that has been demonstrated in the peptide isolated from chymotrypsin-DP[3-5]. Although the position of the acetyl group has not been investigated it seems most likely that it is associated in ester linkage with the serine hydroxyl. Particularly the composition of the small acetylpeptide E (Table II) hardly leaves room for other possibilities.

The present results provide strong evidence that, in the course of the hydrolysis by chymotrypsin of its substrate, the hydroxyl group of the same serine residue is involved as has been found to react with DFP. This peptide structure or at least part of it must therefore participate in the enzymically-active site of chymotrypsin.

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On the enzymic reduction of folic acid by a purified hydrogenase*

The activation of folic acid associated with the cofactor functions of this vitamin involves hydrogenation of PGA**, presumably to the tetra-hydro form[1] and subsequent formylation[2,3,4] or hydroxymethylation[5,6]. The hydroxymethyl compound can be oxidized to the corresponding formyl derivative[7,8]. In the present investigation, folic acid hydrogenase which catalyzes the reduction of PGA was prepared from an acetone powder of chicken liver. The enzyme was purified 8-fold by removal of protein at pH 5.5 and pH 4.5 followed by fractional precipitation of the enzyme with cold acetone. The fractions between 41% and 60% acetone contained most of the activity. By this procedure folic acid hydrogenase was separated from enzymes which convert $PGAH_4$ to its formylated derivatives. The specific requirement for TPNH in the reduction of PGA could be shown, substantiating earlier observations[1,9]. The purification of the enzyme was sufficient to permit study of the stoichiometric relationship between the reduction of PGA and the oxidation of TPNH. Direct evidence is presented that $PGAH_4$ rather than $PGAH_2$ is the product of the enzymic reaction.

The method for the determination of $PGAH_4$ was based on the non-enzymic decomposition of $PGAH_4$ when exposed to air[10,11] yielding PABGA quantitatively[1]; this product was determined by the BRATTON-MARSHALL reaction[12]. The maximum conversion of PGA to $PGAH_4$ was obtained at pH 4.7 in acetate buffer and pH 5.2 in phosphate buffer. As shown in Table I, DPNH was only 20% as active as TPNH in this reaction. DPN^+ and TPN^+ were inactive. The reversibility of the reaction could not be demonstrated; however, the addition of TPN^+ during the incubation inhibited the further conversion of PGA to $PGAH_4$. The formation of diazotizable amine was correlated with the oxidation of TPNH in the experiment shown in Table II. The data indicate that two moles of TPNH are oxidized for each mole of PABGA formed, in accord with the equations:

$$PGA + 2\,TPNH + 2\,H^+ \longrightarrow PGAH_4 + 2\,TPN^+$$

$$PGAH_4 \xrightarrow{\text{non-enzymic}} \text{Pteridine} + PABGA$$

Amethopterin (4-amino-10-methyl PGA) completely inhibited the activity of folic acid hydrogenase at a concentration of $4.2 \cdot 10^{-8} M$ in the presence of $4.4 \cdot 10^{-5} M$ PGA; at a concentration of $4.2 \cdot 10^{-9} M$ Amethopterin, only 10% inhibition was observed.

The colorimetric method for the determination of $PGAH_4$ is limited to those analogues of

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** Abbreviations: PGA, pteroylglutamic acid; $PGAH_2$, dihydropteroylglutamic acid; $PGAH_4$, tetrahydropteroylglutamic acid; TPN^+, triphosphopyridine nucleotide; DPN^+, diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; PABGA, *p*-aminobenzoylglutamic acid.

TABLE I

COFACTOR REQUIREMENT OF FOLIC ACID HYDROGENASE

The system contained: PGA, $6.54 \cdot 10^{-2}$ μmole; DPN^+, TPN^+, DPNH or TPNH, $82.5 \cdot 10^{-2}$ μmole; enzyme, 0.75 ml, in 1.5 ml 0.05 *M* phosphate buffer, pH 5.2; incubated for 3 h under helium at 37°.

Cofactor	*$PGAH_4$ formed* μmole*
none	0
DPN^+	0
TPN^+	0
DPNH	$0.73 \cdot 10^{-2}$
TPNH	$3.90 \cdot 10^{-2}$

* The method for the determination of $PGAH_4$ is described in Table II.

TABLE II

STOICHIOMETRY OF THE REACTION

The system contained: PGA, 0.1308 μmole; TPNH, 1.65 μmoles; enzyme, 1.5 ml; in 3.0 ml 0.05 *M* phosphate buffer, pH 5.2. The incubation was carried out at 37° under a stream of helium in stoppered 15 ml suction flasks. Each sample was duplicated by a control flask to which PGA was not added until the incubation was stopped by chilling the vessels in ice.

Experiment	*Time of incubation*	*TPNH* oxidized*	*PGA** reduced*	*Ratio: TPNH oxid. / PGA red.*
	min	*μmole*		
1	60	0.1407	0.0744	1.89
	120	0.1770	0.0933	1.91
2	120	0.1200	0.0615	1.95

* For determination of TPNH, 1.5 ml aliquots were mixed with 1.0 ml ice-cold acetone and 1.0 ml 1.0 *M* Na_2HPO_4 and then centrifuged. The amount of TPNH oxidized was calculated from the difference in optical density at 340 mμ between the control and the corresponding sample using a molar extinction coefficient for TPNH of $6.22 \cdot 10^3$ [13].

** The determination of $PGAH_4$ was made on the remaining 1.5 ml; 0.5 ml 5 *N* HCl and 1.2 ml acetone were added. After centrifugation for 20 min, the color was developed by a modification of the BRATTON-MARSHALL method[12] allowing minimum dilution of the sample. The absorption of the sample was read against the control at 560 mμ. The molar equivalent of $PGAH_4$ was calculated from a calibration curve of PABGA.

PGA which can yield a *p*-aminobenzoic acid moiety with an unsubstituted amino group, and is, therefore, unsuitable for studying the substrate specificity of folic acid hydrogenase. Such a study is now possible using the purified enzyme and the rate of oxidation of TPNH as a measurement of the enzymic activity.

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The degree of splitting of the enzyme by DPNH, but not by *o*-phenanthroline, has been found to be dependent on the specific activity of the enzyme.