THE COMBINED ACTION OF CARBOXYPEPTIDASE AND *B. SUBTILIS* ENZYME ON OVALBUMIN

Sir:

In a previous communication[1] it was reported that the action of carboxypeptidase (CXY) on ovalbumin was the specific release of free alanine. This observation in itself has been confirmed with four different crystalline CXY preparations (Table I). Up to 0.97 mole alanine/mole ovalbumin are released with only traces of other amino acids (aspartic acid, serine, glutamic acid, glycine, valine, leucine or isoleucine, each 0.01 to 0.05 mole/mole ovalbumin).

However, further studies show that pre-treatment of CXY with diisopropylfluorophosphate (DFP), suggested by Dr. H. Neurath, almost completely inhibits its attack on ovalbumin (Table I). Yet CXY activity measured on a simple substrate (chloroacetyl-L-tyrosine)[2] is not significantly inhibited by DFP.[3] It is concluded that the observed alanine-yielding reaction must depend on the presence of a DFP-sensitive contaminant in the CXY. The conclusion previously drawn,[1] that alanine is C-terminal in the native ovalbumin molecule, must be withdrawn in the light of these new findings. The present experiments show that this C-terminal alanine only becomes available after a preliminary "opening" of the molecule by the contaminating enzyme. Although the latter has not been positively identified, it has been found that its action in unmasking a C-terminal alanine residue can be almost exactly duplicated by the *B. subtilis* enzyme of Güntelberg and Ottesen,[4] recently shown by Ottesen to be highly DFP-sensitive.[5] These experiments have been extended. They suggest that in the proteolytic conversion of ovalbumin to plakalbumin there is a new intermediate protein which has lost no non-protein material but has been altered so as to expose a reactive C-terminal residue.

TABLE I

Ovalbumin 5%, *p*H 7.8; CXY 1 mgm./ml. crystalline suspension; 4 hr. incubation at 23°; stopped with equal vol. 20% trichloroacetic acid (TCA). Precipitate washed with 10% TCA. Supernatants dried *in vacuo* at 23° after removal of TCA.

Carboxypeptidase preparation	No preliminary treatment Yield of free alanine (mole/mole ovalbumin)	Pre-incubated 1 hr. at 23° with 10^{-3} DFP *Total* ninhydrin positive material (leucine color equivalents,[a] mole/mole ovalbumin)
A	0.7[b]	0.04
B	.68[c]	.06
C	.77[c]	.05
C	.97[c]	.05

[a] Reference 6. [b] Paper chromatographic method, ref. 8. [c] Ion exchange chromatographic method, Ref. 7.

Trypsin and chymotrypsin have been encountered as contaminants in CXY[3] and both are DFP-sensitive.[9] Attempts were made at first to duplicate the alanine-yielding reaction with *ad hoc* mixtures of one or both of these enzymes with CXY in which the contaminant had been inhibited by pretreatment with DFP (excess DFP was removed by repeated washing with water or prolonged dialysis against 10% LiCl). Trypsin was used at 0.3 and 0.1 mgm./ml.; chymotrypsin at 0.05 and 0.001 mgm./ml.; DFP-treated CXY at 1 mgm./ml. In none of these preliminary experiments did there appear to be any specific release of alanine.

Then an attempt was made to crystallize the protein remaining after incubation of ovalbumin with untreated CXY. Instead of the flat, cigar-shaped needles of ovalbumin, rectangular plates similar to those of plakalbumin[10] were obtained in high yield (72, 78, 82%). This, together with Ottesen's finding that *B. subtilis* enzyme is DFP-sensitive, led to a study of the combined action of DFP-treated CXY and *B. subtilis* enzyme.

