Similar findings have been reported previously for the rat[2] and the dog[9] but in ruminants (goat[10] and cow[11]) only iodide has been observed in the milk after ^{131}I administration. No metabolically active compounds have been chromatographically detected in milk. This is in agreement with previous results based on biological testing[12].

The relative scarcity or absence of DIT in the presence of large amounts of MIT in milk is puzzling; enzymic deiodination of DIT in the body does not appear to stop at the MIT stage[6]. Failure to iodinate beyond the MIT stage appears to be a more likely explanation and may be simply the consequence of an excess of tyrosine with respect to the available iodine. However, it is of interest that an excess of MIT over DIT appears to be characteristic of the iodinated proteins formed by several "primitive" or "abnormal" biological iodinating systems such as the lactating breast *in vivo*, cell-free preparations of functioning mammary tissue or thyroid *in vitro*[13,14], the developing chick thyroid[15], carcinomas of the thyroid[16,17] and certain adenomas of the thyroid[18].

Thanks are due to Sir CHARLES HARINGTON, Sc.D., F.R.S., and to Dr. R. PITT-RIVERS, F.R.S. for the opportunity afforded to one of us (K. B.-G.) to spend some time as a guest worker at the National Institute for Medical Research.

K. BROWN-GRANT*
National Institute for Medical Research, London (England) VALERIE A. GALTON

1 K. BROWN-GRANT, *J. Physiol.*, 135 (1957) 644.
2 G. D. POTTER AND I. L. CHAIKOFF, *Biochim. Biophys. Acta*, 21 (1956) 400.
3 J. GROSS, *Brit. Med. Bull.*, 10 (1954) 218.
4 J. GROSS AND C. P. LEBLOND, *Endocrinology*, 48 (1951) 714.
5 W. TONG, A. TAUROG AND I. L. CHAIKOFF, *J. Biol. Chem.*, 195 (1952) 407.
6 J. B. STANBURY, A. A. H. KASSENAAR, J. W. A. MEIGER AND J. TERPSTRA, *J. Clin. Endocrinol. and Metabolism*, 16 (1956) 735.
7 J. ROCHE, R. MICHEL AND E. VOLPERT, *Compt. rend. soc. biol.*, 148 (1954) 21.
8 K. BROWN-GRANT AND J. G. GIBSON, *J. Physiol.*, 127 (1955) 341.
9 L. VAN MIDDLESWORTH, *J. Clin. Endocrinol. and Metabolism*, 16 (1956) 989.
10 W. E. WRIGHT, J. E. CHRISTIAN AND F. N. ANDREWS, *J. Dairy Sci.*, 38 (1955) 31.
11 R. F. GLASCOCK, *J. Dairy Research*, 21 (1954) 318.
12 K. L. BLAXTER, *Vitamins and Hormones*, 10 (1952) 217.
13 A. TAUROG, G. D. POTTER, W. TONG AND I. L. CHAIKOFF, *Endocrinology*, 58 (1956) 132.
14 A. TAUROG, G. D. POTTER AND I. L. CHAIKOFF, *J. Biol. Chem.*, 213 (1955) 119.
15 J. B. TRUNNELL AND P. WADE, *J. Clin. Endocrinol. and Metabolism*, 15 (1955) 107.
16 J. R. TATA, J. E. RALL AND R. W. RAWSON, *J. Clin. Endocrinol. and Metabolism*, 16 (1956) 1554.
17 P. G. STANLEY, *Biochem. J.*, 63 (1956) 581.
18 R. PITT-RIVERS, D. HUBBLE AND W. H. HOATHER, *J. Clin. Endocrinol. and Metabolism*, in the press.

Received October 29th, 1957

* Present address: Department of Physiology, University of Birmingham.

Isolation of acetyl peptides from acetylchymotrypsin

During the last few years several papers have been published on the reaction of α-chymotrypsin with diisopropylphosphorofluoridate (DFP). This reaction resulted in a complete inhibition of the enzymic activity and was accompanied by the introduction of the diisopropylphosphoryl group in the enzyme molecule (chymotrypsin-DP). SCHAFFER *et al.*[1] have demonstrated that upon acid hydrolysis of chymotrypsin-DP, the phosphorus moiety of DFP was present in the peptide chain Gly·Asp·phospho Ser·Gly. This sequence could be extended to Gly·Asp·phosphoSer·Gly·Glu. These results were partially confirmed[2]. We found that upon proteolytic digestion of chymotrypsin-DP a peptide could be isolated which contained a diisopropylphosphoryl group substituted to the hydroxyl group of a serine residue[3–5]. The amino-acid sequence in this peptide was shown to be Gly·Asp·Ser·Gly·Gly·Pro·Leu. These results indicate that the attack of DFP on the enzyme is directed against the hydroxyl of a serine residue which is occurring in the peptide sequence Gly·Asp·Ser·Gly.

There is good reason to believe that the site on the enzyme which combines with DFP is identical to that part of the enzyme where substrate hydrolysis is performed[6]. Moreover, it is attractive to consider related mechanisms to govern substrate hydrolysis and inhibition by DFP. In the former case a labile acetyl–enzyme complex is assumed to be formed which breaks down rapidly in water; in the latter case an analogous but stable enzyme–DP complex is formed. These

conclusions, drawn from kinetic evidence[7], were strongly supported by the isolation at low pH of the acetylated enzyme by BALLS AND WOODS[8]. However, the isolation of an acetyl–enzyme intermediate is by itself not sufficient evidence for an analogous mechanism, operating at one and the same site of the enzyme, for both substrate hydrolysis and inhibition by organophosphate. It remains to be demonstrated that the acetyl group in the acetylated enzyme is actually linked to the same known group as the phosphoryl radical in the enzyme–DP complex.

