Purification and Properties of Creatinine Iminohydrolase from *Flavobacterium filamentosum*

Theodore W. Esders† and Shirley Y. Lynn

From the Research Laboratories, Eastman Kodak Company, Rochester, New York 14650

Creatinine iminohydrolase (EC 3.5.4.21), which catalyzes hydrolysis of creatinine to N-methylhydantoin and ammonia, was purified from *Flavobacterium filamentosum*. The average molecular weight of the purified enzyme was 272,480, and the subunit molecular weight was 44,300. Extensive specificity studies indicated that the enzyme utilized cytosine ($K_m$ 0.62 mM; $V_{max}$ 20.1 units/mg) as well as creatinine ($K_m$ 5.00 mM; $V_{max}$ 40.4 units/mg) as a substrate. Each was a competitive inhibitor toward hydrolysis of the other compound.

Dialysis of creatinine iminohydrolase in the presence of 0.01 M Tris phosphate buffer, pH 7.5, containing 1,10-phenanthroline decreased activity by 98%. Reactivation was accomplished by incubating the apoenzyme in the presence of certain divalent metal chlorides, listed in decreasing order of effectiveness: iron(II), zinc, cobalt(II), cadmium, and nickel. The extent of reactivation depended on the substrate and on which metal ion was added to the apoenzyme. Creatinine to cytosine activity ratios varied from 1:3.75 (iron(II) chloride), to 1:0.9 (zinc chloride), to 1:0.06 (nickel chloride). For different preparations of the holoenzyme that ratio ranged from 1:0.45 to 1:1.10. Variable but significant quantities of zinc and iron were present in all preparations of the purified enzyme.

There are two major pathways for the microbial degradation of creatinine. In one, creatinine is first hydrolyzed to creatine in a reversible reaction catalyzed by creatinine amidohydrolase (1). Proteins catalyzing that reaction have been isolated from several microbial sources, and their properties have been described (2–6). Further metabolism via that pathway ultimately leads to production of urea and ammonia through sarcosine and glycine intermediates (7). In a second pathway, creatinine is hydrolyzed to N-methylhydantoin and ammonia in a reaction catalyzed by creatinine iminohydrolase (EC 3.5.4.21) (8). Although a creatinine iminohydrolase was first isolated by Szulmajster in 1958 (9) from the anaerobic bacterium *Clostridium paraputrificum*, these enzymes, as a class, have been little studied. This may be due, at least in part, to the difficulty of isolating organisms that metabolize creatinine or 5-methyl derivative, or 5-methyl derivative was not further metabolized. Also, when the bacterium was cultured in the presence of other nitrogen sources, the creatinine iminohydrolase activity was negligible, although excellent growth was observed. We describe here a procedure for purification of the *F. filamentosum* creatinine iminohydrolase, studies on the physical properties of the enzyme, and kinetic studies that show that this metal-containing enzyme displays dual specificity using either creatinine or cytosine as a substrate.

EXPERIMENTAL PROCEDURES

RESULTS

Purification and Molecular Properties of Creatinine Iminohydrolase—Details of the purification of creatinine iminohydrolase, proof of purity, and molecular properties are presented in the Miniprint Section. Briefly, the specific activity of the purified enzyme was 39.8 units/mg. Also, the average molecular weight was 272,480, and the subunit molecular weight was 44,300, consistent with the native enzyme being composed of six subunits.

Substrate Specificity of Creatinine Iminohydrolase—Several amine-containing compounds were evaluated as potential substrates for creatinine iminohydrolase (Table III). In each case, the substrate concentration given was the highest value tested and represents a saturating concentration for all active compounds. Creatine, arginine, and guanidine, all of which contain the same imino nitrogen functionality as creatinine, were completely inactive. The free base cytosine was utilized efficiently as a substrate (57% creatinine value), whereas the nucleoside, cytidine, and CMP were inactive. Also, 5-fluorocytosine was utilized as a substrate (18% of creatinine value), but negligible activity was observed with either the 3-methyl or 5-methyl derivative (4.6 and 2.3% of creatinine value).

