THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 240, No. 9, September 1965
Printed in U.S.A.

Fructose Diphosphatase from Rabbit Liver

III. NATURE OF THE GROUPS REACTIVE WITH DINITROFLUOROBENZENE*

S. PONTREMOLI, B. LUPPIS, S. TRANIELLO, W. A. WOOD,† AND B. L. HORECKER‡

From the Istituto di Chimica Biologica, Universita di Ferrara, Ferrara, Italy

(Received for publication, January 15, 1965)

In the preceding paper we have described the changes in catalytic properties which result when purified D-fructose 1,6-diphosphatase (EC 3.1.3.11) from rat liver is incubated with 2,4-dinitrofluorobenzene (1). When limiting quantities of DFB[1] are employed, the effect is to produce an increase in activity in the neutral pH range and a general broadening of the pH-activity curve. This is the first report of an increase in activity of an enzyme induced by dinitrophenylation; previously Hirs (2) had reported the inactivation of ribonuclease when it was treated with an excess of DFB, and Rowley, Tchola, and Horecker (3) observed a similar inactivation of aldolase and transaldolase when 1-chloro-2,4-dinitrobenzene was used as the dinitrophenylating agent. With ribonuclease, loss of activity was associated with the dinitrophenylation of lysine residue 41; with transaldolase, inactivation was associated with the dinitrophenylation of 2 lysine residues (4). On the other hand, the inactivation of fructose 1,6-diphosphate aldolase by chlorodinitrobenzene was due to the reaction of approximately 6 cysteine sulfhydryl groups (4). We have now shown that when fructose diphosphatase is treated at pH 9.2 with 4 eq of DFB, 2 cysteine residues and 1 lysine residue are dinitrophenylated. At pH 7.5 the reaction with DFB leads to the dinitrophenylation of 1 cysteine residue (to form *S*-DNP-cysteine); in addition, a small amount of ϵ-DNP-lysine was detected. Evidence is presented to show that the change in catalytic properties is the result of dinitrophenylation of a single cysteine residue. It is suggested that ionization of this cysteine residue is responsible for the conversion of the inactive enzyme at neutral pH to an active form at alkaline pH.

EXPERIMENTAL PROCEDURE

Materials—DFB and other reagents employed were obtained from the sources described in the preceding papers (1, 3, 4). Radioactive DFB labeled with ^{14}C was obtained from the Amersham Radiochemical Centre. The specific activity was 4.37 μC per μmole. Authentic ϵ-DNP-lysine and DNP-glycine were obtained from Calbiochem, and authentic *S*-DNP-cysteine from Gallard-Schlesinger. D-Fructose 1,6-diphosphatase was purified from rabbit liver (5).

Determination of Specific Activity of DFB—In order to check the radiochemical purity and specific activity of the DFB used, a solution containing approximately 0.5 μmole of the radioactive reagent was treated with 3 μmoles of glycine in 0.2 ml of bicarbonate buffer at pH 9.2. After the reaction mixture was incubated at 40° for 90 min, it was extracted three times with 1-ml volumes of ether, and the aqueous solution was then made acid with HCl and extracted with ether. The second ether extract was concentrated and placed on Whatman No. 3MM paper alongside a marker of authentic DNP-glycine. The chromatogram was developed with ascending chromatography with the use of Solvent A (see below). The yellow strip corresponding to DNP-glycine was eluted with 1% ammonia. It was then analyzed spectrophotometrically and counted. The specific activity was found to be 6.1×10^4 cpm per μmole, compared with 5.8×10^4 based on direct determination of the diluted Amersham sample, on the assumption that all the radioactivity present was DFB. It was therefore concluded that the material was radiochemically pure, and that all of the radioactivity in the sample was available for formation of the dinitrophenylated derivatives.

Analytical Procedures—Assays for the hydrolysis of fructose-1,6-di-P and sedoheptulose-1,7-di-P were performed as previously described (1, 5). Radioactivity was measured on planchets with a Selo model LB 1/V low background gas flow counter with a Micromil window. Some difficulty was experienced with loss of volatile DNP-amino acid derivatives, and it was necessary to avoid prolonged heating at high temperature during the drying of the planchets. Chromatography was carried out with Whatman No. 3MM paper, with the use of the following solvent systems: Solvent A, pyridine-isoamyl alcohol-1.6 N ammonia (6:14:20), with the top layer used for development of the chromatogram and the bottom layer for saturation of the atmosphere in the chromatogram tank; Solvent B, butanol-acetic acid-water (4:1:5), with the bottom layer used as the developing solvent.

