enzyme (K_m of Bz-Gly-OEt is about 20-fold smaller than that of Ac-Gly-OEt).

These paradoxical features of the activation of trypsin-catalyzed hydrolysis are certainly related to the ability of the trypsin active-site geometry to be modified by effectors which are more or less related to specific substrates. Our data cannot be completely explained on the basis of a rigid enzymes active site.

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Studies on the Hydrogen-Transfer Reactions Catalyzed by Pyridine Nucleotide Linked Dehydrogenases*

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ABSTRACT: Irreversible one-turnover reactions between GAP-*1-t* (generated from DHAP-*1-t*) and the lobster muscle glyceraldehyde 3-phosphate dehydrogenase–NAD complex have been carried out. Analyses of the products of these reactions have shown that NAD^{3}H is formed and tritium is not incorporated into the enzyme. These results show that the hydrogen-transfer reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase is direct and not mediated through the alternate oxidation and reduction of a tryptophan residue. Yeast alcohol dehydrogenase has been labeled with tritium by adding urea to a reaction mixture which contained yeast alcohol dehydrogenase, ethanol-*1-t*, and NAD at pH 11.0. Fractionation of tryptic digests of the labeled enzyme failed to reveal that a specific tryptophan residue at the active site of alcohol dehydrogenase was labeled. All attempts to label various lactate dehydrogenases with tritium derived from lactate-*2-t* failed.

It has been concluded from these experiments that the hydrogen transfers catalyzed by pyridine nucleotide linked dehydrogenases are direct and not mediated by the alternate oxidation and reduction of tryptophan residues at the active-sites of the enzymes.

It has been suggested that the hydride-transfer reactions catalyzed by pyridine nucleotide dependent dehydrogenases may be mediated through the alternate oxidation and reduction of the methylene group of tryptophanyl side chains in the enzymes (Schellenberg, 1965, 1970). The enzyme-mediated hydride-transfer mechanism is based on the following experimental observations. Tritium is incorporated into the alanine side chains of tryptophan residues of yeast alcohol dehydrogenase (Schellenberg, 1966), rabbit muscle lactate dehydrogenase (Schellenberg, 1967), and the mitochondrial pig heart malate dehydrogenase (Chan and Schellenberg, 1968) when the enzymes are incubated with their respective tritiated substrates under appropriate conditions. The results of studies with model systems have made the proposed mechanism of enzyme-mediated hydride transfer appear attractive indeed. For instance, it has been shown by Schellenberg and McLean (1966) that a model indolenine salt is reduced to the corresponding indole by reduction with 1-benzyl-1,4-dihydronico-

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tinamide. Moreover, with the use of 1-benzyl-1,4-dideuterionicotinamide it was shown that deuterium was transferred to the methylene group at the 3 position of the indole, analogous to the position proposed for the enzymatic tritium-transfer mechanism. Similar nonenzymatic model reactions have been carried out by Huffman and Bruice (1967) and by Hino and Nakagawa (1969).

It has not been clearly demonstrated that the introduction of tritium into the tryptophan residues of alcohol dehydrogenase, lactate dehydrogenase, and malate dehydrogenase from their respective tritiated substrates represents reactions which are on the pathways of the enzyme-catalyzed reactions. As has been pointed out by Schellenberg (1970), the observed incorporation of tritium into these enzymes may be due to side reactions that are not on the enzymatic reaction pathways. The qualitative analysis of the products of an irreversible one-turnover reaction between glyceraldehyde-*1-t* 3-phosphate and the enzyme–NAD complex of glyceraldehyde 3-phosphate dehydrogenase has suggested, that at least for this dehydrogenase, hydride transfer from reduced substrate is direct (Allison *et al.*, 1969). The reaction products of such one-turnover reactions have now been determined quantitatively. These results, as well as the results of some experiments which were designed to determine the specificity of tritium incorporation into yeast alcohol dehydrogenase and various lactate dehydrogenases under conditions described by Schellenberg (1967) are presented in this communication.

Materials and Methods

Enzymes and Substrates. Crystalline lobster muscle glyceraldehyde 3-phosphate dehydrogenase was prepared as described previously (Allison and Kaplan, 1964). Before use in the one-turnover experiments, the reconstituted enzyme–NAD complex was prepared as described in detail elsewhere (Allison *et al.*, 1969). Crystalline yeast alcohol dehydrogenase was purchased from Worthington. Rabbit muscle lactate dehydrogenase was purchased from Boehringer. Crystalline lactate dehydrogenase from dogfish muscle and chicken heart were prepared as described by Pesce *et al.* (1967). Crystalline rabbit muscle aldolase and triosephosphate isomerase were purchased from the Sigma Chemical Co. Trypsin treated with TPCK[1] to inactivate chymotryptic activity was purchased from Calbiochem.

