[CONTRIBUTION FROM BIOPHYSICS RESEARCH LABORATORY OF THE DEPARTMENT OF MEDICINE, HARVARD MEDICAL SCHOOL AND PETER BENT BRIGHAM HOSPITAL]

# Pyridine Nucleotide Dependent Metallodehydrogenases[1]

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The demonstration of zinc as a functional component of four highly purified dehydrogenases had led to the proposition that metals participate in the catalytic activity of other pyridine nucleotide dependent enzymes. This hypothesis was tested by emission spectrochemical analysis for metal content and by inhibition of activity. The following dehydrogenases were examined: the glyceraldehyde 3-phosphate of yeast and of rabbit muscle, the $\alpha$-glycerophosphate of rabbit muscle, the malic of pig heart and the glucose 6-phosphate of yeast (TPN dependent). All of these enzymes were inhibited significantly by exposure to 1,10-phenanthroline, 8-hydroxyquinoline and sodium diethyldithiocarbamate. They contained zinc, iron and copper as the only elements combining with all three chelating agents employed as inhibitors. The data indicate one of these metals, possibly zinc, to be involved in the mechanisms of action of the dehydrogenases, which are now referred to as pyridine nucleotide dependent metallodehydrogenases (PMD). The determination of the metal, specific to each enzyme, awaits further study. This delineation emphasizes the salient role of metals in oxidative, enzymatic catalysis.

The role of metals in enzymatic catalysis has received increasing attention, as methods for their study have become available. Recently, the participation of zinc in dehydrogenation reactions involving the pyridine nucleotide coenzymes has been under investigation in this Laboratory. The alcohol dehydrogenase of bakers' yeast (YADH),[3,4] the alcohol dehydrogenase of horse liver (LADH),[5,6] the glutamic dehydrogenase of beef liver (LGDH),[7] and the lactic dehydrogenase of rabbit muscle (SLDH)[8] contain zinc. Considerations significant to this work and the modes of investigation have been detailed elsewhere.[9]

Compositional studies have related the concentration of metals and the enzymatic activity to a unit weight of protein. Metal-binding reagents have been used to relate the mechanism of action of these enzymes to the chemical reactivity of zinc, preserved to an extent in these enzymes. None of these reagents have been reported to be specific for zinc alone.

The hypothesis that a metal is a functional and structural component of many, if not all, of the pyridine nucleotide dependent dehydrogenases (PND), is a natural extension of the studies on YADH, LADH, LGDH and SLDH. Absolute proof awaits the examination of the remaining enzymes of this group when obtained in purified form. The hypothesis is strengthened if further consistencies are found. The presence of metals in highly active though not necessarily pure preparations, in conjunction with the demonstration of inhibition of activity of each enzyme examined, would validate this suggestion. It therefore follows from these considerations that the metalloprotein nature of the PND systems can be examined by investigation of readily available enzymes, since these precepts apply equally well to purified and partially purified proteins. Delineation of the *specific metal* involved depends upon compositional studies on systems, homogeneous by the accepted physical chemical criteria. The present report extends our knowledge to five additional PND.

## Materials and Methods

Yeast and rabbit muscle glyceraldehyde 3-phosphate dehydrogenases[10,11] and pig heart malic dehydrogenase[12] were obtained from Worthington Biochemical Company. Yeast glucose 6-phosphate dehydrogenase[13] was obtained from the Sigma Chemical Company, and rabbit muscle $\alpha$-glycerophosphate dehydrogenase[11] from Boehringer and Soehne.