To establish the position of the acetyl group in acetylchymotrypsin we have followed a similar procedure as was used for the analysis of chymotrypsin-DP. Although the labile binding of the acetyl group in acetylchymotrypsin above pH 5.0 might interfere with such a procedure, the possibility that upon denaturation of acetylchymotrypsin a stabilisation might occur did not seem unlikely*. Digestion of acetyl chymotrypsin with pepsin at pH 2.0 was indeed found to stabilise the acetyl group sufficiently. This enabled us to continue the digestion at pH 7.8 with pancreatine, a commercial crude pancreas extract.

Acetylchymotrypsin (100 mg) labeled with ^{14}C in the carboxyl group of the acetyl, was prepared following the procedure of BALLS[7], using *p*-nitrophenyl acetate with a specific radioactivity of 0.31 mc/mmole**. The acetylated enzyme was dissolved in 2 ml water and digested with 0.25% pepsin at pH 2.0 and 20°. After 16 h the pH was adjusted to 7.8 and the digestion continued for 16 h at 37° after the addition of 4 mg pancreatine. The ^{14}C-containing derivatives were fractionated and purified by means of high-voltage electrophoresis at pH 6.5 and pH 3.5 followed by paper chromatography in butanol–acetic acid–water (BAW) (4:1:5). One of the peptides was also chromatographed in butanol–water (BW).

The separation of the peptides is shown in Table I. The purified ^{14}C-containing peptides were completely hydrolysed (6*N* HCl at 105° for 24 h) and the amino acids in the hydrolysates were determined by means of two-dimensional paper chromatography in phenol–ammonia and BAW.

TABLE I

ELECTROPHORESIS AND PAPER CHROMATOGRAPHY OF ACETYLPEPTIDES

Paper electrophoresis was performed for 2 h at 3000 V/40 cm. The figures refer to the migration in mm of the radioactive zones towards the anodic (+) or cathodic (—) site.

Peptide	*Electrophoresis*		*Chromatography (R_F)*	
	pH 6.5	*pH 3.6*	*BAW*	*BW*
A	+ 54	— 42	0.49	0.06
B	+ 71	+ 35	0.77	—
C	+ 81	— 32	0.19	—
D	+ 103	— 4	0.19	—
E	+ 122	+ 86	0.44	—

The compositions of the five peptides investigated are summarized in Table II. The recorded numbers of residues are based on visual estimation of the strength of the ninhydrin-coloured spots with respect to the ^{14}C content of the peptides. The actual amino-acid sequences of these five peptides have not been established. However, it is most interesting to observe that their composition is entirely compatible with the requirements of the sequence Gly·Asp·Ser·Gly·Gly·Pro·Leu

TABLE II

AMINO-ACID COMPOSITION OF ACETYLPEPTIDES

A	*B*	*C*	*D*	*E*
Gly			Gly	
Asp		Asp	Asp	
Ser	Ser	Ser	Ser	Ser
Gly	Gly	Gly	Gly	Gly
Gly	Gly	Gly		Gly
Pro	Pro			
Leu	Leu			

* This assumption was based mainly on the observation by Dr. H. S. JANSZ (this laboratory) that the possibility of releasing the DP-group from DFP-inhibited horse-liver ali-esterase with nucleophilic reagents was lost after denaturation.

** We are greatly indebted to Mr. F. BERENDS and Miss I. VAN DER SLUYS from this laboratory for the preparation of this compound.

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.

Medical Biological Laboratory of the National Defence Research Council T.N.O., Rijswijk-Z.H. (The Netherlands)

R. A. OOSTERBAAN
M. E. VAN ADRICHEM

[1] N. K. SCHAFFER, L. SIMET, S. HARSHMAN, R. R. ENGLE AND R. W. DRISKO, *J. Biol. Chem.*, 225 (1957) 197.
[2] F. TURBA AND G. GUNDLACH, *Biochem. Z.*, 327 (1955) 186.
[3] R. A. OOSTERBAAN, P. KUNST, J. VAN ROTTERDAM AND J. A. COHEN, *Biochim. Biophys. Acta*, (in the press).
[4] R. A. OOSTERBAAN, H. S. JANSZ AND J. A. COHEN, *Biochim. Biophys. Acta*, 20 (1956) 402.
[5] J. A. COHEN, R. A. OOSTERBAAN, M. G. P. J. WARRINGA AND H. S. JANSZ, *Discussions Faraday Soc.*, 20 (1955) 114.
[6] I. B. WILSON, in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, (Symposium) Johns Hopkins Press, Baltimore, 1954.
[7] B. S. HARTLEY AND B. A. KILBEY, *Biochem. J.*, 56 (1954) 288.
[8] A. K. BALLS AND H. N. WOOD, *J. Biol. Chem.*, 219 (1956) 245.

Received October 31st, 1957

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

* This work was supported in part by a research grant (CY-2906) from the National Institutes of Health, U.S. Public Health Service.

** 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.