Extremely low levels of crystalline *B. subtilis* enzyme[4] were used, levels which when acting alone release very little NPN and cause almost no plakalbumin formation. In a typical experiment (0.6 micrograms/ml. *B. subtilis* enzyme) the usual peptide splitting reactions[11,12] proceeded to less than 7% completion. Similarly the DFP-treated CXY alone (1 mgm./ml. suspension) split off only minimal amounts of ninhydrin positive material (see Table I). Acting together at these levels, however, these enzymes liberated 0.43 to 0.54 mole alanine/mole ovalbumin. Somewhat higher yield was obtained when the CXY was held in solution in 10% LiCl. Chromatographic analysis shows that the NPN consists almost exclusively of free alanine (Table II). The amounts of peptide present are too low to be the source of the free alanine. This must have its origin then from the body of the altered or "opened" ovalbumin molecule as discussed above. The initial reaction of the *B. subtilis* enzyme (which makes available the C-terminal alanine) thus involves the release of no non-protein material. The possibility of such an initial reaction has been discussed previously by Linderström–Lang and appears to be compatible with the other

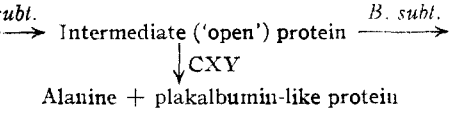
TABLE II

Ovalbumin 4.5%, *p*H 7.8; DFP-treated CXY 0.9 mgm./ml. (held in solution by adjusting final incubation mixture to 10% LiCl concn.); *B. subtilis* enzyme 1.1 microgram/ml. 4 hr. incubation at 24.5°. Analysis by ion exchange chromatographic method; ref. 1.

	Moles amino acid/mole ovalbumin Unhydrolyzed	Hydrolyzed
Aspartic acid	<0.03	0.08
Serine	< .03	.09
Glutamic acid	< .03	.09
Glycine	< .03	.08
Alanine	.65	.94
Valine	< .03	.09
Leucine or isoleucine	< .03	.04

(1) D. Steinberg, THIS JOURNAL, **74**, 4217 (1952).
(2) F. W. Putnam and H. Neurath, *J. Biol. Chem.*, **166**, 603 (1946).
(3) J. A. Gladner and H. Neurath, *Biochem. Biophys. Acta*, **9**, 335 (1952).
(4) A. V. Güntelberg and M. Ottesen, *Nature*, **170**, 802 (1952).
(5) M. Ottesen, unpublished data.
(6) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).
(7) S. Moore and W. H. Stein, *ibid.*, **192**, 663 (1951).
(8) D. Steinberg and C. B. Anfinsen, *ibid.*, **199**, 25 (1952).
(9) E. F. Jansen, M. D. Fellows Nutting, R. Jong and A. K. Ball, *ibid.*, **179**, 189 (1949).
(10) K. Linderström-Lang and M. Ottesen, *Compt. Rend. Lab. Carlsberg, ser. chim.*, **26**, 403 (1949).
(11) M. Ottesen and C. Villee, *ibid.*, **27**, 421 (1951).
(12) M. Ottesen and A. Wollenberger, *Nature*, **170**, 891 (1952).

findings of the Carlsberg group.[13] The following scheme is proposed



Whether the initial reaction involves the opening of a peptidic bond or only represents a configurational alteration has not been determined.

Three of the CXY preparations used in these studies were found to be contaminated with bacteria (aerobic culture on yeast extract agar). The fourth preparation, however, showed no growth although it gave similar results with ovalbumin. Still the absence of viable bacteria does not prove the absence of enzyme.

Even though the *B. subtilis* enzyme so closely mimics the action of the contaminant, the possibility that a different proteolytic enzyme, possibly of pancreatic origin, is the real contaminant must be considered.[14] In any case the importance of recognizing such possible contaminants when using CXY in studies on proteins or peptides is clear. Very low levels of contamination may, as in this case, alter the observed reaction markedly.

A more detailed report will appear in *Comptes rendus du Laboratoire Carlsberg*.

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(13) K. Linderstrom-Lang, *Lane Medical Lectures*, **6**, 73 (1952).

(14) The possibility that CXY itself is able to "open" the ovalbumin molecule and that only this function is inhibited by DFP, while not ruled out, seems unlikely.

(15) Section on Cellular Physiology, National Heart Institute, Bethesda, Md. The author is deeply indebted to Professor K. Linderstrom-Lang and to Professor H. Neurath for their counsel.