Protein was determined gravimetrically after precipitation with trichloroacetic acid.[14]

No estimation of the degree of molecular monodispersity was made on these enzyme preparations for the reasons outlined above. Analyses for metal content of enzyme preparations were performed spectrographically[9]; zinc and copper were also determined chemically.[15,16]

Enzymatic activities were measured spectrophotometrically at 340 m$\mu$ by the rate of DPNH formation or disappearance at 23°. Substrates for the various assays were obtained from commercial sources, including the triose phosphate esters from Boehringer and Soehne. DPN (Pabst Laboratories) was 95% pure by cyanide assay[17] and DPNH (Sigma Chemical Company) was more than 90% pure by measurement of absorbance at 340 m$\mu$. The reaction mixtures for the various enzymatic assays were as follows (light paths were 1 cm. unless otherwise specified): glucose 6-phosphate dehydrogenase: 3.0 ml. containing 30 $\mu M$ $MgCl_2$, 2.5 $\mu M$ potassium glucose 6-phosphate, 0.4 $\mu M$ TPN, 100 $\mu M$ tris buffer *p*H 8.8. $\alpha$-Glycerophos-

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(3) The following abbreviations are used: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; PND, pyridine nucleotide dependent dehydrogenases; PMD, pyridine nucleotide dependent metallodehydrogenases; YADH, yeast alcohol dehydrogenase; LADH, horse liver alcohol dehydrogenase; LGDH, liver glutamic dehydrogenase; SLDH, rabbit skeletal muscle lactic dehydrogenase.

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TABLE I

EMISSION SPECTROGRAPHIC ANALYSES OF VARIOUS DEHYDROGENASES, INHOMOGENEOUS BY PHYSICAL CHEMICAL CRITERIA, NOT PURIFIED MAXIMALLY BY ENZYMOLOGICAL CRITERIA

(All values are given in micrograms per gram of protein.)

| Enzyme | Zn | Cu[a] | Fe | Ni | Mn | Cr | Al | Mg | Ca | Sr | Ba | Pb |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle) | 813 | 55 | 62 | [c] | 12 | [c] | 5 | 88 | 112 | [c] | 7 | [c] |
| Glyceraldehyde 3-phosphate dehydrogenase (yeast) | 295 | 111 | 85 | [c] | 7 | 7 | 15 | 316 | 120 | Trace | 3 | [c] |
| Glucose 6-phosphate dehydrogenase (yeast) | 840 | 810 | 907 | [c] | [c] | 56 | 116 | 141 | 311 | [c] | 26 | 1640 |
| $\alpha$-Glycerophosphate dehydrogenase (rabbit muscle) | 764 | 593 | 259 | [c] | [c] | 41 | 134 | 101 | 1300 | [c] | 63 | [c] |
| Malic dehydrogenase (pig heart muscle) | 2030 | 301 | 1012 | 26 | 74 | [c] | 144 | 539 | 344000[b] | [c] | 10 | [c] |

[a] Cu was determined microchemically. [b] Prepared with $Ca(H_2PO_4)_2$. [c] Not detected and also Ag, Cd, Mo, Co, Sn.

phate dehydrogenase: 3.0 ml. containing 0.75 $\mu M$ triose phosphate esters, 0.8 $\mu M$ DPNH, 50 $\mu M$ triethanolamine buffer $p$H 7.5. Malic dehydrogenase: 0.76 $\mu M$ oxaloacetate, 0.15 $\mu M$ DPNH, 60 $\mu M$ phosphate buffer $p$H 7.0. Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase: 200 $\mu M$ $Na_2HAsO_4$, 18 $\mu M$ triose phosphate esters, 3 $\mu M$ DPN, 1.25 m$M$ triethanolamine buffer $p$H 8.5, 5 cm. light path. Yeast glyceraldehyde 3-phosphate dehydrogenase: 100 $\mu M$ $Na_2HAsO_4$, 18 $\mu M$ triose phosphate esters, 15 $\mu M$ DPN, 1.25 m$M$ triethanolamine buffer $p$H 8.5; 5 cm. light path.

