requirement. Inorganic phosphate or glucose alone, or inorganic phosphate plus glucose together did not activate. Phosphate bound to the hydroxyl of carbon-1 of glucose, fructose, or galactose markedly decreased activation. Glucose-1-phosphate, one of the poorest activators, inhibited at all concentrations of glucose-6-phosphate tested. The inhibition was not competitive

We cannot explain the anomalous lack of activation by erythrose-4-phosphate when other molecules of shorter chain length, such as methyl phosphate and pyrophosphate, did activate. A similar case was observed with 3-phosphohydroxypyruvic acid. No activation was detected with this compound, although 3-phosphoglyceric acid gave a considerable stimulation. The activation by triosephosphate derivatives (with the exception of 3-phosphoglyceric acid) was significant.

Studies with two activators of medium potency such as 2-deoxyglucose-6-phosphate and ribose-5-phosphate were of interest. When these two activators were tested in the presence of glucose-6-phosphate, a competitive inhibition was shown only at the highest concentrations of glucose-6-phosphate. At lower concentrations of glucose-6-phosphate, the activation which they produced increased the activation produced by glucose-6-phosphate. It is possible that this interpretation may also be applied to the activation that was detected with fructose-6-phosphate at low concentrations of glucose-6-phosphate.

The data presented in Table II show a close agreement of the K_i values for all the inhibitors tested (with the exception of inorganic sulfate). Despite the fact that each of the compounds tested had a different half-maximal activation value, the K_i values were very similar. This finding is in keeping with the idea that the K_i reflects the attachment of the phosphate portion of the compound to the enzyme.

The K_i for sulfate was higher than that of phosphate or of the other phosphate compounds tested. It appears that inorganic phosphate may fit best at that site of activation on the enzyme where the phosphate of the sugar phosphate is attached.

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The Amino Acid Sequence of a Hexapeptide Containing an Essential Sulfhydryl Group of Rabbit Muscle Glyceraldehyde-3-Phosphate Dehydrogenase*

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The preferential reactivity of the essential sulfhydryl groups of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase with the yellow sulfhydryl reagent, *N*-(4-dimethylamino-3,5-dinitrophenyl)-maleimide (DDPM) has been utilized for the isolation of peptides containing these groups. After pepsin digestion of the DDPM-treated enzyme, the label was contained predominantly in a single hexapeptide of sequence ala-ser-(DDPM-cys)-thr-thr-aspNH_2. It is thus concluded that the structure of the several active sites of the enzyme are identical, at least in part, and that the reactive sulfhydryl groups are not components of glutathione moieties.

The elucidation of the chemical mechanism of action of an enzyme requires the characterization of transition states, which in turn depends upon a knowledge of the participating atoms in the enzyme molecules and their geometry. The determination of amino acid residues in the active sites of enzymes can provide some information in this regard.

Serine-specific reagents, such as DFP,[1] have been successfully employed for the labeling and isolation of peptide fragments containing part of the catalytically active centers of several proteases and esterases (reviewed in Koshland, 1960). Experiments with a variety of sulfhydryl-specific reagents have demonstrated that the activities of a large number of enzymes are dependent upon the presence of free sulfhydryl groups. Recently the use of DDPM has been de-

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[1] Abbreviations are: DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)-maleimide; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl; PTC, phenylisothiocyanate; PTH, phenylthiohydantoin; DFP, diisopropylphosphorofluoridate; EDTA, ethylenediaminetetraacetic acid.