1 Portions of this paper (including "Experimental Procedures," Fig. 1, and Table I and II) are presented in miniprint at the end of this paper. The abbreviation used is: SDS, sodium dodecyl sulfate. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-2730, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
TABLE III

Substrate specificity of creatinine iminohydrolase

Various compounds at indicated concentrations were substituted for creatinine in the standard reaction mixture. Reaction was initiated by addition of 0.25 μg of creatinine iminohydrolase, and the rate of ammonia production was measured in the standard reaction mixture as described under "Experimental Procedures."

| Compound                  | Creatinine iminohydrolase activity (units/ml) |
|---------------------------|-----------------------------------------------|
| Creatinine (40 mM)        | 111.25                                        |
| Creatine (20 mM)          | 0                                             |
| Arginine (100 mM)         | 0                                             |
| Guanidine (100 mM)        | 0                                             |
| Urea (100 mM)             | 0                                             |
| Cytosine (5 mM)           | 63.02                                         |
| 5-Fluorocytosine (5 mM)   | 20.58                                         |
| 5-Methylcytosine (10 mM)  | 2.57                                          |
| 3-Methylcytosine (10 mM)  | 5.14                                          |
| Cytidine (5 mM)           | 0                                             |
| CMP (10 mM)               | 0                                             |
| Adenine (0.25 mM)         | 0                                             |
| Adenosine (0.25 mM)       | 0                                             |
| AMP (0.25 mM)             | 0                                             |
| Guanine (0.25 mM)         | 0                                             |
| Guanosine (0.25 mM)       | 0                                             |

Fig. 2. Coincident migration of activity against creatinine and cytosine during disc gel electrophoresis. Purified creatinine iminohydrolase (200 μg) was applied to a 7% buffer gel, electrophoresis was carried out, and the gel was sliced into 34 equal fractions. Protein was eluted into 50 μl of 0.01 M Tris phosphate buffer, pH 7.5, and activity was measured with creatinine (○—○) or cytosine (□—□) as substrate in the standard reaction mixture as described under "Experimental Procedures."

Although this dual specificity for creatinine and cytosine was observed with an essentially pure enzyme preparation, it was possible that a very minor component in the preparation was responsible for activity with cytosine. Therefore, electrophoresis was carried out in a 7% buffer gel at pH 8.5, the proteins were eluted from the gel, and enzyme activity was measured. The results in Fig. 2 show that both activities displayed identical migration patterns. Also, a constant creatinine to cytosine activity ratio of 1:0.45 was observed in the three active fractions.

Substrate Saturation Curves—Fig. 3 shows substrate saturation curves for creatinine and cytosine. Normal hyperbolic kinetics were observed with both substrates, and Lineweaver-Burk treatment of the data yielded $K_m = 5.00$ mM for creatinine and 0.62 mM for cytosine. For this preparation, the $V_{max}$ for creatinine was 203 units/ml, whereas that for cytosine was 190 units/ml. Thus, the creatinine to cytosine activity ratio was 1:0.50; for other purified preparations this value was...
1:045–1.10. $K_m$ for 5-fluorocytosine (not shown) was 0.42 mM and the $V_{max}$ 42 units/ml.

The data in Fig. 4 show the results of testing each substrate as an inhibitor toward activity with the other substrate. With cytosine as the substrate, activity was determined by monitoring the decrease in cytosine concentration at 280 nm, and with creatinine as the substrate, activity was measured by the colorimetric procedure based on the Jaffe method (12). Preliminary experiments showed that in each case the inhibitor had no effect on the assay procedure. Creatinine displayed competitive inhibition when cytosine was the substrate, and cytosine displayed competitive inhibition when creatinine was the substrate. These data are consistent with both activities resulting from catalysis at one active site.