Protein was determined as described in the preceding paper (5). For the dinitrophenylated protein, in which color interfered with the usual procedures for spectrophotometric analysis, assays were carried out by the ninhydrin reaction after alkaline hydrolysis according to the procedure of Hirs, Moore, and Stein (6). Crystalline serum albumin was used as a standard.

Protein Hydrolysis—Hydrolysis was carried out with 5.7 N HCl at 100° for 16 hours in evacuated sealed tubes. The tubes were removed from the oven and cooled before opening. The

* Supported by grants from the United States Public Health Service (GM 09663), the National Science Foundation (GB 1465 and GB 2648), and the Italian Consiglio Nazionale della Richerche, Impresa Enzimologia.

† Senior Research Fellow, National Institutes of Health. Permanent address, Department of Biochemistry, Michigan State University, East Lansing, Michigan.

‡ Recipient of a travel grant from the American Commission for Cultural Exchange with Italy under the authorization of the Fulbright-Hays Act.

[1] The abbreviations used are: DFB, 2,4-dinitrofluorobenzene; DNP-, dinitrophenyl-.

TABLE I

Dinitrophenylation with DFB-^{14}C and protection by substrate

The incubation mixtures (0.5 ml) contained 0.2 M sodium carbonate buffer at pH 9.2 in Experiments 1 and 2, 0.2 M triethanolamine buffer, pH 7.5, in Experiment 3, and 0.005 mM D-fructose 1,6-diphosphatase (0.325 mg; specific activity, 120 units per ml), and 0.02 mM DFB-^{14}C (4 eq per mole of protein). Where indicated, 0.5 mM fructose-1,6-di-P (FDP) or 5 mM sedoheptulose-1,7-di-P (SDP) were also present. The reactions were carried out in the dark at room temperature with mechanical stirring. Samples were removed at intervals for determination of enzymatic activity at pH 7.5. After 15 to 30 min at pH 9.2, or 80 to 120 min at pH 7.5, when no further changes in enzyme activity were observed, 10% trichloroacetic acid was added, and the sample was chilled and centrifuged for 5 min at 7000 rpm. The precipitates were washed three times by resuspending them in 0.5 ml of 5% trichloroacetic acid. The washed precipitates were dissolved in 0.5 ml of 1 N ammonia. Aliquots were plated, dried, and counted. The effectiveness of washing with trichloroacetic acid for removal excess labeled DFB was established in a control in which the radioactive reagent was added immediately after the first addition of trichloroacetic acid.

Additions	Activity	Radioactivity	Ratio of DNP-^{14}C to enzyme
	units/mg	*cpm/mg*	*moles/mole*
Experiment 1			
None	23		
DFB-^{14}C*	66	607	2.9
DFB-^{14}C + FDP	26	428	2.0
DFB-^{14}C + SDP	60		
Experiment 2			
None	15		
DFB-^{14}C†	70	1,440	2.8
DFB-^{14}C + FDP	21	1,010	2.0
Experiment 3			
None	22		
DFB-^{14}C‡	51	29,500	0.77
DFB-^{14}C + FDP	22	5,900	0.14

* Specific activity, 2.7×10^4 cpm per μmole.
† Specific activity, 6.7×10^4 cpm per μmole.
‡ Specific activity, 5.3×10^6 cpm per μmole.

hydrolysates were evaporated to dryness under reduced pressure in a rotary evaporator, dissolved in water, and again evaporated to dryness; this was repeated approximately five times. The yield of radioactivity after these procedures was variable, but approximately 70% of that present in the original protein was recovered after hydrolysis. Aliquots were acidified and extracted with ether, and the ether extracts were tested for radioactivity to exclude the presence of α-DNP-amino acids which would have been derived from the NH_2-terminal groups.