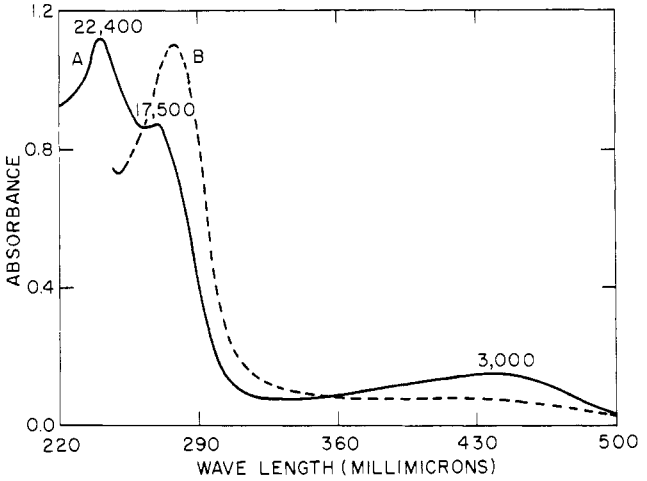

FIG. 1.—Absorption spectra of DDPM-*N*-acetylcysteine (curve A) and of alkylated enzyme (curve B). (A) DDPM-*N*-acetylcysteine was dissolved in 0.1 M $NaPO_4$, *p*H 7.0, to give a final concentration of 5×10^{-5} M. The molar extinction coefficients of the peaks at 240 mμ, 270 mμ, and 440 mμ are given on the figure. (B) The enzyme was treated with 3.3 equivalents of DDPM, then precipitated with ammonium sulfate, as described in the text. The precipitate was washed with saturated ammonium sulfate solution, then dissolved in 0.1 M $NaPO_4$, *p*H 7.0, containing 0.01 M EDTA. Spectra were measured with a Beckman DU spectrophotometer in the laboratory of Professor Hans Tuppy, who kindly provided the sample of DDPM-*N*-acetylcysteine.

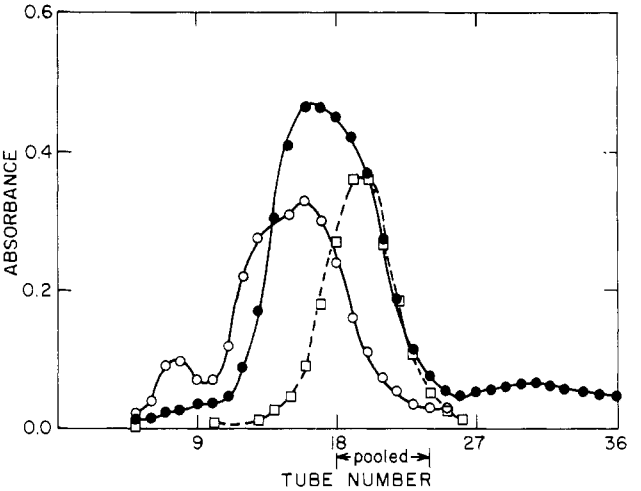

FIG. 2.—Sephadex G-25 chromatography of first pepsin digestion mixture. Fractions (20-ml) were collected. □, 440 mμ absorbance; ○, 470 mμ (ninhydrin) absorbance; ●, 280 mμ absorbance. The 280-mμ-absorbancy values were divided by 10 for plotting.

veloped for the labeling of sulfhydryl groups and for the isolation of sulfhydryl-containing peptides from serum albumins and tobacco mosaic virus protein (Witter and Tuppy, 1960; Tuppy, 1961). The yellow color of this reagent greatly facilitates the purification of labeled peptides. In addition, the adsorbability of DDPM-peptides on talc provides a rapid and convenient method for separating the labeled material from the bulk of the contaminating peptides in a single step. In a preliminary communication we described the isolation of a peptide from the active center of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase with the use of DDPM (Segal and Gold, 1963). This report presents further details of these experiments and the amino acid sequence of this peptide. During the course of our investigations, Harris *et al.* (1963) briefly described the amino acid sequence of a tryptic peptide of the enzyme labeled with radioactive iodoacetate or *p*-nitrophenyl acetate, which appears to contain the peptic peptide discussed in the present study. Cunningham and Schepman (1963) have isolated a tripeptide from *p*-nitrophenyl acetate–labeled enzyme which may be a component of the hexapeptide described here.

EXPERIMENTAL AND RESULTS

Materials—Crystalline rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, DFP-treated carboxypeptidase A, pepsin, and DL-glyceraldehyde-3-phosphate, as the barium salt of the diethyl acetal derivative, were obtained from Sigma Chemical Co., St. Louis, Mo. DDPM was kindly supplied by Professor H. Tuppy. DDPM-cysteine was prepared as described by Witter and Tuppy (1960). The purest quality of FDNB and PTC were obtained from Eastman Organic Chemicals. Anhydrous hydrazine was obtained from Matheson, Coleman and Bell.