**Creatinine Iminohydrolase Activity at Various pH Values**

Enzyme activity with creatinine or cytosine as substrate was measured at various pH values. The enzyme displayed a broad pH optimum at pH 7.0–8.5 with a constant ratio of activity with the two substrates throughout the optimum (Fig. 5). However, at lower or higher pH, activity with cytosine decreased more rapidly than that for creatinine. The reason for this is not known.

**Metal Ion Dependence of Creatinine Iminohydrolase**

When creatinine iminohydrolase was dialyzed in 0.01 M Tris phosphate buffer, pH 7.5, containing 10 mM 1,10-phenanthroline for 48 h at 5 °C, only 2% of the initial activity remained. In other experiments, as much as 20% of the original activity remained; the extent of inactivation was dependent on several factors, including the duration of dialysis and the enzyme concentration. As shown in Table IV, the salts of certain divalent metal ions restored activity, when added to the apoenzyme. The indicated concentration of each metal ion was the optimum concentration for reactivation of the enzyme and was the result of evaluating each over a wide range of concentrations. Zinc chloride and cobalt(II) chloride restored activity with either creatinine or cytosine as substrate to values similar to those observed with the holoenzyme. The
creatinine to cytosine activity ratio in the presence of zinc chloride was 1:0.9. Iron(II) chloride greatly enhanced activity with cytosine (485% of activity with holoenzyme), whereas activity with creatinine was again similar to that of the holoenzyme. The creatinine to cytosine activity ratio was 1:3.75. Nickel chloride, on the other hand, only slightly reactivated the enzyme with cytosine (4% of activity with holoenzyme), whereas with creatinine, significant reactivation was observed (38% of activity of holoenzyme). The creatinine to cytosine activity ratio was 1:0.62. Thus, the relative activity toward a particular substrate is a function of the metal ion present and can be varied by a factor of at least 50, as judged by creatinine to cytosine activity ratios.

Table IV also shows $K_m$ values determined for each substrate using creatinine iminohydrolase activated by the various divalent metal ion salts. Minor variations in the $K_m$ values were observed, but the major effect was on $V_{max}$ values.

Several other salts were ineffective at reactivating the apoenzyme: barium chloride, calcium chloride, copper(II) chloride, magnesium chloride, tin(II) chloride, strontium chloride, iron(III) chloride, lead acetate, and mercury(II) chloride.

**Metal Ion Binding to Creatinine Iminohydrolase—Nickel chloride and zinc chloride were chosen to determine the effect of the order of addition of metal ions on reactivation of the apoenzyme. When the apoenzyme was preincubated first in the presence of 0.1 mM nickel chloride and then in the presence of 0.1 mM zinc chloride in addition to the nickel chloride, the activity was the same as that obtained in the presence of nickel chloride alone (Table V). Also, the reverse experiment was carried out; when the enzyme was first preincubated in the presence of 0.1 mM zinc chloride, nickel chloride had no effect on the extent of reactivation. These data are consistent with the interpretation that the metal ions compete for the same or similar sites on the enzyme, and once a particular metal ion is bound to the enzyme, it is not easily displaced.

These experiments were carried out with creatinine as substrate, but identical results were obtained with cytosine as substrate and different divalent metal ion salt combinations.

### Table IV

| Effector | Creatinine | Cytosine | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ |
|----------|------------|----------|-----------|------|-----------|------|-----------|------|-----------|------|
|          | units/ml   | mM       | units/ml  | mM   | units/ml  | mM   | units/ml  | mM   | units/ml  | mM   |
| Holoenzyme None | 258.06 | 5.0 | 172.41 | 0.5 | 1:0.67 |
| Apoenzyme None | 3.77 | 5.0 | 2.70 | 0.5 | 1:0.91 |
| Iron(II) chloride | 222.22 | 3.33 | 833.33 | 1.0 | 1:3.75 |
| Zinc chloride | 200.00 | 3.85 | 181.33 | 1.0 | 1:0.91 |
| Cobalt(II) chloride | 192.31 | 3.33 | 166.67 | 0.71 | 1:0.96 |
| Cadmium chloride | 105.26 | 3.75 | 12.20 | 0.50 | 1:0.11 |
| Nickel chloride | 100.00 | 3.75 | 6.45 | 0.71 | 1:0.06 |

*Ratio is $V_{max}$ in presence of cytosine divided by $V_{max}$ in presence of creatinine.