RESULTS

Number of Groups Dinitrophenylated—When the protein was treated with 4 eq of DFB at pH 9.2, and aliquots of the solution were tested at pH 7.5, we observed the expected increase in specific activity (Table I). In the two experiments shown, the activity at this pH was increased approximately 3-fold in the first case, and nearly 5-fold in the second case. Dinitrophenylation at pH 7.5 was considerably slower than at pH 9.2 and required nearly 2 hours to reach a plateau of enzyme activity. The presence of 0.5 mM fructose-1,6-di-P during the treatment with DFB at either pH prevented the change in catalytic properties, but 5 mM sedoheptulose-1,7-di-P was without effect. When the dinitrophenylated proteins were collected by precipitation with trichloroacetic acid, dissolved in alkali, and counted, it was

TABLE II

Effect of fructose 1,6-diphosphate on rate of dinitrophenylation

Dinitrophenylation was carried out at pH 7.5, as described in the *legend* to Fig. 2, with 4 eq of DFB per mole of enzyme. At 40 min, when the reaction was still linear, aliquots were tested for enzyme activity at pH 7.5 with fructose-1,6-di-P with the use of the spectrophotometric assay (5). Aliquots were also tested for dinitrophenylation, as in the *legend* to Fig. 3.

Fructose 1,6-diphosphate	Activity	Increase	DNP uptake
M	*ΔA/min*	%	*eq/mole*
Control	0.014		0
10^{-3}	0.014	0	0.14
10^{-4}	0.016	14	0.12
10^{-5}	0.026	86	0.31
10^{-6}	0.033	138	0.44
10^{-7}	0.037	160	0.48
0	0.037	160	0.47

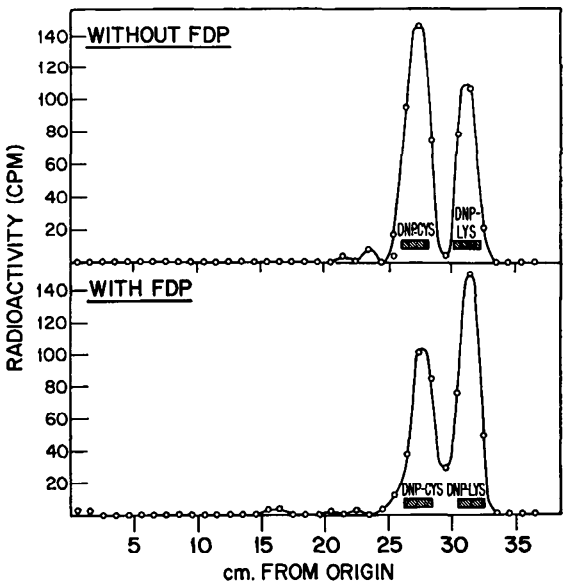


FIG. 1. Chromatography of hydrolyzed DNP-fructose diphosphatase (pH 9.2). The DNP-protein was prepared at pH 9.2, as described in Table I, with DFB having a specific activity of 1.8×10^6 cpm per μmole and was hydrolyzed with HCl as described in "Experimental Procedure." Aliquots corresponding to about 0.1 mg of the original DNP-protein were streaked on Whatman No. 3MM paper in a 2-cm band. In the experiment shown, 3.2×10^3 cpm and 2.6×10^3 cpm (total counts) were chromatographed in the experiments without and with fructose-1,6-di-P (*FDP*), respectively. Before application to the paper, each sample (0.1 ml) received 50 μg of ε-DNP-lysine and 100 μg of *S*-DNP cysteine. In addition, samples containing the same amount of ε-DNP-lysine and *S*-DNP-cysteine were spotted alongside the 2-cm streaks. Chromatography was carried out in the dark for 10 hours with Solvent B. The top layer of the solvent mixture was used to equilibrate the tank, and the bottom layer was used as developing solvent. The yellow areas corresponding to ε-DNP-lysine and *S*-DNP-cysteine were located and the paper was cut into 1-cm strips, each 2.5 cm wide. The radioactivity of each strip was measured in the counter. Visualization of the yellow DNP-amino acid spots was aided by exposing the paper to ammonia vapor.

found that the radioactivity corresponded to nearly 3 eq per mole when dinitrophenylation was carried out at pH 9.2 and only 1 eq per mole when the treatment was carried out at pH 7.5. The presence of fructose-1,6-di-P in either case blocked the dinitrophenylation of 1 group; under these conditions, 2 dinitrophenyl groups were taken up by the protein at pH 9.2 and essentially none at pH 7.5.

