bumin. When the sulfhydryl group in denaturing medium is inactivated by addition of 0.68 mole of silver nitrate or potassium ferricyanide, the reaction ratio of copper(II) to BSA was again found to be unity.

Our experiments have been carried out under conditions quite different from those of Klotz, *et al.*,[4] and no comparison with their results is made here. Our results show that in the *p*H range 8–10, one copper(II) is bound to the BSA molecule extremely tightly, and that the sulfhydryl group is not the reactive group. It is of interest to note that nickel(II), even in very small concentrations, and also, to a lesser extent, cobalt(II) greatly interfere with the copper(II) BSA reaction while even large amounts of zinc(II) have very little effect. Thus, the group in the BSA molecule responsible for the binding of copper(II), the nature of which is at present unknown, apparently also forms stable complexes with nickel(II) and cobalt(II) but not with zinc(II). One unique site in the BSA molecule is the N-terminal aspartyl group, and further work may show whether this group is the site of copper(II) binding.

There is very little (if any) reaction between copper(II) and human $\gamma$-globulin under our conditions. The method has been applied to the determination of albumin in blood serum, and preliminary results are promising.

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(4) I. M. Klotz and H. G. Curme, THIS JOURNAL, **70**, 939 (1948); H. A. Fiess and I. M. Klotz, *ibid.*, **74**, 887 (1952); I. M. Klotz, J. M. Urquhart and H. A. Fiess, *ibid.*, **74**, 5537 (1952); I. M. Klotz, J. M. Urquhart, T. A. Klotz and J. Ayers, *ibid.*, **77**, 1919 (1955); I. M. Klotz, I. L. Faller and J. M. Urquhart, *J. Phys. Colloid Chem.*, **54**, 18 (1950); I. M. Klotz and H. A. Fiess, *ibid.*, **55**, 101 (1951).

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## ENZYME CATALYSIS AND ENZYME SPECIFICITY—COMBINATION OF AMINO ACIDS AT THE ACTIVE SITE OF PHOSPHOGLUCOMUTASE[1]

*Sir:*

In the classical template model for enzyme action the substrate is assumed to be absorbed with a precise fit into an area called the "active site" of the protein.[2] Presumably the amino acids at this site provide not only the catalytic action but also the specificity of the enzyme. From the widely varying specificity patterns of enzymes it would be anticipated that the amino acid composition at the active site would vary to fit the particular substrate. On the other hand, stereochemical and other evidence had led to the hypothesis that a few bond-breaking mechanisms might be common to reactions of widely different specificities[3] and hence there might be some common amino acid sequences. The sequence of a portion of the active site of chymotrypsin has been established[4–6] and hence a comparison could be made if similar information could be obtained for an enzyme of very different specificity. Such an opportunity arose when it was demonstrated that the active site of phosphoglucomutase was marked by a serine phosphate.[7,8]

$P^{32}$-labeled enzyme was prepared by exchange with radioactive substrates (glucose-1-$PO_4^{32}$ and glucose-6-$PO_4^{32}$) under conditions similar to those of the standard assay.[9] It had been established that the enzyme is labeled only at the active site by this procedure.[8] The enzyme was then degraded, either with acid alone, with proteolytic enzymes alone, or with proteolytic enzymes followed by acid hydrolysis. Radioactive phosphopeptides were isolated by use of Dowex 50 columns[10,11] and paper chromatography. The compositions of the phosphopeptides are shown in Table I.

TABLE I

$P^{32}$-PHOSPHOPEPTIDES ISOLATED FROM PHOSPHOGLUCOMUTASE

| Peptide | Phosphopeptide obtained by treatment with | Amino acid compn. |
|---|---|---|
| 1 | Proteolytic + acid | (Asp,Ser,Gly,Glu) |
| 2 | Proteolytic + acid | (Asp,Ser,Gly,Glu,Ala) |
| 3 | Proteolytic + acid | (Asp,Ser,Gly,Glu,Ala,Val,Thr) |
| 4 | Proteolytic only | (Asp,Ser,Gly,Glu) |
| 5 | Proteolytic only | (Asp,Ser,Gly,Glu Ala,Val,Thr, Leu) |
| 6 | Acid only | (Asp,Ser,Gly,Glu,Ala) |
| 7 | Acid only | (Asp,Ser,Gly,Glu,Ala,Val Thr, Leu) |

While the detailed sequence has not been completely established, it is to be noted that the composition of each peptide and the order from comparison of lower and higher peptides is in perfect agreement with the sequence for chymotrypsin which is Asp Ser Gly Glu Ala Val.[4–6] The probability that the 7 peptides isolated from phosphoglucomutase would agree with this sequence by chance alone is very small. Moreover the concordance of the acid derived and proteolytic enzyme plus acid derived peptides is strong evidence against the sequence being an artifact of the separation procedure. The data therefore indicate that the amino acid sequence of the active site for at least 6 amino acids is the same for an enzyme which specifically hydrolyzes peptide bonds and an enzyme that specifically transfers phosphate between carbohydrate molecules.