### Table V

| Enzyme activity | units/ml |
|----------------|----------|
| Enzyme activity | 15.43 |

### Table VI

| Metal | g atom metal/mol of enzyme $^a$ | Range $^b$ |
|-------|--------------------------------|-----------|
| Zinc  | 1.5                             | 0.87-2.5  |
| Iron  | 0.62                            | 0.1-1.2   |
| Nickel| 0.48                            | 0.0-1.0   |
| Cobalt| 0                               |           |
| Cadmium| 0                               |           |

*Average from five different preparations of the enzyme.

The presence of metals in creatinine iminohydrolase was determined by atomic absorption analysis as described under "Experimental Procedures." The kinetic constants were determined by Lineweaver-Burk treatment of the data.

**Effect of order of metal ion addition on activation of apocreatinine iminohydrolase**

The apoenzyme (15 mg/ml), prepared as described in Table III, was incubated in a solution containing 0.1 mM metal ion at room temperature for 5 min. The numbers (1) and (2) refer to order of metal ion additions. The time between (1) and (2) was 5 min. Five minutes after the last metal ion addition, 10 $\mu l$ of that solution was used for measurement of enzyme activity in the standard reaction mixture with creatinine as substrate as described under "Experimental Procedures."
F. filamentosum Creatinine Iminohydrolase

Discussion

Creatinine iminohydrolase was purified from extracts of F. filamentosum, a microorganism which, when grown in the presence of creatinine, produces large amounts of that enzyme. The specific activity of the purified preparation (39.8 units/mg) was similar to that reported for two other creatinine iminohydrolase preparations, one from C. lilium (34.0 units/mg) (10) and one from the anaerobe C. paraputrefaciens (36.1 units/mg) (9). However, the extent of purity of the latter enzyme is not known. The $K_m$ of the F. filamentosum enzyme (5.00 mM) for creatinine was higher than that value for the C. lilium enzyme (1.27 mM), whereas the iminohydrolase from the anaerobe had a significantly higher $K_m$ of 18.0 mM. No physical characterization studies of the C. paraputrefaciens enzyme were reported. The molecular weight of the C. lilium enzyme was estimated at 200,000, whereas we determined an average molecular weight of 272,480 and a subunit molecular weight of 44,300 for the F. filamentosum enzyme, consistent with the enzyme existing as a hexamer.

Extensive substrate specificity studies revealed that the purified F. filamentosum creatinine iminohydrolase also displayed cytosine deaminase activity. The enzyme from C. lilium, on the other hand, did not utilize cytosine as substrate. All kinetic data reported herein are consistent with both activities being catalyzed by the same protein and indeed at the same active site. At first the structural relationship between creatinine and cytosine is not immediately obvious, but, as shown by examination of the possible tautomeric forms of cytosine, similarity does exist with three positions on the ring, including the same reactive imino nitrogen functionality in both molecules.

The 5-position on the cytosine ring has a marked effect on activity. Substitution of the hydrogen at that position by fluorine (5-fluorocytosine) decreases the activity to 33% of the cytosine value with no apparent affect on the $K_m$ and substitution with a methyl group in that position virtually eliminates activity. Indeed, 5-methylcytosine inhibits reaction with either cytosine or creatinine substrate. At the same time, the analogous position on the creatinine molecule does contain a methyl group (N-methyl).