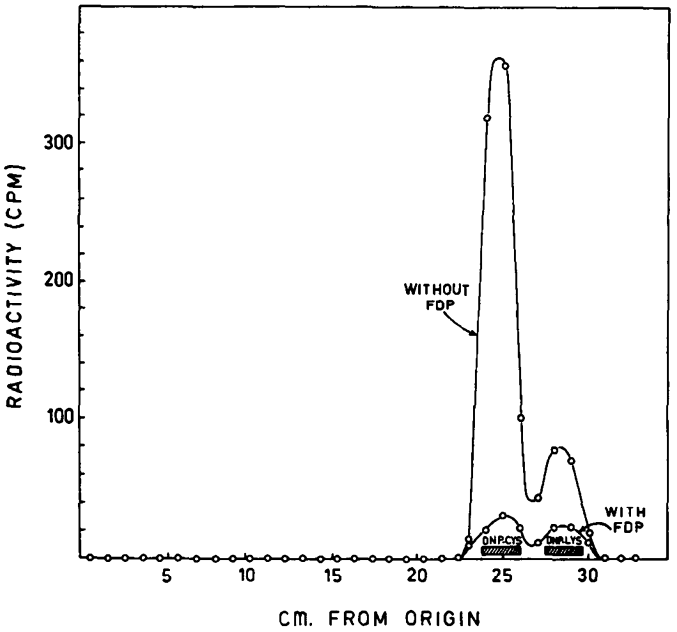


FIG. 2. Chromatography of hydrolyzed DNP-fructose diphosphatase (pH 7.5). The DNP-protein was prepared at pH 7.5, as described in Table I, and was hydrolyzed and chromatographed as described in Fig. 1. In the experiment without fructose-1,6-di-P (*FDP*), an aliquot corresponding to about 0.07 mg of protein (3.18×10^3 cpm) was located with *S*-DNP-cysteine and ε-DNP-lysine carrier and chromatographed. The specific activity of the DFB was 5.3×10^6 cpm per μmole.

TABLE III

Quantitative estimation of DNP-amino acids formed in dinitrophenylated D-fructose 1,6-diphosphatase

The areas under the curves in Fig. 2 were estimated by plotting the results on uniform paper, cutting out the portions represented by the peaks, and weighing them. The values in the table are calculated from the total amount of radioactivity placed on the paper and the proportion of the total in each radioactive area.

pH	Fructose-1,6-di-P	*S*-DNP-cysteine	ε-DNP-lysine	Ratio
		cpm	*cpm*	
9.2	−	2050	1150	1.78
	+	1220	1330	0.92
7.5	−	2600	580	
	+	440	350	

Protection by Fructose-1,6-di-P—In the presence of fructose-1,6-di-P, the rate of dinitrophenylation was decreased and the changes in catalytic activity were prevented (Table II). This protective effect could be detected with as little as 10^{-6} M fructose-1,6-di-P and was essentially complete at 10^{-4} M. Even with the highest concentrations of fructose-1,6-di-P, some dinitrophenylation was observed without change in catalytic activity (see also Table I and Fig. 2).

Nature of Groups Reactive with DFB—In order to determine which amino acids had reacted with the reagent, the proteins were hydrolyzed with HCl and the hydrolysates were examined