Enzyme Assay.—Glyceraldehyde-3-phosphate dehydrogenase activity was determined by measuring the rate of DPN reduction at 340 mμ with a Cary recording spectrophotometer. The 3-ml reaction mixture contained 18 μmoles DPN, 45 μmoles Na_2HAsO_4 (adjusted to *p*H 8.5), 18 μmoles DL-glyceraldehyde-3-phosphate, 30 μmoles sodium pyrophosphate, *p*H 8.5, and 18 μmoles, EDTA *p*H 7. A suitable dilution of the enzyme was added to the mixture to initiate the reaction. The specific activity of the crystalline enzyme was comparable to the highest values reported in the literature when assayed under the prescribed conditions (Koeppe *et al.*, 1956). In the absence of cysteine, approximately 75% of maximum activity was observed.

Preparation of DDPM-peptide.—The ammonium sulfate suspension of the enzyme was centrifuged at 10,000 rpm for 20 minutes. The pellet was dissolved in cold 0.1 M pyrophosphate buffer, *p*H 6.7, containing 0.01 M EDTA to an enzyme concentration of approximately 10^{-4} M. A value of ϵ_{280} of 1.43×10^5 (M) $(cm)^{-1}$ (Koeppe *et al.*, 1956; Velick *et al.*, 1953) was used for enzyme molarity calculations, and a value of 135,000 (Velick, 1958) for molecular weight. To label the reactive sulfhydryl groups, an aliquot of a 50 mM solution of DDPM in methyl Cellosolve was added to the enzyme at a molar ratio of 3.3 moles of DDPM per mole of glyceraldehyde-3-phosphate dehydrogenase. Alkylation of the reactive sulfhydryl groups was allowed to proceed for 2.5 hours at 0°. A comparison of activity before and after alkylation indicated that DDPM treatment of the enzyme resulted in a loss of 86–98% of activity (Segal and Gold, 1963). The absorption spectra of the alkylated enzyme and of DDPM-*N*-acetylcysteine are presented in Figure 1.

Solid ammonium sulfate was added to the DDPM-enzyme solution to 90% saturation and the mixture was centrifuged at 10,000 rpm for 20 minutes. The bright-yellow pellet was dissolved in cold deionized water and dialyzed against several changes of water overnight. The *p*H of the yellow dialyzed solution was adjusted to 2 with dilute HCl, and pepsin, equivalent to 2% of the calculated weight of glyceraldehyde-3-phosphate dehydrogenase and dissolved in 0.01 N HCl, was added to the solution. The mixture was allowed to incubate at 37° for 24 hours with periodic adjustment to *p*H 2. The solution was evaporated to a volume of 10–15 ml in a rotary flash evaporator and applied to a G-25 Sephadex column, 5 × 26 cm, previ-

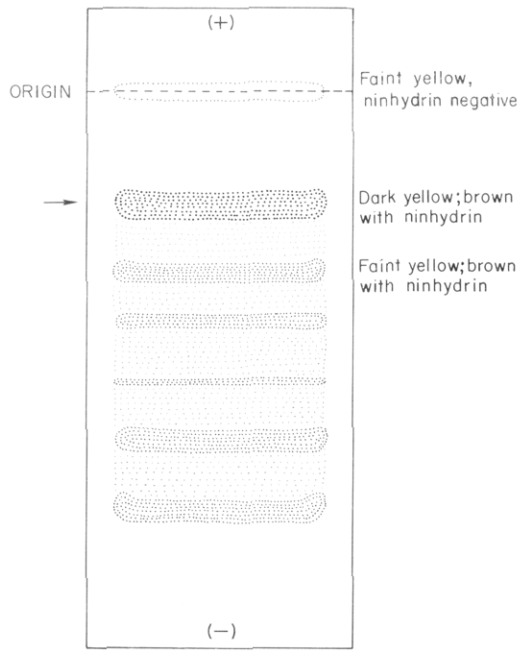

FIG. 3.—Ionophoresis (*p*H 2) of the talc eluate. Ionophoresis was at 5 kv for 3 hours. The main yellow band, indicated by the arrow, was cut out and the rest of the paper was dipped in 0.25% ninhydrin in acetone (Silman *et al.*, 1962). The ninhydrin-positive bands are shown in heavy dots. The light shading represents a background of ninhydrin-positive material.