The dicyclohexylammonium salt of the dimethyl ketal of dihydroxyacetone phosphate was purchased from the Sigma Chemical Co. Pyruvate was purchased from Sigma. NAD^+ and NADH were P-L Biochemical products.

Synthesis of Radioactive Substrates. DHAP-*1-t* was prepared by the aldolase-catalyzed exchange reaction essentially by the procedure described by Rose and Rieder (1958). DHAP was prepared from the dicyclohexylammonium salt of its dimethyl ketal as described by Ballou (1960) with the exception that the solution was more concentrated (0.1 M rather than 0.03 M). The exchange reaction mixture contained 1.0 ml of 0.1 M DHAP, adjusted to pH 7.0 with solid NH_4HCO_3, 1.0 ml of tritiated water (1 Ci), and 0.1 ml of aldolase (10 mg/ml). The reaction mixture was incubated for 1 hr at 25° at which time 1 ml of 0.02 M cupric acetate was added to inactivate the aldolase. This solution was applied to a 1.5 × 20 cm column of DEAE-Sephadex which was equilibrated with 0.005 M Tris-HCl (pH 7.5). The column was washed with 300 ml of the equilibration buffer to remove tritiated water and then with 0.05 M HCl to remove the DHAP-*1-t*. The DHAP-*1-t* was concentrated by precipitation as the barium salt of its hydrazone. The salt of the hydrazone was converted into free DHAP-*1-t* in a small volume as described by Hall (1960). The resulting DHAP-*1-t* (46 μmoles) contained 9.8×10^5 cpm/μmole of tritium and was stored frozen at pH 3.0.

D,L-Lactate-*2-t* with a specific activity of 1.07×10^7 cpm/μmole was synthesized by the reduction of pyruvate with NaB^3H_4 (Lowenstein, 1963).

Scintillation Counting Techniques. Samples of 1–100 μl were counted in 10 ml of the scintillation fluid described by Bray (1960) or in 20 ml of the scintillation fluid described by Schellenberg (1965) with the use of a Nuclear-Chicago Unilux counter. Radioactivity was located on paper strips with the use of a Tracerlab 4π strip scanner.

Experiments with Glyceraldehyde 3-Phosphate Dehydrogenase. Irreversible one-turnover reactions were carried out at pH 7.8 and 10.0 using DHAP-*1-t* and triosephosphate isomerase to generate glyceraldehyde-*1-t* 3-phosphate (GAP-*1-t*). The reaction mixture at pH 7.8 contained in 3.0 ml: the reconstituted glyceraldehyde 3-phosphate dehydrogenase–NAD complex (10 mg), DHAP-*1-t* (1 μmole), Na_2HAsO_4 (30 μmoles), NH_4HCO_3 (850 μmoles), and triosephosphate isomerase (0.1 mg). The reaction mixture at pH 10.0 contained the same components except that 500 μmoles of glycine–NaOH buffer (pH 10.0) replaced the NH_4HCO_3 used at the lower pH. At both pH values the reaction was initiated by the addition of the isomerase.

At both pH 7.8 and 10.0, the reduction of the enzyme-bound NAD in the reconstituted enzyme–NAD complex was nearly complete, as assessed by the increase in optical density at 340 mμ, immediately after the addition of the isomerase. To insure that the reactions were complete under the nonsaturating conditions employed, the reaction mixtures were incubated for 5 min at room temperature at which time the enzymes in the one-turnover reaction mixtures were denatured by the addition of 2.4 g of solid urea. These solutions were then submitted to gel filtration in 6 M urea on Sephadex G-25. The results of the fractionation of the pH 7.8 reaction mixture are shown in Figure 1. The radioactive peak of each of the reaction mixtures obtained after chromatography on Sephadex G-25, was subjected to anion-exchange chromatography to resolve the NAD, NAD^3H, and unreacted DHAP-*1-t*. The results of this fractionation for the reaction mixture incubated at pH 7.8 are presented in Figure 2.

Experiments with Yeast Alcohol Dehydrogenase. Yeast alcohol dehydrogenase was labeled with tritium derived from ethanol-*1-t* in two separate reaction mixtures. The reaction mixtures contained in a final volume of 6 ml: yeast alcohol dehydrogenase (50 mg), Tris-HCl (pH 8.1, 600 μmoles), NAD^+ (25 μmoles), diethylamine-HCl buffer (pH 11.0, 2400 μmoles), and ethanol-*1-t* (156 μmoles, 0.83 mCi). The ethanol-*1-t* in 1.6 M diethylamine buffer prepared at 0° was added to the other components of the reaction mixture at 0°. The reaction mixtures were incubated for 1 hr at 0° at which time 4.8 g of recrystallized urea was added to denature the enzyme.