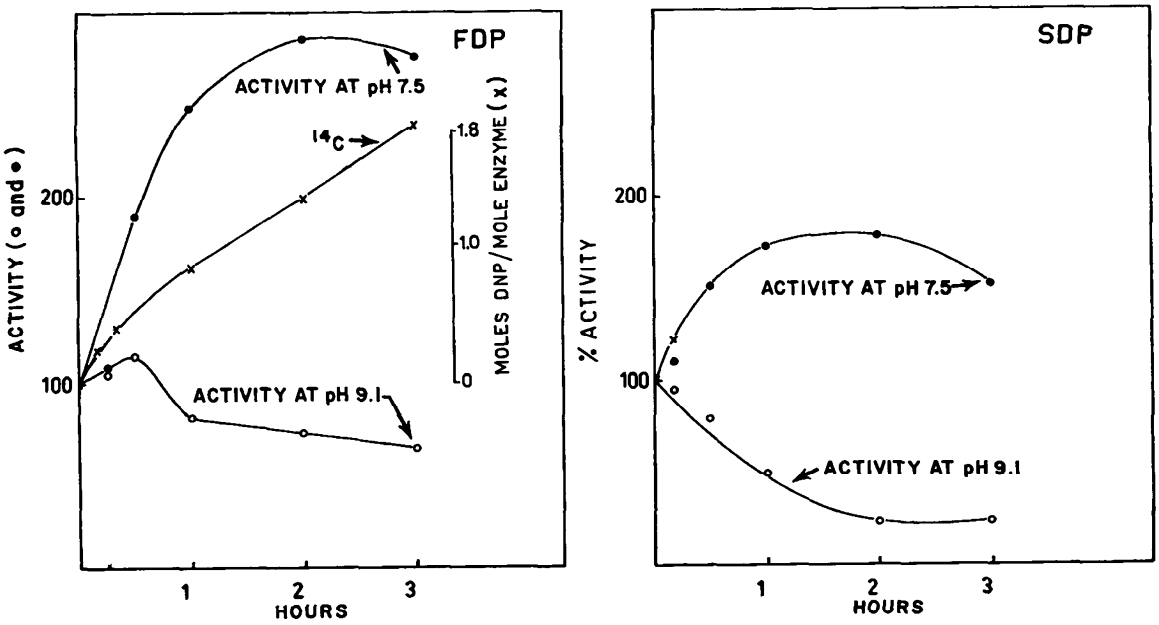


FIG. 3. Dinitrophenylation at pH 7.5 with excess DFB-^{14}C. The reaction mixture (6.0 ml) contained 3.9 mg of D-fructose 1,6-diphosphatase (0.005 mM), 0.02 M triethanolamine buffer, pH 7.5, and 0.12 mM DFB-^{14}C. It was incubated in the dark at 25° with mechanical stirring. Aliquots (0.75 ml) were removed, treated with 0.75 ml of 10% trichloroacetic acid, cooled, and centrifuged; the precipitates were washed three times with 5% trichloroacetic acid. The washed precipitates were dissolved in 0.175 ml of 1 N NH_4OH, plated, and counted. Protein was measured by the ninhydrin method after alkaline hydrolysis (see "Experimental Procedure"). Other aliquots were removed at the same time for enzyme assay, by the use of the method based on release of P_i (5), for both substrates. Activity with fructose-1,6-di-P is shown on the *left* (*FDP*), and with sedoheptulose-1,7-di-P on the *right* (*SDP*). The extent of dinitrophenylation is shown by the *inset ordinate*.

in paper chromatography. With the protein dinitrophenylated at pH 9.2, radioactivity was observed in two separate areas corresponding in location to *S*-DNP-cysteine and ε-DNP-lysine. Therefore, in subsequent runs, these two DNP-amino acid derivatives were added as carriers. Following chromatography, the strips were cut into 1-cm sections and counted (Fig. 1). The radioactivity was confined to two areas, one of which corresponded precisely to the yellow spot corresponding to DNP-cysteine, and the second to the spot corresponding to DNP-lysine. The same procedure was used to analyze the protein dinitrophenylated at pH 7.5 (Fig. 2). Calculation of the areas under the curves showed that in the experiment at pH 9.2 approximately twice as much *S*-DNP-cysteine as ε-DNP-lysine was formed in the absence of fructose-1,6-di-P, and nearly equivalent amounts of the two dinitrophenyl derivatives were formed in the presence of fructose-1,6-di-P (Table III). At pH 7.5, in the absence of substrate, 82% of the radioactivity was in the *S*-DNP-cysteine area; very little dinitrophenyl was incorporated in the presence of substrate.

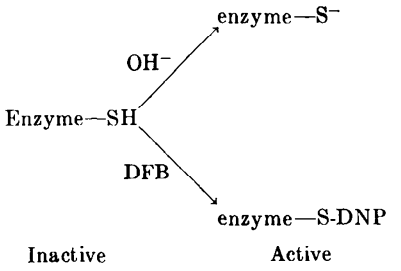
Reaction at pH 7.5 with Excess DFB—When the enzyme was treated at pH 7.5 with 24 eq of DFB-^{14}C, there was a progressive increase in enzyme activity measured at pH 7.5 which reached a maximum at about 2 hours (Fig. 3). At this time, the activity with fructose-1,6-di-P had increased by 2.9-fold while that with sedoheptulose-1,7-di-P had increased by 1.8-fold. These changes were comparable to those observed when the enzyme was dinitrophenylated at pH 9.2 (1). At this time, slightly more than 1 DNP eq had been taken up by the protein. Further incubation resulted in continued dinitrophenylation, which eventually resulted in a decrease in phosphatase activity. Hydrolysis of the DNP-protein and chromatography of the hydrolysates showed that the major product was *S*-DNP-cysteine. This accounted for 81%, 77%, and 75% at 1, 2, and 3 hours, respectively. The remaining radioactivity was present as ε-DNP-lysine.