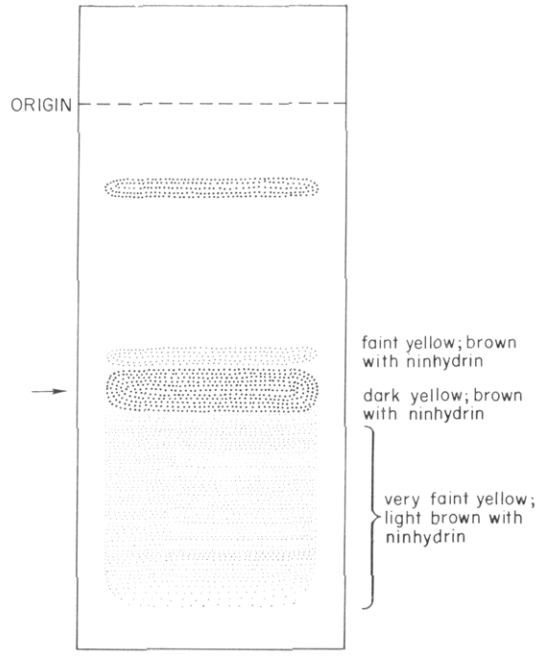

FIG. 4.—Paper chromatography of major yellow band from *p*H 2 ionophoresis. The chromatogram was developed for 24 hours. The major yellow band, indicated by the arrow, was cut out and the rest of the paper was dipped in 0.25% ninhydrin in acetone (Silman *et al.*, 1962). The ninhydrin-positive bands are shown in heavy dots. The light shading represents a background of ninhydrin-positive material.

ously washed with water. Fractions of 20 ml each were eluted with water. Ninhydrin analysis (Troll and Cannon, 1953) and 280 mμ and 440 mμ absorbancy determinations (absorption maximum of DDPM-*N*-acetylcysteine, Fig. 1) were performed (Fig. 2). There was a retardation of the 440-mμ-absorbing material with respect to the ninhydrin-positive and 280-mμ-absorbing substances. Approximately 50–60% of the 440-mμ-absorbing material was collected, containing about 10% of the original ninhydrin value. The fractions were pooled, evaporated to 25–30 ml, and subjected to a second pepsin digestion with a quantity of the protease equivalent to 1% of the originally calculated weight of glyceraldehyde-3-phosphate dehydrogenase. The solution was then evaporated to a 5-ml volume, placed on a talc column, 2 × 16 cm, washed with several column volumes of water, and eluted with a 50% ethanol solution containing 2% acetic acid (Witter and Tuppy, 1960). In a typical experiment 86% of the 440-mμ-absorbing material was recovered from the talc column with only 18% of the ninhydrin-positive material applied. The ethanol–acetic acid eluate was evaporated to dryness, spotted on Whatman No. 3 MM paper, previously dampened with acetic acid–formic acid buffer, *p*H 2,[2] and subjected to paper ionophoresis at a 5-kv potential as described by Atfield and Morris (1961). A major ninhydrin-positive yellow band, along with two minor yellow bands, and a considerable quantity of colorless ninhydrin-positive material were obtained, as shown in Figure 3. The major yellow band was cut from the paper and the material was eluted with a 10% acetic acid solution. The eluate was evaporated to dryness over P_2O_5 *in vacuo*, and subjected to descending paper chromatography with butanol–acetic acid–water (4:1:5) (Fig. 4). The central portion of the resulting major band of yellow material was eluted from the paper with 10% acetic acid solution, evaporated to dryness over P_2O_5 *in vacuo*, and subjected to descending paper chromatography with butanol–acetic acid–water (4:1:5) (Fig. 4). The central portion of the resulting major band of yellow material was eluted from the paper with 10% acetic acid solution, evaporated to dryness over P_2O_5 *in vacuo*, and subjected to paper ionophoresis at *p*H 4.7.[2] After ionophoresis, a single yellow band was present at the origin and no other ninhydrin-positive material appeared. The ionophoretic behavior of the yellow peptide at *p*H 4.7 indicates that it is a neutral compound. Based on a value of ϵ_{440} of 3000 $(M)^{-1}$ $(cm)^{-1}$ for DDPM-N-acetylcysteine (Fig. 1), the 400 mμ absorbance of the purified material indicated a yield of about 5% of DDPM-peptide from the original starting material.[3]