1,10-Phenanthroline (G. Frederick Smith Co., Columbus, Ohio), 8-hydroxyquinoline and sodium diethyldithiocarbamate (Eastman Kodak Chemicals) were dissolved and titrated to appropriate $p$H values before use. The cleaning of glassware, chemicals and water has been described.[4]

## Results

The metal contents, in micrograms per gram of protein, of the following dehydrogenases are shown in Table I: glyceraldehyde 3-phosphate of rabbit muscle, glyceraldehyde 3-phosphate of yeast, glucose-6-phosphate of yeast, $\alpha$-glycerophosphate of rabbit muscle, malic of pig heart. Zinc, iron and copper are found in all these enzyme preparations. The molecular weight of glyceraldehyde phosphate dehydrogenase of rabbit muscle is 137,000[18] and that of the glyceraldehyde phosphate dehydrogenase of yeast has been reported as 119,000.[19] The former binds 2 or 3 moles of DPN,[20] the latter 2 moles of DPN.[21] The measured mole ratios of zinc to protein are 1.7 and 0.54, respectively, while for iron they are 0.15 and 0.18, and for copper 0.12 and 0.21. For the rabbit muscle enzyme, the zinc:protein ratio approaches the coenzyme:protein ratio of the pure enzyme.[18] The measured zinc contents are taken to reflect the state of impurity. The iron and copper contents are lower than those of zinc in all enzymes except glucose 6-phosphate of yeast. Since the molecular weights of the other enzymes are not known, no meaningful molar ratios can be presented.

The high calcium content of malic dehydrogenase can be accounted for by its method of preparation.[12] The lead in the glucose 6-phosphate dehydrogenase is presumed to represent contamination.

All of these enzymes contain aluminum, magnesium, calcium and barium. The amounts of magnesium in glyceraldehyde 3-phosphate dehydrogenase of yeast, that of calcium in glucose 6-phosphate dehydrogenase and that of both these elements in malic dehydrogenase are of the same order of magnitude as those of zinc, copper and iron in the same enzymes. Nickel, manganese, cobalt and strontium occur sporadically and in insignificant quantity.

The activity of the five enzymes was measured in the presence of three chelating agents known to have great affinity for the transition and group IIb elements: 1,10-phenanthroline, 8-hydroxyquinoline and sodium diethyldithiocarbamate. The effect of these reagents was assayed in two ways. (a) The enzyme alone was held in contact with the reagent to be tested and its activity was measured as a function of the time of *preincubation;* and (b) the enzyme was added unmodified to a reaction mixture containing substrate, coenzyme and the chelating agent to be tested and activity was measured *immediately.*

Table II shows the first-order *rate* constants for the inactivation of five enzymes preincubated with the stated concentrations of chelating agents. The initial rate of inactivation has been shown to be first order in all instances. Temperatures were selected to give adequate rates without significant reduction of control activity. All the enzymes

TABLE II

FIRST-ORDER RATE CONSTANTS OF THE INHIBITION OF SEVERAL DEHYDROGENASES BY METAL-BINDING AGENTS

The preincubation of all enzymes with reagents was at $p$H 7.0 in 0.05 $M$ potassium phosphate buffer, with the exception of the glyceraldehyde phosphate dehydrogenases, which were preincubated at $p$H 8.5 in 0.05 $M$ triethanolamine. The rate constants were determined at inhibitor concentrations: 1,10-phenanthroline (OP) $5 \times 10^{-3}$ $M$, 8-hydroxyquinoline (8OHQ) $5 \times 10^{-3}$ $M$, and diethyldithiocarbamate (DDC) $2 \times 10^{-1}$ $M$.

| | Enzyme | Preincubation temp., °C. | $k$ (per minute/mole inhibitor) OP | 8OHQ | DDC |
|---|---|---|---|---|---|
| 1 | Glyceraldehyde phosphate of skeletal muscle | 23 | 7.2 | 4.0 | 0.34 |
| 2 | Glyceraldehyde phosphate of yeast | 23 | 0.29 | 0.14 | .029 |
| 3 | Malic of pig heart | 23 | .33 | 7.1 | .0083 |
| 4 | Glucose-6-phosphate of yeast | 37 | .51 | 1.5 | .44 |
| 5 | $\alpha$-Glycerophosphate of skeletal muscle | 40 | 135.0 | 17.7 | 1.70 |

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TABLE III

INHIBITION OF SEVERAL DEHYDROGENASES BY METAL BINDING AGENTS

Concentrations shown are those required to produce 25 and 50% inhibition of activity by reagents present in the reaction mixture cuvettes.