After the pH of the reaction mixtures was lowered to pH 7.8 by the addition of 2.0 ml of 4.0 M Tris-HCl (pH 7.0), 100 μmoles of neutral iodoacetate was added to carboxymethylate the cysteine residues of the yeast alcohol dehydrogenase. Carboxymethylation was allowed to proceed for 1 hr at room temperature, at which time the first of three dialyses against

[1] The abbreviations used are: DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; ADH, alcohol dehydrogenase; and LDH, lactate dehydrogenase; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

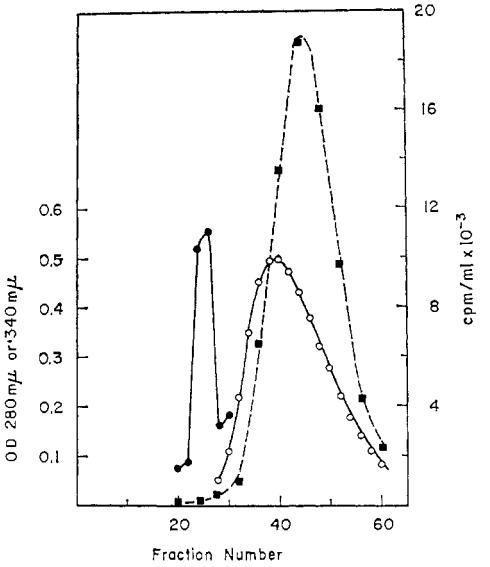

FIGURE 1: Separation of glyceraldehyde 3-phosphate dehydrogenase from reaction products following a one-turnover reaction. The enzyme in the one-turnover reaction mixture (described in the Materials and Methods) was denatured by the addition of urea to a final concentration of 8 M. Carrier DPN and DPNH, 5 mg each, were added and the mixture was applied to a 1.8 × 100 cm column of Sephadex G-25 coarse which was equilibrated and eluted with 0.05 M Tris-HCl containing 6 M urea. The effluent was collected in 5.0-ml fractions. (●) Optical density at 280 mμ; (○) optical density at 340 mμ; and (■) ^3H counts per minute per milliliter.

3.0 l. of 10^{-3} M HCl was begun to remove excess reagents. In a separate experiment three dialyses were shown to adequately remove the dialyzable radioactivity.

Trypsin (1 mg) and NH_4HCO_3 (100 mg) were added to the dialyzed protein solutions. Tryptic digestion was allowed to proceed for 6 hr at 37°. The lyophilized tryptic digests were dissolved in 2 ml of 0.05 M NH_4OH and subjected to gel filtration on a 2.5 × 100 cm column of Sephadex G-25. The optical density at 280 mμ and the radioactivity of the 5-ml collected fractions were monitored. The fractionation of the tryptic peptides by gel filtration is illustrated in Figure 3.

Experiments with Lactate Dehydrogenase. The components of the reaction mixtures which are described in Table III were added in the following sequence at 0°; 0.1 ml of 17.0 mM D,L-lactate-*2-t* which had a specific activity of 10^7 cpm/μmole was added to 0.10 ml of the various enzymes which had been dialyzed against 5 mM NaEDTA (pH 9.2). A few grains of the sodium salt of NAD were then added where indicated, after which 0.1 ml of 0.5 or 1.7 M NaEDTA (pH 10.2) was added. Where indicated, 0.05 ml of 2.0 M hydrazine (pH 10.0) was added. The reaction mixtures were incubated for 15 min at 0° at which time 0.3 g of urea was added. The reaction mixtures were incubated an additional 10 min at 0° and then warmed to room temperature to dissolve the urea. The urea solutions were then applied to a 2.0 × 30 cm column of Sephadex G-25 which was equilibrated and eluted with 8 M urea in 0.05 M EDTA (pH 10.0) to separate the protein from the radioactive substrate.

Results

Experiments with Glyceraldehyde 3-Phosphate Dehydrogenase. The analysis of the products of irreversible one-turnover reactions carried out by adding GAP-*1-t* (generated from DHAP-*1-t* to the glyceraldehyde 3-phosphate–NAD complex should distinguish between the direct hydride-transfer mechanism described by eq 1, or the tryptophan-mediated hydride-transfer mechanism described by eq 2. After the one-turnover reactions described in Materials and Methods had gone to completion as assessed by NADH formation, the component enzymes were denatured by the addition of urea. The resulting solutions were subjected to gel filtration in 6 M urea to remove the glyceraldehyde 3-phosphate dehydrogenase from unreacted DHAP-*1-t* and NADH produced by the reactions. Figure 1 shows the separation of the protein components from the radioactive components of the one-turnover reaction carried out at pH 7.8. Identical results were obtained when the reaction mixture which was carried out at pH 10.0 was subjected to gel filtration under the same conditions. Since tritium was not incorporated into the enzyme during the one-turnover reactions at both pH values, the only way to explain the results in terms of a tryptophan-mediated hydride transfer mechanism is to propose that a tryptophan residue at the active site of the enzyme exists in an oxidized form as shown in eq 3. Equation 3 suggests that an indolenine residue exists in glyceraldehyde 3-phosphate which is thermodynamically favored. When it is reduced by an aldehyde substrate under irreversible conditions, the tryptophan residue formed will donate a hydride ion to the enzyme-bound NAD to form NADH and revert to the more stable idolenine form. Although such a mechanism cannot be ruled out completely, spectral evidence does not support the presence of an indolenine residue in glyceraldehyde 3-phosphate dehydrogenase. The absorption spectra of indolenine salts in nonaqueous solvents, which have been reported (Schellenberg and McLean, 1966; Huffman and Bruice, 1967) suggest that a protein which contains an indolenine residue might have unusual absorption characteristics in the near-ultraviolet region. The examination of the absorption spectrum of lobster muscle glyceraldehyde