This apparently surprising result is good support for the above mentioned hypothesis that common bond-breaking mechanisms exist despite vary-

(1) Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

(2) E. Fischer, *Ber.*, **27**, 2985 (1894).

(3) D. E. Koshland, Jr., "Mechanism of Enzyme Action," edited by McElroy and Glass, Johns Hopkins Press, Baltimore, Md., 1954, p. 608.

(4) J. A. Cohen, R. A. Oosterbann, M. G. Warringa and H. S. Jansz, *Disc. Faraday Soc.*, **20**, 114 (1955).

(5) N. K. Schaffer, S. Harshman, R. R. Engle and R. W. Drisko, *Federation Proc.*, **14**, 275 (1955).

(6) F. Turba and G. Gundlach, *Biol. Z.*, **327**, 186 (1955).

(7) G. R. Jolles and L. Anderson, Am. Chem. Soc. Abstracts, Sept. 1955, p. 23c.

(8) E. P. Kennedy and D. E. Koshland, Jr., *J. Biol. Chem.*, in press.

(9) V. A. Najjar, *ibid.*, **175**, 280 (1948).

(10) M. Flavin, *ibid.*, **210**, 771 (1954).

(11) N. K. Schaffer, S. Harshman and R. R. Engle, *ibid.*, **214**, 799 (1955).

ing specificity type. It suggests further that (a) the common sequence around serine is involved in making the otherwise inert $CH_2OH$ side chain reactive and (b) the amino acids responsible for the specificity lie further from the reactive serine, perhaps in adjacent coils. While there must be an intimate steric relation between the amino acids responsible for the catalytic action and those responsible for the specificity, these results indicate that the two may be separable. In this case common sequences related to specificity as well as to the bond-breaking action might be expected, a result which would be of enormous value in relating enzyme structure to function.

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## ENZYMATIC INTRODUCTION OF DOUBLE BONDS INTO STEROID RING A

*Sir:*

The introduction of unsaturation into ring A of steroids is of interest in connection with the aromatization involved in biosynthesis of estrogens,[1] and in the microbiological formation of $\Delta^1$-dehydrosteroids,[2] some of which possess interesting physiological properties. *Pseudomonas testosteroni* (A.T.C.C. No. 11996)[3] can utilize certain steroids as a sole source of carbon and degrade these compounds to $CO_2$ and $H_2O$ by a series of reactions catalyzed by adaptive enzymes. During the course of a study of the pathway involved in the oxidative degradation of steroids by this microörganism, it was found that early steps were the interconversion of 17$\beta$-hydroxyl and 17-ketone functions and the introduction of $\Delta^1$-double bonds and $\Delta^4$-double bonds into steroids of both the 5$\alpha$-H and 5$\beta$-H series. Such reactions have been reported in a variety of microbiological systems.[1,2] The enzymatic interconversion of 17$\beta$-hydroxy- and 17-ketosteroids is catalyzed by the diphosphopyridine nucleotide-linked enzyme $\beta$-hydroxysteroid dehydrogenase and has been analyzed in detail.[4] The purpose of the present communication is to report on the enzymatic mechanism of the introduction of double bonds into steroids. This constitutes, to our knowledge, the first demonstration of these reactions by means of soluble enzymes.