Cytosine deaminases have been isolated from several microbial sources and purified preparations obtained from Serratia marcescens (25), Pseudomonas aureofaciens (26), and bakers' yeast (27). The bacterial enzymes are very high-molecular-weight multisubunit proteins of 580,000 and 630,000 respectively, whereas the yeast enzyme has a molecular weight of 34,400. Kinetic data were available only for the yeast enzyme, which showed a $K_m$ of 5.0 mM for cytosine. This should be compared with the value of 0.62 mM obtained for the F. filamentosum enzyme. In terms of substrate specificity, none of these cytosine deaminases were tested with creatinine. Based upon the results reported herein, it is possible that one or more of these proteins might utilize that compound as a substrate.

The F. filamentosum creatinine iminohydrolase is a metal-ion-activated and metal-containing enzyme. The apoenzyme was prepared by dialysis against 1,10-phenanthroline, and rapid reactivation was obtained by addition of specific divalent metal ion salts to the apoenzyme. The relative activity with creatinine or cytosine was dependent on the metal ion present, such that creatinine to cytosine activity ratios could be varied from 1:3.75 (iron(II) chloride) to 1:0.9 (zinc chloride) to 1:0.06 (nickel(II) chloride). We are aware of no reports on metal ion involvement in catalysis by known creatinine iminohydrolases or cytosine deaminases. However, the specificity of several well-characterized metalloenzymes is also dependent on the metal used to reactivate the apoenzyme. For example, the protease to esterase ratio of carboxypeptidase A is a function of the metal ion present (28), and the same is true for the phosphate ester to o-phosphorothioate ester activity ratio of alkaline phosphatase (29). Both of these enzymes are zinc metalloenzymes.

Studies on the metal content of purified preparations of creatinine iminohydrolase show that zinc and iron were present in all preparations. However, several lines of evidence suggest that the metal content in the purified enzyme does not reflect the stoichiometry in the native enzyme. First, the amounts of each metal varied considerably from preparation to preparation. Variation in metal content as a function of the purification procedure has been observed with other metalloenzymes (30-32). Second, of the five preparations, two were metal-iron deficient; addition of zinc chloride to the preparation increased the activity by 45%. The third point relies on the fact that the ratio of activities with creatinine or cytosine as substrate is a function of the metal ion present. When activity in the crude extract was measured with both substrates, the creatinine to cytosine ratio was 1:3. As noted above, in the purified preparations that ratio was 1:0.45-1:1.10. Thus, this purified enzyme does not reflect what is observed in the crude extract. When the apoenzyme was reactivated by various metal ions, a creatinine to cytosine ratio of 1:3.75 was observed with the iron-activated enzyme (Table IV). Determination of the metal content of the native enzyme requires modification of the purification procedure to lessen the possibility of dissociation of the metal ion(s) from the enzyme during the purification, and the ratio of the activities with the two substrates can serve as a guide for those studies.

Acknowledgments—We acknowledge early contributions by C. A. Michrina and the technical assistance of G. D. Noto. We also thank Dr. J. R. Schaeffer for preparation of materials for chromatographic studies and E. McLean for atomic absorption analyses.

References

1. Akamatsu, S., and Kanai, Y. (1951) Enzymologia 15, 122-125
2. Kaplan, A., and Naugler, D. (1974) Mol. Cell. Biochem. 3, 9-15
3. Kaplan, A., and Szabo, L. (1974) Mol. Cell. Biochem. 3, 17-25
4. Forde, A., and Johnson, D. (1974) Biochem. Soc. Trans. 2, 1342-1344
5. Donnelly, W. (1975) Biochem. Soc. Trans. 3, 1215-1216
6. Donnelly, W., and Johnson, D. B. (1977) Int. J. Biochem. 8, 11-16
7. Akamatsu, R., and Miyashita, R. (1951) Enzymologia 15, 173-176
8. Szulmajster, J. (1958) J. Bacteriol. 75, 633-639
9. Szulmajster, J. (1958) Biochim. Biophys. Acta 30, 154-163
10. Ulbajima, T., and Terada, O. (1976) Agric. Biol. Chem. 40, 1055-1066
F. filamentosum Creatinine Iminohydrolase