DISCUSSION

Treatment of purified fructose diphosphatase from rabbit liver with 4 eq of DFB at pH 9.2 yields a protein containing 3 DNP residues, two of which are *S*-DNP-cysteine and one of which is ε-DNP-lysine. When the dinitrophenylation reaction is carried out at this pH in the presence of 5 mM fructose 1,6-diphosphate, which prevents the change in catalytic activity induced by DFB, two amino acids are dinitrophenylated, one *S*-DNP-cysteine and one ε-DNP-lysine. It is thus clear that the change in catalytic activity is due entirely to dinitrophenylation of a single cysteine residue. This conclusion is supported by the experiments carried out at pH 7.5. Under these conditions, the dinitrophenylation is limited to a single cysteine —SH residue and similar activation is observed. At pH 7.5, even with a large excess of DFB, the dinitrophenylation is largely due to the formation of a single *S*-DNP-cysteine.

Fructose-1,6-di-P protects more effectively against the action of DFB at pH 7.5 than at pH 9.2. At the lower pH, 0.10 mM fructose-1,6-di-P protects completely against the changes in catalytic activity, and under these conditions, dinitrophenylation is reduced by 75%.

The changes in catalytic activity produced by dinitrophenylation (1) resemble those observed when the pH is raised, suggesting that in either case one sulfhydryl group is involved. It is noteworthy that the point of inflection of the pH curve is 8.6, which is the expected pK of a protein sulfhydryl group. On the basis of these results, we suggest the following model.



The enzyme with the undissociated sulfhydryl group is inactive or shows low catalytic activity. Raising the pH leads to the ionization of this sulfhydryl group and the appearance of catalytic activity. A similar conversion of the enzyme to an active form apparently results from dinitrophenylation of one sulfhydryl group. Since enzyme activity is not blocked in the DNP-protein, this sulfhydryl group cannot be part of the active site itself but must be located at an allosteric site, the ionization or dinitrophenylation of which results in a change in conformation of the protein. It will be of interest to seek a physiological substance capable of reacting with this sulfhydryl group and producing a comparable change in enzyme activity at neutral pH. Such an allosteric effector would be responsible for the control of the activity of this enzyme in living cells.

Through the use of other sulfhydryl reagents, we have obtained independent evidence supporting the hypothesis that activation of the enzyme results when the sulfhydryl groups are modified. These results will be reported in a paper to follow.

SUMMARY

The reaction of fructose diphosphatase with 2,4-dinitrofluorobenzene at pH 9.2 leads to the preferential dinitrophenylation of two sulfhydryl groups and one lysine ε-amino group. At pH 7.5, one sulfhydryl group is preferentially dinitrophenylated. Dinitrophenylation of this sulfhydryl group is accompanied by a change in the catalytic properties of the enzyme, and particularly by a large increase in activity at pH 7.5. The reaction of the enzyme with dinitrofluorobenzene is modified or prevented by the presence of the substrate fructose 1,6-diphosphate, but not by sedoheptulose 1,7-diphosphate. The effect of fructose 1,6-diphosphate is specifically to prevent dinitrophenylation of the sulfhydryl group involved in changes in catalytic activity. A comparison of these results with the effect of increasing pH suggests that the active form of the enzyme is one in which the sulfhydryl group is either ionized or blocked with a suitable reagent.

REFERENCES

1. Pontremoli, S., Luppis, B., Wood, W. A., Traniello, S., and Horecker, B. L., *J. Biol. Chem.*, **240**, 3464 (1965).
2. Hirs, C. H. W., *Brookhaven Symposia in Biol.*, **15**, 154 (1962).
3. Rowley, P. T., Tchola, O., and Horecker, B. L., *Arch. Biochem. Biophys.*, **107**, 305 (1964).
4. Kowal, J., Cremona, T., and Horecker, B. L., *J. Biol. Chem.*, **240**, 2485 (1965).
5. Pontremoli, S., Traniello, S., Luppis, B., and Wood, W. A., *J. Biol. Chem.*, **240**, 3459 (1965).
6. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.*, **219**, 623 (1956).