Under suitable conditions *Ps. testosteroni* carries out efficient conversions of androstane-3,17-dione (I), testosterone, $\Delta^4$-androstene-3,17-dione (II), $\Delta^1$-androstene-3,17-dione (III) and etiocholan-17$\beta$-ol-3-one to principally $\Delta^{1,4}$-androstadiene-3,17-dione (IV) and to a lesser extent to $\Delta^{1,4}$-androstadien-17$\beta$-ol-3-one (V). In a typical conversion, this microörganism was grown in 450 ml. of a medium containing 0.5% sodium lactate in a mineral base, at 30° and an initial *p*H of 7.0, and at the end of 24 hours 45 mg. of II was added in 2 ml. of acetone and the incubation continued for 24 hours longer. Extraction of the culture with ethyl acetate, followed by chromatography on silicic acid, crystallization from hexane–acetone, and sublimation at 90–115° (0.0005 mm.) afforded 20.5 mg. of IV, m.p. 140.5–141° (no depression on admixture with an authentic sample); $[\alpha]^{25}D$ +123° (*c* 1.04, in $CHCl_3$); $\lambda_{max}^{alc}$ 243 m$\mu$ ($\epsilon$ 16,200); sulfuric acid chromogens[5] were identical with a known sample (peaks at 265, 305 and 390 (broad) and a minimum at 288 m$\mu$); (*Anal.* Found: C, 80.08; H, 8.46. Calcd. for $C_{19}H_{24}O_2$: C, 80.24; H, 8.51); infrared analysis showed $\lambda_{max}^{KBr}$ 5.77 $\mu$ (17-ketone), 6.04, 6.17 and 6.25 $\mu$ ($\Delta^{1,4}$-3-ketone), identical with an authentic sample. Mixed paper chromatography showed no separation from authentic IV.

In similar experiments, conversions in good yield of I and III to IV were demonstrated and the products rigorously identified by comparison paper chromatography, m.p. and infrared spectra. Evidence for the presence of small amounts of V in each case was obtained. Conversion of 19-*nor*-testosterone by the same system in 72 hours gave a 67% yield of estrone m.p. 253–256°; $[\alpha]^{25}D$ +154° (*c* 0.644, in dioxane); $\lambda_{max}^{alc}$ 282 m$\mu$ ($\epsilon$ 1960); on addition of NaOH, $\lambda_{max}^{alc}$ 243 m$\mu$ ($\epsilon$ 8000) and 300 m$\mu$ ($\epsilon$ 2500). The infrared spectrum showed peaks at 5.83 $\mu$ (17-ketone), 6.32, 6.18 $\mu$ (—C=C—) and 3.06 $\mu$(OH group), and was identical with that of authentic estrone. On paper chromatography the substance migrated as estrone and gave the phenolic reaction with the Turnbull reagent.[6] Estradiol-3,17$\beta$ was also found in smaller amounts.

Cell-free extracts of *Ps. testosteroni* were prepared from cultures grown on 0.5% sodium lactate as described above. After 24 hours growth, II was added to give a final concentration of 0.01% and incubation continued for 24 hours longer. Cells were harvested and washed by centrifugation. The washed cells efficiently converted I to IV. After rupture by sonic oscillation in a 9 KC Raytheon oscillator for 20 minutes, the sonicate also converted I to IV vigorously.

The unwashed residue from centrifugation of the sonicate for 20 minutes at 10,000 × *g* was also active in this conversion. The supernatant alone was inactive and its activity could be restored by adding phenazine methosulfate (PMS), an oxidation reduction dye which reacts with certain flavoprotein enzymes[7] but not by the addition of di- and triphosphopyridine nucleotides. The supernatant upon the addition of PMS still introduced $\Delta^1$- and $\Delta^4$-double bonds after centrifuging for 30 minutes at 105,000 × *g*. The enzymatic activities were demonstrated in a variety of ways including paper chromatographic analysis of products and ultraviolet spectrophotometry of products formed from I.

A convenient and specific assay of the $\Delta^1$-dehydrogenase activity was the measurement of estrone formation from $\Delta^4$-estrene-3,17-dione with the Folin

(1) P. Talalay, *Physiol. Revs.*, in press.

(2) S. H. Eppstein, P. D. Meister, H. C. Murray and D. H. Peterson, *Vitamins and Hormones*, **14**, 359 (1956).

(3) P. Talalay, M. M. Dobson and D. F. Tapley, *Nature*, **170**, 620 (1952).

(4) (a) P. Talalay and M. M. Dobson, *J. Biol. Chem.*, **205**, 828 (1953); (b) P. Talalay and P. I. Marcus, *ibid.*, **218**, 675 (1956).

(5) A. Zaffaroni, *Rec. Prog. in Hormone Research*, **8**, 51 (1953).

(6) G. M. Barton, R. S. Evans and J. A. F. Gardner, *Nature*, **170**, 249 (1952).

(7) T. P. Singer, E. B. Kearney and V. Massey, in O. H. Gaebler, "Enzymes: Units of Biological Structure and Function," Academic Press, New York, N. Y., 1956, p. 417.