11. Goodhue, C., and Masurekar, P. (1981) Am. Soc. Microbiol. Natl. Meet. (Abstr. 042)
12. Jaffe, H. (1886) Z. Physiol. Chem. 10, 391
13. Hedrick, J. L., and Smith, A. J. (1968) Arch. Biochem. Biophys. 126, 155–164
14. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
15. Martin, R. G., and Ames, B. N. (1961) J. Biol. Chem. 236, 1372–1379
16. Margolis, J., and Wrigley, C. W. (1975) J. Chromatogr. 106, 204–209
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
18. Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S. (1948) J. Biol. Chem. 174, 961–977
19. Beers, R. F., Jr., and Sizer, I. W. (1952) J. Biol. Chem. 195, 133–140
20. Lamprecht, W., and Trautschold, I. (1965) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) Vol. 1, p. 543, Academic Press, New York
21. Vallee, B. L., and Hoch, F. L. (1955) Proc. Natl. Acad. Sci. U. S. A. 41, 327
22. Hohorst, H. (1965) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) Vol. 1, p. 215, Academic Press, New York
23. Hugget, A., and Nixon, D. A. (1957) Biochem. J. 66, 12
24. Hjerten, S. (1973) J. Chromatogr. 87, 325–331
25. Sakai, T., Yu, T., Tabe, H., and Omata, S. (1975) Agric. Biol. Chem. 39, 1623–1629
26. Sakai, T., Yu, T., Taniguchi, K., and Omata, S. (1975) Agric. Biol. Chem. 39, 2015–2020
27. Ipata, P. L., Marmocchi, F., Magni, G., Felicioli, R., and Polidoro, G. (1971) Biochemistry 10, 4270–4276
28. Coleman, J. E., and Vallee, B. L. (1961) J. Biol. Chem. 236, 2244–2249
29. Chlebowski, J. F., and Coleman, J. E. (1974) J. Biol. Chem. 249, 7192–7202
30. Simpam, R. T., Vallee, B. L., and Tait, G. H. (1968) Biochemistry 7, 4336–4542
31. Drum, D. E., Li, T.-K., and Vallee, B. L. (1969) Biochemistry 8, 3783–3791
32. Lee, M. H., and Dawson, C. R. (1973) J. Biol. Chem. 248, 6596–6602
Purification and Properties of Creatinine Inimhydrase from Flavobacterium filamentosum

Theodore W. Eders and Shirley Y. Lyon

SUPPLEMENTARY MATERIAL TD

**Purification of Creatinine Inimhydrase from Flavobacterium filamentosum**

**EXPERIMENTAL PROCEDURES**

**Materials**—Sigma Chemical Company supplied the proteins used as standards for characterization. NADPH, 5-hydroxytryptophan, 
-ketoglutaric acid, L-glutamic acid dehydrogenase (ammonia free), Tris base, and many of the other reagents used for specificity studies. Other organic chemicals were from Kodaik Laboratory Chemicals. Bio Rad. Pharmacia supplied Sephadex G-200, Sepharose 4B, and other reagents used for gel exclusion chromatography. All other metal ions and other inorganic reagents were purchased from Baker and Adam, Allied Chemical Company, and other suppliers.