Amino Acid Analysis.—The isolated peptide was hydrolyzed in 5.7 N HCl containing 1% thioglycolic acid at 105° for 24 hours and analyzed on a Moore-

[2] The *p*H 2 ionophoresis medium contained 15.5 ml of 88% formic acid and 66.5 ml of glacial acetic acid per liter. The *p*H 4.7 ionophoresis medium contained 10 ml of pyridine and 10 ml of glacial acetic acid per liter.

[3] Although the final yield of pure peptide was low, it was clear from the several purification steps that no labeled peptide was present other than the trace contaminants shown in Figures 3 and 4. In the Sephadex chromatography, some 40–50% of the yellow material was discarded with 90% of the ninhydrin-positive material. Since the yellow peptides emerged as a single symmetrical peak (Fig. 2), it may be assumed that the fraction taken for further purification was a representative sample of the total material. Also in the ionophoretic and chromatographic steps, in an intentional sacrifice of yield for purity, only the central portion of the main yellow band was eluted. It may be mentioned that Witter and Tuppy (1960) and Tuppy (1961) have shown that, owing to the formation of rearrangement products of DDPM-cysteine moieties in peptides, a major component and two minor components of a single DDPM-peptide characteristically appear on ionophoretograms.

Stein amino acid analyzer.[4] The composition of the peptide, as reported earlier (Segal and Gold, 1963), was (ala, ser, DDPM-cys, thr_2, $aspNH_2$).[5,6] A rapid semiquantitative amino acid analysis was performed by subjecting an acid hydrolysate of the peptide to *p*H 2 paper ionophoresis together with a standard solution containing alanine, serine, threonine, and aspartic acid. The paper was developed with a solution of 0.25% ninhydrin in acetone, then warmed at 60° for 10 minutes, and the ninhydrin-positive spots were cut out. The blue material was eluted with a 65% ethanol solution and the 570-mμ absorbance was determined (Silman *et al.*, 1962). The relative ratios of 570-mμ-absorbing material were similar to the molar ratios of amino acid residues determined with the Moore-Stein amino acid analyzer. The peptide was devoid of tryptophan as determined by the method of Spies and Chambers (1949).

NH_2-terminal Amino Acid by the FDNB Method.—The amino-terminal residue of the peptide was determined by the FDNB method of Sanger as described by Fraenkel-Conrat (1955). The DNP-peptide was hydrolyzed in constant-boiling HCl (5.7 N) for 2–3 hours. The DNP-amino acid was extracted with ether and subjected to descending paper chromatography with 1.5 M potassium phosphate buffer, *p*H 6, as the developing solvent. The chromatographic procedure resulted in the appearance of a single yellow spot corresponding to the position of DNP-ala. Since histidine, arginine, and lysine were not component residues of the peptide, the aqueous fraction was not examined for DNP-amino acids. The results of the amino acid analysis and the NH_2-terminal residue determination indicated that the isolated peptide is a single hexapeptide with an NH_2-terminal alanine.

NH_2-terminal Sequence by the PTC Method.—The sequential arrangement of the first three amino acid residues at the amino-terminal end of the peptide was determined by a modification of the Edman reaction as described by Konigsberg and Hill (1962). The procedure employed the semiquantitative estimation of amino acid residues remaining after each cycle of the reaction rather than the chromatographic isolation of the PTH-amino acid derivatives. To 0.3 μmole of the peptide in water, 0.3 ml of 1% PTC in pyridine and 50 μl of 25% triethylamine were added. After a reaction period of 3 hours at 37°, the excess PTC was removed with several extractions with thiophene-free benzene and the aqueous fraction was evaporated to dryness over P_2O_5 *in vacuo*. The dried residue was suspended in 1 ml of trifluoroacetic acid and allowed to stand at 25° for 1 hour for cyclization and cleavage of the PTH-amino acid. The trifluoroacetic acid was removed in a vacuum dessicator over P_2O_5, the dried residue was suspended in 1 ml of 0.2 M HCL, and the PTH-amino acid was extracted with thiophene-free benzene. The aqueous layer was dried, resuspended in a known volume of water from which an aliquot was withdrawn, dried, dissolved in 5.7 N HCl with thioglycollate, and hydrolyzed at 105° for 24 hours. The hydrolysate was subjected to ionophoresis for semi-