| | Enzyme | % Inhibition | Concentration of inhibitor ($M$) OP | 8OHQ | DDC |
|---|---|---|---|---|---|
| 1 | Glyceraldehyde phosphate of skeletal muscle | 25 | $1.0 \times 10^{-2}$ | $5.6 \times 10^{-4}$ | $7.5 \times 10^{-4}$ |
| | | 50 | $1.5 \times 10^{-2}$ | $7.5 \times 10^{-4}$ | $2.0 \times 10^{-2}$ |
| 2 | Glyceraldehyde phosphate of yeast | 25 | $5.0 \times 10^{-3}$ | $7.8 \times 10^{-4}$ | $1.9 \times 10^{-2}$ |
| | | 50 | $6.9 \times 10^{-3}$ | $8.7 \times 10^{-4}$ | $2.1 \times 10^{-2}$ |
| 3 | Malic of pig heart | 25 | $9.0 \times 10^{-3}$ | $3.5 \times 10^{-3}$ | $5.0 \times 10^{-2}$ |
| | | 50 | $2.1 \times 10^{-2}$ | $3.9 \times 10^{-3}$ | $8.4 \times 10^{-2}$ |
| 4 | Glucose 6-phosphate of yeast | 25 | $8.0 \times 10^{-3}$ | [a] | $3.5 \times 10^{-2}$ |
| 5 | $\alpha$-Glycerophosphate of skeletal muscle | 25 | $3.0 \times 10^{-4}$ | ........ | $3.3 \times 10^{-2}$ |
| | | 50 | ........ | $2.0 \times 10^{-3}$ | ........ |

[a] Complete inhibition with $2 \times 10^{-3}$ $M$ 8OHQ; a precipitate of Mg-8OHQ was formed.

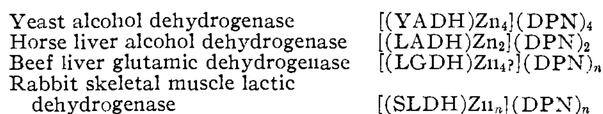
were inhibited by the three reagents; the rate constants vary widely. The time required for 50% inhibition varied from one minute for the interaction of $\alpha$-glycerophosphate with $5 \times 10^{-3}$ $M$ 1,10-phenanthroline to 14.5 hours for the interaction of yeast glyceraldehyde 3-phosphate dehydrogenase with $5 \times 10^{-3}$ $M$ 8-hydroxyquinoline. This inhibition with these three reagents does not follow any readily discernible pattern.

When enzyme was added directly to the reaction mixture containing a chelating agent, the *degree* of inhibition could be measured as a function of inhibitor concentration. Table III presents the concentration of chelating agents required for 25 and 50% inhibition of these enzymes by the three reagents. Effective inhibition for the three reagents—1,10-phenanthroline, 8-hydroxyquinoline and sodium diethyldithiocarbamate—was obtained with concentrations in the range $5 \times 10^{-4}$ to $5 \times 10^{-2}$ $M$. The rates of reaction in the presence and absence of inhibitor remained linear during the assay period. Failure of inhibition with these reagents was not observed under the experimental conditions employed.

## Discussion

The examination of the alcohol, glutamic and skeletal lactic dehydrogenases indicated several generalities with respect to all of these enzymes. The purified protein can be isolated with its full zinc complement and full activity; the ratio of gram atoms of zinc to mole of protein is an integral number; the ratio of zinc to coenzyme bound, when known, is an integral number; the ratio of gram atoms of zinc to mole of protein (or of coenzyme) is a small number, in conformity with the law of multiple proportions; exposure of *apo-PMD* to zinc-binding agents, under suitable conditions, inhibits enzymatic activity; the coenzyme may protect against these inhibitors; metal ions, including zinc, and sulfhydryl-reacting reagents, inhibit enzymatic activity.