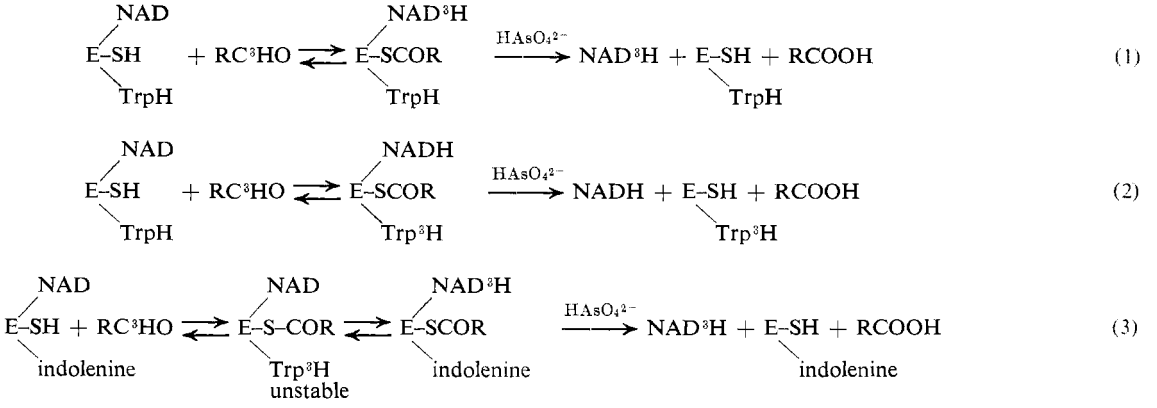

TABLE I: Summary of the Product Analysis of the One-Turnover Reaction Experiments with Lobster Muscle Glyceraldehyde 3-Phosphate Dehydrogenase.[a]

	Expt at pH 7.8	Expt at pH 10.0
μmoles of enzyme subunit	0.28	0.28
DHAP-*l-t* added (cpm)	9.7×10^5	9.6×10^5
μmoles of NADH formed	0.25[b]	0.29[b]
DHAP-*l-t* recovered (cpm)	8.4×10^5[c]	8.6×10^5[c]
NAD^{3}H recovered (cpm)	1.5×10^5	1.2×10^5
Total ^{3}H recovered (cpm)	9.9×10^5	9.8×10^5
μg/atoms ^{3}H transferred	0.15	0.12
μg-atoms ^{3}H transferred per μmole of NADH formed	0.60	0.41

[a] The composition and fractionation of each of the reaction mixtures is described in the text. [b] The concentration of NADH was determined with the use of the molar extinction coefficient of 6.2×10^3. [c] Recovered as an equilibrium mixture of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate.

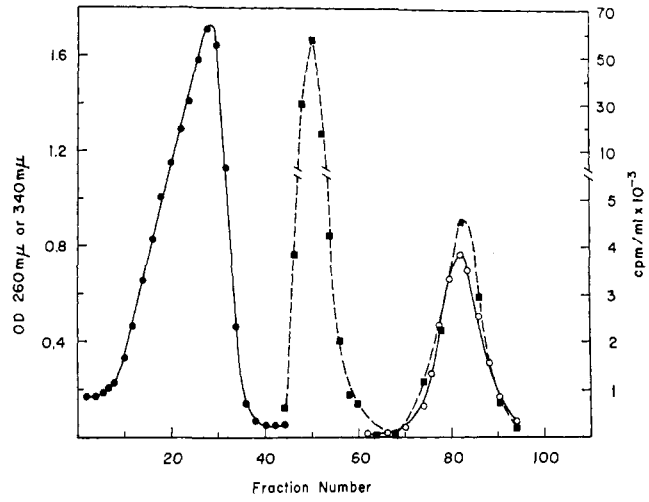

FIGURE 2: Fractionation of the products of the one-turnover reaction on DEAE-Sephadex. The pooled fractions 32–58 illustrated in Figure 1 were applied to a 1.5 × 20 cm column of DEAE-Sephadex A25 which was equilibrated with 0.01 M NH_4HCO_3. The column was washed with a linear bicarbonate gradient with the use of a mixing chamber containing 400 ml of 0.01 M NH_4HCO_3 and a reservoir chamber containing 400 ml of 0.7 M NH_4HCO_3. The effluent was collected in 4.0-ml fractions. (●) Optical density at 260 mμ; (○) optical density at 340 mμ; and (■), ^{3}H counts per minute per milliliter.