**Measurement of Creatinine Inimhydrase Activity**—Creatinine inimhydrase activity was measured by three methods. For the standard method, contained in a total volume of 1.0 ml. 100 μmol Tris-phosphate buffer, pH 7.5, containing 1 M potassium phosphate buffer, pH 7.5, and 2.0 umol creatinine. Reaction was initiated by enzyme addition (0.15 U) after equilibration at room temperature in 0.5 M

**RESULTS**

**Purification of Creatinine Inimhydrase**—All procedures were carried out according to the method of Weber and O’Mara (14). Proteins were disassociated for 2 min at 100 °C rather than 30 min at 4 °C and then dialyzed overnight against 0.05 M sodium phosphate buffer, pH 7.2, containing 1% SDS and 1 M potassium phosphate buffer. After dialysis, the proteins were removed electrophoretically with a constant current of 10 μA. The electrophoretic mobility was measured. Dots were sampled for at least 1 h in 0.5% naphthol blue in 5% acetic acid. and excess dye was removed with a Calonite quick drier. Final bands obtained were identified by the mobility of creatinine inimhydrase was compared with that of bovine serum albumin and ovalbumin (subunit M, 64,000).

**DISCUSSION**

**Purification of Creatinine Inimhydrase**—All procedures were carried out according to the method of Hedrick and Smith (13). Samples containing 25-150 μg of protein were dialyzed overnight against 50 mM Tris-phosphate buffer, pH 7.5, and 0.5 M Tris-phosphate buffer, pH 7.5. Active fractions were pooled, concentrated, and then eluted with 0.01 M Tris-phosphate buffer, pH 7.5, and stored frozen.

**DISCUSSION**

**Purification of Creatinine Inimhydrase**—All procedures were carried out according to the method of Hedrick and Smith (13). Samples containing 25-150 μg of protein were dialyzed overnight against 50 mM Tris-phosphate buffer, pH 7.5, and 0.5 M Tris-phosphate buffer, pH 7.5. Active fractions were pooled, concentrated, and then eluted with 0.01 M Tris-phosphate buffer, pH 7.5, and stored frozen.

**DISCUSSION**

**Purification of Creatinine Inimhydrase**—All procedures were carried out according to the method of Hedrick and Smith (13). Samples containing 25-150 μg of protein were dialyzed overnight against 50 mM Tris-phosphate buffer, pH 7.5, and 0.5 M Tris-phosphate buffer, pH 7.5. Active fractions were pooled, concentrated, and then eluted with 0.01 M Tris-phosphate buffer, pH 7.5, and stored frozen.

**DISCUSSION**

**Purification of Creatinine Inimhydrase**—All procedures were carried out according to the method of Hedrick and Smith (13). Samples containing 25-150 μg of protein were dialyzed overnight against 50 mM Tris-phosphate buffer, pH 7.5, and 0.5 M Tris-phosphate buffer, pH 7.5. Active fractions were pooled, concentrated, and then eluted with 0.01 M Tris-phosphate buffer, pH 7.5, and stored frozen.

**DISCUSSION**

**Purification of Creatinine Inimhydrase**—All procedures were carried out according to the method of Hedrick and Smith (13). Samples containing 25-150 μg of protein were dialyzed overnight against 50 mM Tris-phosphate buffer, pH 7.5, and 0.5 M Tris-phosphate buffer, pH 7.5. Active fractions were pooled, concentrated, and then eluted with 0.01 M Tris-phosphate buffer, pH 7.5, and stored frozen.

**DISCUSSION**

**Purification of Creatinine Inimhydrase**—All procedures were carried out according to the method of Hedrick and Smith (13). Samples containing 25-150 μg of protein were dialyzed overnight against 50 mM Tris-phosphate buffer, pH 7.5, and 0.5 M Tris-phosphate buffer, pH 7.5. Active fractions were pooled, concentrated, and then eluted with 0.01 M Tris-phosphate buffer, pH 7.5, and stored frozen.