An empirical structural formula contains many of the essential facts of these considerations



| | |
|---|---|
| Yeast alcohol dehydrogenase | $[(YADH)Zn_4](DPN)_4$ |
| Horse liver alcohol dehydrogenase | $[(LADH)Zn_2](DPN)_2$ |
| Beef liver glutamic dehydrogenase | $[(LGDH)Zn_4?](DPN)_n$ |
| Rabbit skeletal muscle lactic dehydrogenase | $[(SLDH)Zn_n](DPN)_n$ |

where $n = 1, 2, 3 \ldots$

The following complexing agents with high affinity for zinc ions, as well as other members of the group IIB and transition metals, were employed to inhibit the enzymatic activity of these four enzymes: 1,10-phenanthroline, $\alpha,\alpha'$-dipyridyl, 8-hydroxyquinoline, diethyldithiocarbamate, dithizone, 2,3-dimercapto-1-propanol, thiourea, thioacetamide, semicarbazide, ammonium phenylnitrosohydroxylamine and azide. All these enzymes are inhibited by these metal-binding agents (*vide infra*). In the case of 1,10-phenanthroline, inhibition of their activity can be *prevented* by the addition of zinc ions to the chelating agent prior to incubation with the enzyme and can be *reversed* by the addition of zinc ions. The coenzyme protects these enzymes against such inhibition. Competitive inhibition between DPN and 1,10-phenanthroline has been shown with yeast alcohol dehydrogenase[22] and more recently with glutamic dehydrogenase.

Zinc, copper and iron are the only metals found in significant concentrations in the glyceraldehyde 3-phosphate dehydrogenase of yeast and muscle, $\alpha$-glycerophosphate dehydrogenase of muscle, malic dehydrogenase of pig heart and the glucose 6-phosphate dehydrogenase of yeast. These three elements form strong complexes with 1,10-phenanthroline, 8-hydroxyquinoline and sodium diethyldithiocarbamate. Magnesium, calcium, barium and aluminum, present in all preparations, form complexes only with 8-hydroxyquinoline among the three reagents employed. Thus, the inhibition data are best explained by an interaction of the complexing agents with zinc, iron or copper.

The concentrations in which these elements occur can obviously be no more than a guide to the probable functional metal, since the enzymes are not homogeneous with respect to protein. Low concentrations of metal may be due to inadequate purification, while high concentrations might be attributed to contamination. The consistently high concentrations of zinc should be noted. Further study is particularly indicated to explain the presence of the iron and copper in glucose 6-phosphate dehydrogenase and malic dehydrogenase. A final conclusion as to the functional metal cannot be derived from these analytical data alone. The results are not inconsistent, however, with the hypothesis that a metal, most probably zinc, is a struc-

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tural component of these and possibly many additional pyridine nucleotide dependent dehydrogenases. It is to be re-emphasized that this hypothesis can be accepted with finality only on the basis of detailed experimental examination of all the enzymes of this group. In this regard, the present data constitute an interim report.

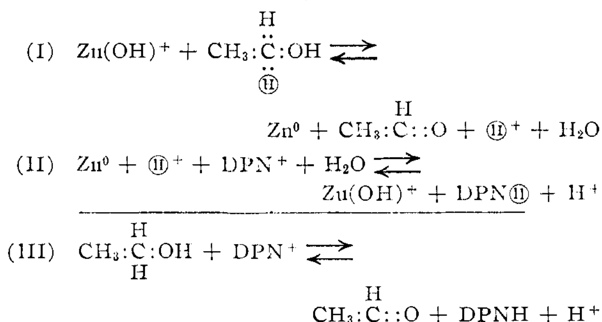
All the PND of unknown purity examined in these studies are inhibited by chelating agents. Glucose 6-phosphate dehydrogenase of yeast is the only TPN specific dehydrogenase represented in this study; it is inhibited. The first-order inactivation constants vary widely. In several instances inhibition could be prevented partially by the addition of zinc to OP prior to its contact with the enzyme. The degree and rate of inhibition of each one of these enzymes is a function of *p*H and temperature, as well as the concentration and type of inhibitor. The optimal pattern of physical conditions resulting in maximal inhibition is presumably characteristic of each one of these enzymes. In general, the same concentration of inhibitor in a reaction mixture inhibited less than under the conditions of preincubation. The inhibition produced by these agents in the presence or absence of substrate and coenzyme differs and is currently under investigation.