3-phosphate dehydrogenase after removal of the bound NAD with charcoal or after treatment of the enzyme with sulfhydryl reagents to destroy the absorption band that is characteristic of the active enzyme–NAD complex (Racker and Krimsky, 1952) failed to reveal unusual absorption characteristics in the near-ultraviolet region (W. S. Allison, unpublished experiments, 1964). Since the mechanism described by eq 3 is unlikely, it is quite probable that the hydride transfer catalyzed by glyceraldehyde 3-phosphate dehydrogenase is direct.

Figure 2 illustrates the fractionation of NAD, NAD^{3}H, and unreacted DHAP-*l-t* in the radioactive peak shown in Figure 1 which was obtained by anion-exchange chromatography on DEAE-Sephadex. Table I summarizes the product analysis of both one-turnover reactions. In both experiments all of the radioactivity introduced as DHAP-*l-t* was recovered as the equilibrium mixture of DHAP-*l-t* plus GAP-*l-t*, and NAD^{3}H. The material in the first radioactive peak in Figure 2 was identified as the equilibrium mixture of DHAP-*l-t* and GAP-*l-t* by assay with lobster muscle glyceraldehyde 3-phosphate dehydrogenase in the presence and absence of triosephosphate isomerase.

The hydride-transfer reactions proceeded with isotope discrimmination as is shown in Table I. The ratio of the microgram atoms of ^{3}H transferred to the amount of NADH which was observed to be formed spectrophotometrically was 0.60 at pH 7.8 and was 0.41 at pH 10.0.

Experiments with Yeast Alcohol Dehydrogenase. Tritium was incorporated into yeast alcohol dehydrogenase after incubation with ethanol-1-*t* and NAD followed by denaturation with urea as described in Materials and Methods. Table II shows that 0.050 and 0.055 μg-atom of tritium were associated with each μmole of enzyme subunit present in the duplicate reaction mixtures which could not be removed by exhaustive dialysis.

Figure 3 shows that the tryptic digest of the yeast alcohol dehydrogenase which was labeled with tritium could be resolved into at least two radioactive components which absorbed at 280 mμ. One of the major radioactive peaks which absorbed at 280 mμ was not retained on Sephadex G-25. This material, in the separate digests was fractionated further by gel filtration on Sephadex G-50 in expt 1 and by paper electrophoresis at pH 6.5 in expt 2. In expt 1 the material in fractions 51–70 was freeze-dried and then applied to a 3.0 × 100 cm column of Sephadex G-50 and was eluted with 0.05 *m* NH_4OH. The peptides in this peak were fractionated into four peaks

TABLE II: Summary of the Yeast ADH Labeling Experiments.

	Expt 1	Expt 2
Ethanol-*l-t;* cpm/μmole (stereospecific)	3.2×10^6	2.5×10^6
cpm of ^{3}H incorporated	30,860	25,990
μg-atoms of ^{3}H incorporated	9.6×10^{-3}	11.0×10^{-3}
μg-atoms incorporated per μmole of ADH subunit	6.2×10^{-3}	7.9×10^{-3}
Total ^{3}H in peak I, cpm[a]	12,650	8460
μg-atoms of ^{3}H in peak I	4.0×10^{-3}	3.5×10^{-3}
μg-atoms of ^{3}H in peak I per μmole of ADH subunit	3.0×10^{-3}	2.6×10^{-3}
Total ^{3}H in peak II, cpm[b]	9270	6625
μg-atoms of ^{3}H in peak II	2.9×10^{-3}	2.9×10^{-3}
μg-atoms of ^{3}H in peak II per μmole of ADH subunit	2.1×10^{-3}	2.1×10^{-3}

[a] Peak I is the radioactive material which was not retained by Sephadex G-25 as shown in Figure 1. [b] Peak II is the radioactive material which was retained by Sephadex G-25 as shown in Figure 1.