**DISCUSSION**

**Purification of Creatinine Inimhydrase**—All procedures were carried out according to the method of Hedrick and Smith (13). Samples containing 25-150 μg of protein were dialyzed overnight against 50 mM Tris-phosphate buffer, pH 7.5, and 0.5 M Tris-phosphate buffer, pH 7.5. Active fractions were pooled, concentrated, and then eluted with 0.01 M Tris-phosphate buffer, pH 7.5, and stored frozen.
Metal ion Analysis of Creatinine Iminohydrolase—Purified creatinine iminohydrolase was dialyzed against 10 mM Tri-
phosphate buffer, pH 7.5. A 1.0 ml sample, containing 3-20 mg of protein, and 1.0 ml of the corresponding dialysis buffer 
were used for metal analysis. The analyses were performed on the Perkin-Elmer model 5000 atomic absorption spectrophotometer with the HGA 500 heated graphite furnace and AAS-40 automatic sequence analyzers. Atomic absorption 
matrix reagent blanks were used to determine contamination in the reagent. Low-temperature ashing was used to oxidize the 
samples. In each case, values obtained for the dialysis buffer alone were subtracted from those obtained for a 
particular sample.

Miscellaneous Enzyme Assays—Lactate dehydrogenase 
was assayed by a mixture of 10 mM pyruvate and 180 mM NADH 
in 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.0, at 37 °C, 140 nm. Malate dehydrogenase was measured with 
180 mM NADH and 180 mM malic acid in 1.0 ml of 0.1 M 
kissium phosphate buffer, pH 7.5. at 37 °C, 240 nm (18). 
Catalase was assayed with 180 mM H2O2 in 1.0 ml of 0.05 M 
kissium phosphate buffer, pH 7.0, at 30 °C, 240 nm (19). 
Hexokinase was determined by a modification of the procedure 
to quantify ATP (20). Reaction mixtures contained 100 nmol 
glucose, 5 umol ATP, 5 umol magnesium sulfate, 0.3 umol NADP, 
and 10 U glucose-6-phosphate dehydrogenase in 1.0 ml of 0.05 M 
Tri-HEC buffer, pH 7.5, at 30 °C, 430 nm (21). L-=-Glycerophosphate dehydrogenase 
was assayed with 200 nmol 
NAD' in 10 ml of 0.02 M sodium pyrophosphate, pH 9.0, at 
30 °C, 340 nm. Alcohol 
dehydrogenase was assayed with 0.05 M Tris-HCl buffer, pH 7.0, at 37 'C, 430 nm (22). Reaction mixtures contained 200 umol 
d-l-glycerophosphate and 0.3 umol NAD+ in 0.1 M Tris-HCl buffer, pH 7.5, at 30 °C, 340 m. Alcohol 
dehydrogenase was assayed with 200 umol 
NAD' in 10 ml of 0.02 M sodium pyrophosphate, pH 9.0, at 
30 °C, 340 nm (21). L-=-Glycerophosphate dehydrogenase 
was assayed by a simplified version of the method described by 
Roberts (21). Reaction mixtures contained 200 umol 
D,L-a-glycerophosphate and 0.3 umol NAD' in 0.1 M Tri-hec buffer, pH 6.5, at 37 °C, 340 nm. Blue dextran and bovine 
serum albumin were determined by absorbance at 600 and 280 
respectively. Glucose was determined with 20 U glucose 
oxidase, 10 purpurogallin units peroxidase, and 0.21 umol 
creatinine iminohydrolase. The propanol fractionation served 
for a final step, DEAE-cellulose chromatography, 
was visible when electrophoresis 
was performed at pH 8.5. One band 
was visible in the final product. In other preparations, occasionally a minor, faster migrating band was observed, but 
Table II

| Molecular weight |
|------------------|
| Gel filtration  | 288,400 |
| Sucrose gradient centrifugation | 278,500 |
| Disc gel electrophoresis | 278,000 |
| Gradient electrophoresis | 245,000 |
| SDS electrophoresis | 44,300 |

**Figure 1:** Electrophoresis of various fractions from creatinine iminohydrolase purification procedure. Samples (50 µl) were applied to 7% buffer gels, and electrophoresis was carried out at pH 8.5 as described in "Experimental Procedures." Samples were (I) 100 mg crude extract, (II) 150 mg propanol product, (III) 25 mg Sepharose 4B hexylamine product, and (IV) 25 mg DEAE-cellulose product.