The data suggest that yet additional PND may be inhibited under the appropriate constellation of experimental conditions. A review of the literature fails to reveal similar results with such dehydrogenase systems with the exception of the DPN-dependent formic dehydrogenase of peas and the DPN-dependent isocitric dehydrogenase of yeast; the former has been inhibited with azide, cyanide and 8-hydroxyquinoline. Formic dehydrogenase was not inhibited with Versene, sodium diethyldithiocarbamate, thiourea, *o*-phenanthroline and $\alpha,\alpha'$-dipyridyl.[23] Isocitric dehydrogenase has been inhibited by cyanide and azide.[24] The inhibition of other dehydrogenases with cyanide and semicarbazide is ambiguous because of the presumed interaction of these reagents with either the pyridine nucleotide or the substrate. Interpretation of these findings must now include the possibility that these inhibitors could combine with a functional metal atom of the apoenzyme under appropriate conditions. In the absence of stated conditions of exposure of enzyme to inhibitor, the reported lack of inhibition of various other dehydrogenases with metal-complexing agents cannot be interpreted.

These studies do not bear upon the manner in which zinc plays its role in the molecular events of enzymatic dehydrogenation. Previous investigations have indicated that zinc is an active center of YADH by virtue of the demonstrable competitive inhibition between DPN and 1,10-phenanthroline. While preliminary *p*H titrations indicated a complex between DPN and zinc ions in solution,[4] experiments with $Zn^{65}(OH)_2$ in this Laboratory have now shown the absence of complexation.[25] Similar evidence has been obtained independently by ionophoresis.[26]

Although there is no evidence for the existence of a simple zinc–DPN complex in solution, the participation of the cation as an orienting center for apoenzyme, coenzyme and possibly substrate is not to be discounted, especially in view of the competition between DPN and a chelating agent. The ability of electrophilic $Zn^{++}$ to coördinate with radicals and polar groups containing oxygen, nitrogen and sulfur makes it highly suitable to serve as an organizing center for coenzymes and other small molecules in the active catalytic complex. The "no-bond model"[27] has not thus far been subjected to experimental verification but represents a basis for further study.

Investigations of oxidizing metalloenzymes which contain copper, iron and molybdenum have provided considerable information on molecular processes of electron transfer, in which the oxidation–reduction characteristics of the metal atoms play a critical part. As zinc has only one stable valence state in solution, it is unlikely that it participates directly in oxidation–reduction phenomena through stepwise reactions of the type



although this possibility cannot be overlooked.

Many of the PND, as well as many other enzymes, are readily inhibited by low concentrations of metal ions. When such an enzyme is a metalloenzyme, it may be inhibited, among others, by ions of the very metal which is an integral, functional part of the apoenzyme molecule (*viz.*, zinc in PMD). It would appear that the mode and site of binding differ significantly for the *integral* metal and for the added metal ion.[27] The bond between the *integral* metal and protein is probably quite specific and is a functional determinant. The degree of specificity attributed to the inhibitory action of metal ions has been extrapolated to deny the participation of the metal in enzymatic activity—a *non-sequitur*.

These data reported indicate additional common properties of some PND enzymes. These observations add to the known characteristic similarities of these enzymes and re-emphasize the analogy of their mechanism of action. These studies do not finally settle the question of the metalloenzyme nature of these dehydrogenases. The implication that zinc or another metal is a functional component in several enzymes of this class serves to indicate the existence of a significant number of

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pyridine nucleotide dependent metallodehydrogenases (PMD). This group of PMD is now joined with other members of the respiratory metalloenzyme group: hemes, metalloflavoproteins, copper oxidases and carbonic anhydrase. The salient position of metals in oxidative catalysis is thereby extended and the major oxidative pathway thus seems to involve a metal in each enzymatic step.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