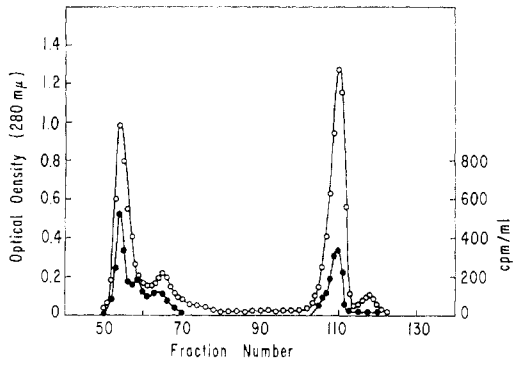

FIGURE 3: Fractionation of the radioactive tryptic digest of yeast ADH on Sephadex G-25. The tryptic digest, prepared as described in Materials and Methods, was applied to a 2.5 × 100 cm column of Sephadex G-25 in 0.05 M NH_4OH and was eluted with the same solvent. The effluent was collected in 5.0-ml fractions (○), optical density at 280 mμ; (●), ^3H counts per minute per milliliter.

which absorbed at 280 mμ by this procedure. However, not one of these peaks contained tritium radioactivity which was significantly above background. In expt 2 the material in fractions 51–61 was freeze-dried and applied to a sheet of Whatman No. 3MM chromatography paper in a 10-cm band. The paper was subjected to electrophoresis at pH 6.5 for 50 min at 2.5 kV. After electrophoresis a guide strip was stained with the Ehrlich reagent to detect tryptophan peptides while the remainder of the strip was monitored for radioactivity with the use of a Tracerlab 4π strip scanner. While three tryptophan-positive peptides were detected by the Ehrlich reagent, no radioactivity was detected by strip scanning.

Since the material in the larger radioactive peak shown in Figure 3 could be separated into peptide components which contained tryptophan by both gel filtration on Sephadex G-50 which did not contain detectable radioactivity, it is concluded that the radioactivity in this peak does not represent tritium which was specifically incorporated into a tryptophan residue.

The peptide material in the peak which was retained on Sephadex G-25 shown in Figure 3 was fractionated further by high-voltage paper electrophoresis. Table III summarizes the characteristics of a tryptophan-positive, radioactive peptide that was purified by a combination of high-voltage paper electrophoresis at pH 6.5 and 3.5. After electrophoresis at pH 6.5 strip scanning revealed the presence of a single radioactive band which migrated with a tryptophan-positive peptide which was acidic. This material was cut out as a band and was resubmitted to electrophoresis at pH 3.5. After the second electrophoresis step radioactivity could not be located on the paper with the use of a Tracerlab 4π strip scanner operating at its highest sensitivity. However, an acidic peptide which contained tryptophan was located on a guide strip with the use of the Ehrlich reagent. After elution 10% of the material was subjected to scintillation counting and was found to contain a small amount of tritium as shown in Table III. The remainder of the peptide, which was obtained in both expt 1 and 2 was hydrolyzed at 108° with constant-boiling HCl in evacuated, sealed tubes (Moore and Stein, 1963). The hydrolysate from expt 1 was subjected to high-voltage paper electrophoresis at pH 1.9 (Ambler, 1963). The amino acids in the peptide were qualitatively identified after electrophoresis with the use of the collidine–ninhydrin reagent (Bennett, 1967). Qualitatively, the amino acid composition of the peptide was Lys^+,Asp^+,Thr^+,Ser^{2+},Glu^{2+},Gly^{4+},Ala^+,CM-Cys^+,Leu^+,-Ile^+. The hydrolysate from expt 2 was subjected to amino acid analysis on a Spinco 120B amino acid analyzer which was equipped with 6.6-mm flow cells. With the exception of the glycine peak, the amino acid peaks were barely above the base line and could not be calculated with certainty. The qualitative composition of the peptide obtained in expt 2 was Lys^+,Asp^+,Thr^+,Ser^{2+},Glu^{2+},Gly^{4+},Ala^+,CM-Cys^+,Leu^+,-Ile^+.

TABLE III: The Characteristics of a Peptide Derived from Yeast ADH that Contains Tritium and Tryptophan.

	Expt 1	Expt 2
Mobility relative to Asp at pH 6.5[a]		
Radioactivity[b]	0.64	0.56
Tryptophan[c]	0.64	0.56
Mobility relative to Asp at pH 3.5[d]		
Radioactivity[b]	Not detected	Not detected
Tryptophan[c]	1.57	1.34
^3H radioactivity after elution (cpm)	625	625

[a] Electrophoresis was carried out at pH 6.5 for 50 min at 2.5 kV with the use of the buffer described by Ambler (1963). [b] Radioactivity was determined with the use of a Tracerlab 4π Strip Scanner. [c] Tryptophan was detected on a guide strip with the use of the Ehrlich reagent. [d] After electrophoresis at pH 6.5 the radioactive peptide was cut out, sewn onto another sheet of Whatman No. 3MM, and subjected to electrophoresis at pH 3.5 for 50 min at 2.5 kV with the use of the buffer described by Ambler (1963).