TABLE I

FRACTION OF AMINO ACIDS REMAINING AFTER THREE CYCLES OF THE EDMAN DEGRADATION

Amino acid	Cycle[a] 1st	2nd	3rd
Alanine	*0.25*	0.13	0.17
Serine	0.85	*0.18*	0.22
Threonine	0.80	0.82	0.86
Aspartic acid	1.00	0.73	0.84
2-Amino-2-carboxyethylmercaptosuccinic acid[b]	1.00	0.90	*0.40*

[a] The fraction of the amino acid lost in each cycle is italicized. [b] The hydrolysis product of DDPM-cysteine.

quantitative estimation. The remainder of the solution was subjected to subsequent cycles of the PTC reaction. The results of three cycles of the reaction are shown in Table I, where the fraction of 570-mμ-absorbing material remaining for each residue is tabulated after each cycle. From the results, the sequence of amino acid residues at the amino-terminal end of the peptide is ala-ser-(DDPM-cys)-(thr_2, $aspNH_2$).

COOH-terminal Sequence by the Carboxypeptidase Method.—Incubation of the peptide with DFP-treated carboxypeptidase A resulted in the release of threonine and asparagine from the peptide. The method employed was a modification of the procedure described by Fraenkel-Conrat (1955). A suspension of carboxypeptidase A was added to cold H_2O and solubilized by the dropwise addition of 0.1 N NH_4OH. The *p*H was quickly adjusted to 8 with dilute acetic acid. An aliquot of the enzyme solution was added to 0.1–0.2 μmole of an aqueous solution of the peptide such that a molar ratio of substrate to enzyme of 100:1 would result (based on a molecular weight of 34,400 for carboxypeptidase). After an incubation period of 12 hours the reaction was stopped by heating the mixture in a 100° water bath for 1 minute. The mixture was cooled and centrifuged, and the resultant clear supernatant was removed and evaporated to dryness *in vacuo* over P_2O_5. The dried residue was subjected to *p*H 2 paper ionophoresis and compared to a standard solution of threonine and asparagine. Semiquantitative determination of threonine and asparagine indicated a ratio of approximately 2 threonine to 1 asparagine. No other free amino acids were present. Thus carboxypeptidase A fails to cleave the DDPM-cysteinyl bond under these conditions. The results substantiate the conclusion from the amino-terminal-sequence determinations regarding the positions of the DDPM-cysteine residue and the threonine and asparagine residues.

An analysis of the rate of release of asparagine and threonine by carboxypeptidase hydrolysis indicated further that asparagine was the COOH-terminal residue. Incubation of the enzyme with the peptide was allowed to proceed for 0.5-hour and 12-hour periods. The reaction was stopped as described above at the end of each period, and the supernatant fraction of the centrifuged reaction mixture was subjected to quantitative amino acid analysis on the Moore-Stein amino acid analyzer. The results are presented in Table II. It is seen that asparagine is rapidly removed from the peptide followed by a slower release of threonine. At the end of the 12-hour incubation period there is a quantitative yield of both residues from the peptide. These results indicate the COOH-terminal sequence, -thr-thr-$aspNH_2$.

[4] We are indebted to Mr. Mas Yamada of the Botany Department, Washington University, for these analyses.

[5] The hydrolysis product of DDPM-cysteine is 2-amino-2-carboxyethylmercaptosuccinic acid. This compound is eluted before aspartic acid on the Moore-Stein amino acid analyzer.