# Synthesis of a Protected Tetrapeptide Amide Containing the Carboxyl Terminal Sequence of Lysine–Vasopressin[1]

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The synthesis of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-N$^\epsilon$-tosyl-L-lysylglycine ethyl ester and related compounds starting from N$^\epsilon$-tosyl-L-lysine is described. This protected tetrapeptide ester was obtained by two different routes, one involving as the final step the coupling of S-benzyl-N-carbobenzoxy-L-cysteine with L-prolyl-N$^\epsilon$-tosyl-L-lysylglycine ethyl ester and the other, the coupling of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-proline with N$^\epsilon$-tosyl-L-lysylglycine ethyl ester. The product obtained after conversion of the protected tetrapeptide ester to the corresponding amide and removal of the carbobenzoxy group has been used as an intermediate in studies on the synthesis of lysine–vasopressin.

In connection with synthetic studies on the peptide hormone lysine–vasopressin, isolated from hog posterior pituitaries,[2,3] the tetrapeptide derivative S-benzyl-L-cysteinyl-L-prolyl-N$^\epsilon$-tosyl-L lysylglycinamide was desired. A primary consideration in the selection of this derivative was the protection of the $\epsilon$-amino group of the lysine molecule by a group which would remain unaffected under the conditions of a peptide synthesis involving carbobenzoxy intermediates, but which could be conveniently removed later in the synthetic steps to vasopressin. The tosyl (*p*-toluenesulfonyl) group was chosen, since it is not attacked by any of the usual decarbobenzoxylating reagents except sodium in liquid ammonia, which cleaves it readily.[4] Since the employment of the latter reagent was already anticipated for effecting the final stage of the vasopressin synthesis, the tosyl group was particularly convenient for our purpose.

The series of reactions employed for the synthesis of the protected tetrapeptide amide is given in Fig. 1. N$^\epsilon$-Tosyl-L-lysine (I) was prepared in high yield by treatment of the copper complex of L-lysine with tosyl chloride in aqueous acetone and removal of the copper with hydrogen sulfide, essentially according to the procedure employed by Kurtz[5] for the preparation of analogous benzenesulfonyl derivatives of lysine and ornithine. Erlanger, Sachs and Brand[6] have recently prepared N$^\delta$-tosyl-L-ornithine by similar means and have used this compound in synthetic peptide work.

The $\alpha$-carbobenzoxy derivative of N$^\epsilon$-tosyl-L-lysine was coupled with glycine ethyl ester according to the procedure of Young, *et al.*,[7] with ethylene chlorophosphite as the coupling reagent. Removal of the carbobenzoxy group was effected with hydrogen bromide in glacial acetic acid[8] at room temperature to give the crystalline hydrobromide of N$^\epsilon$-tosyl-L-lysylglycine ethyl ester (II) in 90% yield. This substance was used directly in the next synthetic step, but the tosyl dipeptide ester IIa could be obtained by treating the hydrobromide with potassium carbonate. This ester underwent slow decomposition in solution to give 3-($\omega$-tosylaminobutyl)-2,5-piperazinedione. A dipeptide ester identical with IIa in melting point and optical rotation was also obtained from N$^\epsilon$-tosyl-L-lysine-N$^\alpha$-carboxy anhydride and glycine ethyl ester essentially by the method of Bailey,[9] *i.e.*, by reaction in chloroform at low temperature.

II was coupled with N-carbobenzoxy-L-proline to give crystalline N-carbobenzoxy-L-prolyl-N$^\epsilon$-tosyl-L-lysylglycine ethyl ester (III). The coupling reagent used with best results was *o*-phenylene chlorophosphite,[10] and it is noteworthy that in this case the use of this reagent resulted in higher yields (70–75%) than when either ethylene chlorophosphite or tetraethyl pyrophosphite was used (45–50%).

Decarbobenzoxylation of III with a 2 *M* solution of hydrogen bromide in glacial acetic acid gave the hydrobromide of L-prolyl-N$^\epsilon$-tosyl-L-lysylglycine ethyl ester, from which the tosyl tripeptide ester IV was obtained by treatment with triethylamine. The conversion of IV to S-benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-N$^\epsilon$-tosyl-L-lysylglycine ethyl ester (V) was accomplished by coupling with S-ben-

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