Experiments with Lactate Dehydrogenase. Table IV summarizes the reaction conditions which were employed in attempts to incorporate tritium into lactate dehydrogenase derived from D,L-lactate-*2-t*. These experimental conditions are similar to those described by Schellenberg (1967) under which he demonstrated the incorporation of 0.59 atom of ^3H/molecule of rabbit muscle LDH. The enzymes in the reaction mixtures described in Table IV were denatured by the addition of urea to 8 M and then subjected to gel filtration on Sephadex G-25 in 8 M urea as described in Materials and Methods. Tritium was not incorporated into the denatured enzyme in any of the experiments.

NAD and hydrazine were added to some of the reaction mixtures as indicated to promote tritium incorporation if the postulated tryptophan-mediated hydride-transfer reaction were correct. In expt 1 and 2 the protein which was isolated from the Sephadex G-25 column was dialyzed, freeze-dried, hydrolyzed with 6 M HCl in evacuated, sealed tubes, and counted. This procedure failed to reveal the incorporation of tritium into the enzyme.

Discussion

The analyses of the products of the one-turnover reactions described for glyceraldehyde 3-phosphate dehydrogenase clearly indicate that the hydride transfer catalyzed by this dehydrogenase is direct and not tryptophan mediated. Since glyceraldehyde 3-phosphate dehydrogenase catalyzes the

TABLE IV: Summary of the Reaction Conditions Employed in Attempts to Label Lactate Dehydrogenase.

Expt	LDH	Enzyme Present (mg)	D,L-Lactate-2-*t* (μmoles)	NaEDTA (pH 10.2, μmoles)	NAD (μmoles)	Hydrazine (μmoles)	Vol (ml)
1	Chicken H_4[a]	11.2	1.7	50			0.30
2	Chicken H_4[a]	11.2	1.7	50	~3		0.30
3	Dogfish M_4[b]	2.1	1.7	50			0.30
4	Dogfish M_4[b]	2.1	1.7	50	~3		0.30
5	Dogfish M_4[b]	2.1	1.7	50		100	0.35
6	Dogfish M_4[b]	2.1	1.7	50	~3	100	0.35
7	Dogfish M_4[b]	3.5	1.7	170	~3	100	0.35
8	Rabbit M_4[c]	11.2	1.7	170			0.30

[a] The specific activity of the chicken H_4 LDH was 200 U/mg in the units described by Pesce *et al.* (1967). [b] The specific activity of the dogfish M_4 LDH was 900 U/mg in the units described by Pesce *et al.* (1967). [c] The specific activity of the rabbit M_4 LDH was 500 U/mg in the units described by Pesce *et al.* (1967).

oxidative phosphorylation of an aldehyde to form an acyl phosphate *via* a thiol ester intermediate, it may be argued that the reaction pathway for the hydride transfer catalyzed by this dehydrogenase is not the same as the reaction pathways catalyzed by alcohol, lactate, and malate dehydrogenases. In the latter reactions carbonyl groups and alcohols are interconverted during hydride transfer without the formation of covalent enzyme–substrate intermediates. However, Wang and Gabriel (1970) have provided evidence that the hydride transfers catalyzed by thymidine disphosphate–D-glucose oxidoreductase are direct. This enzyme catalyzes the conversion of TDP–glucose into TDP–4-keto-6-deoxyglucose, a reaction which proceeds with the alternate reduction and oxidation of enzyme-bound pyridine nucleotide coenzyme. In the first half-reaction TDP–glucose is oxidized to TDP–4-ketoglucose and enzyme bound NADH is produced. In the second half-reaction TDP–4-keto-5,6-glucoseen, arising from TDP–4-ketoglucose by spontaneous β elimination and rearrangement, is reduced by enzyme-bound NADH to form TDP–4-keto-6-deoxyglucose. Wang and Gabriel (1970) have also shown that TDP–6-deoxy-D-glucose, a structural analog of the substrate, can be oxidized in the first half-reaction to form TDP–4-keto-6-deoxyglucose and enzyme-bound NADH. Since TDP–4-keto-6-deoxyglucose cannot participate in the second half-reaction, enzyme-bound NADH accumulates. By carrying out this reaction with TDP–6-deoxy-D-glucose-*t* enzyme-bound NAD^{3}H was formed directly. That the hydride transfer proceeded directly was shown by dissociating the enzyme–NAD^{3}H complex by heat and then separating the denatured enzyme from the NAD^{3}H by gel filtration.

The results of our experiments with yeast ADH agree, in part with those reported by Schellenberg (1965). We have observed the incorporation of tritium into the enzyme derived from ethanol-*1-t*, albeit the efficiency of the incorporation is much lower than that reported by Schellenberg (1965). When considered in terms of a tryptophan-mediated hydride-transfer mechanism, more than half of the radioactivity was nonspecifically incorporated. This is the radioactivity which was associated with the peptide components in the tryptic digests, which were not retained by Sephadex G-25.