[6] The presence of asparagine rather than aspartic acid is indicated by the behavior of the peptide as a neutral compound at *p*H 4.7 and is confirmed by the results of the carboxypeptidase and hydrazinolysis experiments (*vide infra*).

TABLE II
CARBOXYPEPTIDASE-CATALYZED RELEASE OF AMINO ACIDS[a]

Amino Acid	Time of Incubation	
	0.5 hr	12 hr
Threonine	0.035	0.190
Asparagine	0.068	0.120

[a] 0.1 μmole of peptide was incubated for each time period. Results are expressed as μmoles of amino acid released.

COOH-terminal Residue by Hydrazinolysis.—Confirmation of the identification of the COOH-terminal residue was effected by subjecting the peptide to hydrazinolysis according to the procedure described by Carlton and Yanofsky (1963). Anhydrous hydrazine was added to 0.1–0.2 μmole of the dried peptide and the solution was heated at 105° for 10 hours in sealed evacuated hydrolysis tubes. The material was removed from the tubes and evaporated to dryness over H_2SO_4 *in vacuo*. The dried material was resuspended in water and the amino acid hydrazides were precipitated by the addition of *iso*-valeraldehyde at 0°. After centrifugation the resultant aqueous layer was removed, extracted with ethyl acetate, and evaporated to dryness over P_2O_5 *in vacuo*. The dried residue was subjected to *p*H 2 paper ionophoresis. No free amino acids were detected. As a control for the procedure, two dipeptides, glycylglycine and glycyl-L-threonine (0.1–0.2 μmole), were subjected to the same treatment. Free glycine and threonine, respectively, were obtained from the hydrazinolyzed dipeptides, as expected. If threonine were COOH-terminal, this residue should be obtained upon hydrazinolysis of the DDPM-peptide. Since COOH-terminal amino acid amides are converted to the respective hydrazides (Carlton and Yanofsky, 1963), the absence of a free amino acid after hydrazinolysis of the peptide indicates that the COOH-terminal residue is asparagine. The results of the COOH-terminal sequence investigations indicate the order -thr-thr-aspNH_2.

DISCUSSION

The ready visual localization of DDPM-peptides on columns and on paper, together with the separability of DDPM-peptides from a large fraction of the contaminating peptides by means of talc columns, offer important advantages of this reagent for the labeling of sulfhydryl peptides. The hydrolysis product of DDPM-cysteine residues is clearly separated from the other amino acids both by ion-exchange chromatography and paper ionophoresis.

Earlier studies with rabbit muscle glyceraldehyde-3-phosphate dehydrogenase had indicated that not all the sulfhydryl groups present in the molecule are essential for activity (Segal and Boyer, 1953; Boyer and Segal, 1954). There is ample evidence for the existence of three or four active sites per molecule, each containing at least one essential sulfhydryl group (Koeppe *et al.*, 1956; Velick, 1953, 1958; Segal and Boyer, 1953; Boyer and Segal, 1954; Racker *et al.*, 1959; Park *et al.*, 1961). The fact that stoichiometric quantities of DDPM reacted preferentially with the sulfhydryl groups at the active centers (Segal and Gold, 1963) greatly facilitated the labeling of these moieties.

The results of these studies and those of Harris *et al.* (1963) demonstrate that the amino acid sequences of the peptide chains containing the sulfhydryl groups are identical in the several active centers, for a chain length of at least eighteen amino acids. This fact, together with the finding of Velick and Udenfriend (1953) of a single species of NH_2-terminal amino acid in the protein (valine), suggest that the enzyme is composed of identical subunits.

The earlier proposal of a glutathione moiety as the sulfhydryl-containing component of the active site (Krimsky and Racker, 1952) is inconsistent with the amino acid sequence studies.

The concentration of hydroxyl amino acids in immediate proximity to the sulfhydryl group participating in the enzymatic reaction is noteworthy. The hydrophilic nature of these groups and the amide group would be expected to orient this portion of the peptide chain on the surface of the protein, consistent with the marked reactivity of the sulfhydryl group with the substrates and with sulfhydryl reagents. In addition they may play a specific role in offering points of attachment of one or another of the three substrates, glyceraldehyde-3-phosphate, DPN, and inorganic phosphate.

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