The first was a difference spectrum between native urocanase and urocanase modified with phenylhydrazine. This spectrum revealed a difference peak with an absorption maximum at 312 nm, similar to that seen for keto acid phenylhydrazones. We now believe that this peak could also have been due to a phenylhydrazine-NAD adduct, analogous to the well known reaction product between NH\(_2\)OH or hydrazine and NAD\(^+\) (24). Because of instability of such adducts at neutral pH unless bound to an enzyme (25), we have been unable to confirm or disprove the identity of the 312 nm absorbing species. A second property, namely formation of a \(p\)-bromophenacyl ester derivative of the \(^3\)H material isolated from reduced and hydrolyzed urocanase, has been re-examined by us. While such a derivative can be prepared, its chromatographic properties do not conform to those reported earlier. In fact, the bulk of the \(^3\)H derivative remains at the origin in the solvent system originally used (as does the \(p\)-bromophenacyl ester of nicotinic acid), while only a trace quantity (<10%) of the radioactivity can be found in the area corresponding to the migration position of \(\alpha\)-hydroxybutyric \(p\)-bromophenacyl ester. We have therefore concluded that this evidence can no longer be used to support an identification of \(\alpha\)-keto- butyrate as the urocanase coenzyme.

The extensive data for the presence of NAD\(^+\) in urocanase, coupled with data on the stoichiometry of tritium incorporation upon NaB\(^3\)H\(_4\) reduction, the microbiological assay for nicotinic acid (or nicotinamide coenzymes) and the incorporation of \(^{14}\)C nicotinic acid into urocanase during biosynthesis, together provide strong evidence for a 1:1 NAD\(^+\):urocanase ratio and point to the existence of one binding site for NAD\(^+\) with an extremely low dissociation constant at neutral pH. However, urocanase has been shown to consist of two apparently identical subunits (26) and the question can be posed as to whether a second NAD\(^+\) site of reduced affinity might exist. Preliminary experiments involving incubation of urocanase with concentrations of NAD\(^+\) up to 1 mM reveal that no increase in specific activity can be detected. Thus the presence of another NAD\(^+\) site has not been experimentally verified. Furthermore, treatment of urocanase with charcoal leads to irreversible binding of the enzyme to charcoal, thereby

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**DISCUSSION**

The results described clearly indicate the presence of 1 mol of NAD\(^+\) firmly bound to each mole of urocanase and illustrate how nicotinic acid produced from the degradation of NAD\(^+\) was mistakenly identified as \(\alpha\)-hydroxybutyrate in our earlier investigations. The almost complete reliance upon chromatographic and electrophoretic similarities between \(\alpha\)-hydroxybutyrate and the \(^3\)H unknown obtained from reduced and hydrolyzed urocanase can now be seen to have been inadequate for purposes of structural analysis of the urocanase coenzyme, in that the criteria did not differentiate between \(\alpha\)-hydroxybutyrate and nicotinate. Two other chemical properties of the urocanase coenzyme or its reduction product were examined in the original study but have not been dealt with here. The first was a difference spectrum between native urocanase and urocanase modified with phenylhydrazine. This spectrum revealed a difference peak with an absorption maximum at 312 nm, similar to that seen for keto acid phenylhydrazones. We now believe that this peak could also have been due to a phenylhydrazine-NAD adduct, analogous to the well known reaction product between NH\(_2\)OH or hydrazine and NAD\(^+\) (24). Because of instability of such adducts at neutral pH unless bound to an enzyme (25), we have been unable to confirm or disprove the identity of the 312 nm absorbing species. A second property, namely formation of a \(p\)-bromophenacyl ester derivative of the \(^3\)H material isolated from reduced and hydrolyzed urocanase, has been re-examined by us. While such a derivative can be prepared, its chromatographic properties do not conform to those reported earlier. In fact, the bulk of the \(^3\)H derivative remains at the origin in the solvent system originally used (as does the \(p\)-bromophenacyl ester of nicotinic acid), while only a trace quantity (<10%) of the radioactivity can be found in the area corresponding to the migration position of \(\alpha\)-hydroxybutyric \(p\)-bromophenacyl ester. We have therefore concluded that this evidence can no longer be used to support an identification of \(\alpha\)-keto- butyrate as the urocanase coenzyme.

The extensive data for the presence of NAD\(^+\) in urocanase, coupled with data on the stoichiometry of tritium incorporation upon NaB\(^3\)H\(_4\) reduction, the microbiological assay for nicotinic acid (or nicotinamide coenzymes) and the incorporation of \(^{14}\)C nicotinic acid into urocanase during biosynthesis, together provide strong evidence for a 1:1 NAD\(^+\):urocanase ratio and point to the existence of one binding site for NAD\(^+\) with an extremely low dissociation constant at neutral pH. However, urocanase has been shown to consist of two apparently identical subunits (26) and the question can be posed as to whether a second NAD\(^+\) site of reduced affinity might exist. Preliminary experiments involving incubation of urocanase with concentrations of NAD\(^+\) up to 1 mM reveal that no increase in specific activity can be detected. Thus the presence of another NAD\(^+\) site has not been experimentally verified. Furthermore, treatment of urocanase with charcoal leads to irreversible binding of the enzyme to charcoal, thereby

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**Fig. 4.** Polyacrylamide gel electrophoresis of \[^{14}\text{C}]\text{urocanase isolated from a \[^{14}\text{C}]\text{nicotinic acid-grown auxotroph. The gel was sliced into 2-mm sections and the radioactivity in each determined.**

**Fig. 5.** Electrophoresis of the \[^{14}\text{C}\text{labeled material isolated from \[^{14}\text{C}]\text{urocanase by perchloric acid treatment. The isolation procedure was as described under ‘Experimental Procedures’ and the electrophoresis was as in Fig. 3.**

**Fig. 6.** Elution profiles of standard pyridine nucleotide coenzymes and \[^{14}\text{C}\text{ material isolated from \[^{14}\text{C}]\text{urocanase. Chromatography was performed at 25° on a column (1 \times 25 cm) of DE52 (bicarbonate form). After sample application, the column was washed with 25 ml of water, then eluted with a linear gradient (800 ml) of 0 to 0.2 M NH\(_4\)HCO\(_3\), plus 0.1 mM dithiothreitol; 5-ml fractions were collected. Radioactivity (C) is expressed as counts per min per ml.**
Presence of NAD$^+$ in Urocanase

precluding any attempts to prepare apoenzyme for direct titration with NAD$^+$ and to show a direct catalytic requirement for NAD$^+$.

A central consideration concerns the essentiality for NAD$^+$ in the catalytic process. The strongest evidence presently available comes from the potent inhibition of enzymatic activity by so-called "carbonyl-attacking reagents" (1), most of which are also known to modify NAD$^+$ (21, 24, 27). In the case of NaBH$_4$, it has now been shown that this inhibition is accompanied by the formation of NADH on the enzyme. The recognized enzymatic function of NAD$^+$ is believed to be as a hydride ion acceptor for the oxidation of substrates. In urocanase, the mechanism must also include the reoxidation of enzyme-bound NADH so that active enzyme is re-established upon product release. The mechanism proposed for this enzyme (1, 28) requires that the hydration of urocanic acid result from proton abstractions and additions rather than an intramolecular hydride ion transfer as would be implied by the presence of NAD$^+$. Indeed, it is difficult to envision a hydride ion transfer mechanism for urocanase since the newly added side chain hydrogen atoms of imidazolone propionate were shown to have a solvent origin (28). Work in our laboratory is currently directed toward clarifying this possibly novel NAD$^+$-mediated mechanism.

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