The observation that the tritium in the peptide peak which was retained by Sephadex G-25 migrated as a single radioactive component which gave a tryptophan-positive reaction with the Ehrlich reagent when submitted to electrophoreses at pH 6.5, indicates that some of the tritium bound to the enzyme may have been incorporated into a tryptophan residue. The extremely low yield of this peptide which was obtained by elution after the purification procedure described indicates that the peptide was a minor component in the tryptic digest of the yeast ADH. Such a minor component could represent a tryptic peptide of a protein contaminant of the yeast ADH, or represent a tryptic peptide of ADH which contained a tryptophan residue the indole ring of which had been partly oxidized prior to or after the incubation of the yeast ADH with ethanol-*1-t* at pH 11.0.

It is surprising that we could not repeat the labeling of any of the lactate dehydrogenases with tritium derived from lactate-*2-t*. If 0.01 g-atom of ^{3}H had been incorporated per 36,000 g of LDH, it would have been detected in the experiments described.

Since the hydride-transfer reactions catalyzed by glyceraldehyde-3-phosphate dehydrogenase and thymidine diphosphate–D-glucose oxidoreductase are direct, and no evidence has been obtained to indicate that a specific tryptophan residue participates in the hydride transfer catalyzed by yeast alcohol dehydrogenase, we conclude that direct hydride transfer is a general mechanism for pyridine nucleotide linked dehydrogenases. This conclusion is supported by recent observations in several laboratories. Mildvan and Weiner (1969) have suggested a model for the ternary complex of liver alcohol dehydrogenase with NAD and ethanol. The model is based on studies with a spin-labeled analog of NAD. In the model it is difficult to fit a tryptophan side chain between the NAD and ethanol in an orientation favorable for tryptophan-mediated hydride transfer. L. Bernstein (personal communication, 1971) has provided convincing evidence that the mitochondrial malate dehydrogenase isolated from chicken heart does not contain tryptophan. Allen and Wolfe (1970) have not been able to confirm that tryptophan residues of the supernatant malate dehydrogenase are labeled with tritium under conditions described by Chan and Schellenberg (1968).

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Bovine Pepsinogens and Pepsins. III. Composition and Specificity of the Pepsins*

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ABSTRACT: This investigation is concerned with the properties of several pepsins derived from bovine fundic mucosa. The bovine pepsins do not appear to differ among themselves in their amino acid composition, but differences previously found in organic phosphate content are confirmed. Bovine pepsins show similarity in composition to porcine and human pepsins with respect to the large number of acidic and small number of basic residues. However, the content of individual amino acids varies considerably among these pepsins. Bovine pepsins, like human pepsin, have no lysine.

The bovine pepsins do not differ from each other in activity. Comparative studies with porcine pepsin showed rather marked quantitative differences. Both species have similar pH optima. Bovine pepsins have about 25 and 40% of the activity of porcine pepsin toward *N*-acetyl-L-phenylalanyl-L-diiodotyrosine and *N*-acetyl-L-phenylalanyl-L-tyrosine, respectively. Bovine pepsins showed very little activity toward benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-tryptophan ethyl ester, a good substrate for porcine pepsin. Bovine pepsins have 60–70% of the activity of porcine pepsin with hemoglobin as substrate. Bovine and porcine pepsins have similar milk clotting action. The phosphate content has no effect on the activity of the bovine pepsins on any of the substrates tested.

Bovine pepsin was isolated from gastric juice and crystallized by Northrop (1933). Bovine pepsinogen was isolated by Chow and Kassell (1968). Previous studies from this laboratory have demonstrated the large number of differences in amino acid composition between bovine and porcine pepsinogen (Chow and Kassell, 1968) and the constancy of composition, except for changes in organic phosphate, among the major and minor bovine pepsinogens (Meitner and Kassell, 1970). The present report extends these studies to the bovine pepsins prepared from purified pepsinogen. It includes the composition of the major and minor pepsins and their action on proteins and synthetic substrates.

Experimental Section

Substrates. *N*-Acetyl-L-phenylalanyl-L-diiodotyrosine, *N*-acetyl-L-phenylalanyl-L-tyrosine, and benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-tryptophan ethyl ester were products of Cyclo Chemical Corp., Los Angeles, Calif., and were reported by the manufacturer to be chemically pure by chromatography in three different solvent systems.

Enzymes. Crystalline porcine pepsinogen (lot PG 117) was obtained from Worthington Biochemical Corp., Freehold, N. J. Porcine pepsin used for comparative studies was prepared by activation of crystalline porcine pepsinogen in a manner similar to that of Rajagopalan *et al.* (1966).

Activation of Bovine Pepsinogen and Chromatographic Separation of the Pepsins on Hydroxylapatite. Bovine pepsinogen was purified according to the method of Chow and Kassell

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