THE SEPARATION OF THE FOUR PLATINUM GROUP METALS PALLADIUM, RHODIUM, IRIDIUM AND PLATINUM

Sir:

The separation of the four platinum group metals palladium, rhodium, iridium and platinum has been accomplished very simply at room temperature by the use of Dowex-50 cation exchange resin. Acting on some preliminary observations on rhodium by C. I. Browne (private communication) we have found that a mixture of these elements may be separated as follows: the solution is taken near dryness repeatedly with a mixture of nitric and perchloric acids until every trace of halide ion is removed and the ions are left in a small volume (0.2–0.5 ml.) of fuming perchloric acid. The solution is then diluted to a volume of approximately ten milliliters with distilled water and is run into the top of an ion-exchange column packed with Dowex-50 resin. Under these conditions platinum will pass through the column while the other three elements adhere. The column may be washed with distilled water to remove the last traces of platinum. If any halide ions remain in the solution, the platinum fraction will contain small amounts of the other three elements.

Palladium is then stripped from the column with dilute (0.05 to 0.5 *M*) hydrochloric acid. This process occurs quite readily.

Rhodium elutes gradually from the column with 2 *M* hydrochloric acid. This process is rather slow. Experiments are in progress to determine the effect of raising the temperature of the eluting solution to increase the rate of the reactions involved.

Iridium is removed with 4 to 6 *M* hydrochloric acid. This process, like the elution of rhodium, is rather slow.

Our experiments appear to show that sulfate ion prevents the adsorption of rhodium and iridium by the resin, presumably by forming neutral or anionic complexes with the metal cations.

This separation has been used in conjunction with other simple chemical steps for the radiochemical separation of pure rhodium in good yield from uranium fission products.

Further research will be done on this process.

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PEROXIDE-INDUCED ADDITIONS OF METHYL FORMATE TO OLEFINS

Sir:

Preliminary studies with the peroxide-induced reaction of ethyl formate with ethylene gave complex products which contained both the ethyl esters of telomeric aliphatic acids, $H-(CH_2CH_2)_x-COOC_2H_5$, and formate esters of telomeric secondary alcohols, $H-COOCH(CH_3)-(CH_2CH_2)_x-H$. Methyl formate was successfully used in the work reported here to reduce to a minimum products of the latter type, and telomeric methyl esters, $H-(CH_2CH_2)_x-COOCH_3$, have been obtained.

A solution of *t*-butyl peroxide (8.8 g., 0.061 mole) in methyl formate (600 g., 10.0 moles) was held at 130° in a glass-lined, stainless steel autoclave under a pressure of ethylene (methyl formate vapor pressure plus ethylene, 340–440 p.s.i.) until its absorption ceased (20 hours). Distillation of the reaction mixture gave unreacted methyl formate and a higher boiling product (70–80 g.). Its further distillation through a small fractionating column packed with a wire spiral gave the following products: (1) a series of fractions containing *t*-butyl alcohol and methyl propionate (5.78 g., b.p. 72–84°, $n^{20}D$ 1.3761–1.3827, m.p. of *p*-toluidide 123°)[1,2]; (2) methyl valerate (6.91 g., b.p. 130°, $n^{20}D$ 1.3980, m.p. of *p*-toluidide 69–69.5°)[2]; (3) methyl enanthate (7.94 g., b.p. 113–116° at 100 mm., $n^{20}D$ 1.4165, m.p. of *p*-toluidide 79.5–80°, m.p. of mixture with an authentic sample gave no depression, enanthic acid made by carbonation of *n*-hexyl-magnesium bromide); (4) methyl pelargonate (6.24 g., b.p. 88–91° at 8 mm., $n^{20}D$ 1.4262, m.p. of *p*-toluidide 83.5°)[2]; (5) methyl hendecanoate (5.02 g.,

(1) All toluidides were prepared by the method of C. F. Koelsch and D. Tenenbaum, THIS JOURNAL, **55**, 3049 (1933).

(2) R. L. Shriner and R. C. Fuson, "Identification of Organic Compounds," John Wiley and Sons, Inc., New York, N. Y., 1948, p. 222.