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Carboxypeptidase in Mammalian Tissue.* (20249)

ROBERT N. FEINSTEIN AND JOHN C. BALLIN.† (Introduced by J. M. Coon.)
From the USAF Radiation Laboratory and the Departments of Biochemistry and Pharmacology, University of Chicago, Ill.

The term "carboxypeptidase" is used in two distinctly different senses. On the one hand, it refers specifically to the enzyme entity first isolated in crystalline form from pancreas by Anson(1). On the other hand, it is used in more general reference to an enzyme or group of enzymes found in any of several tissues and classified by Fruton, Irving, and Bergmann(2) as Cathepsin IV, and later by Tallan, Jones, and Fruton(3) as carboxypeptidase. The pancreatic carboxypeptidase is usually assayed at pH 7.3-7.8 in the absence of cysteine, which Smith and Hanson(4) found actually to inhibit this enzyme. The carboxypeptidase (Cathepsin IV) of spleen and kidney was measured by Fruton, Irving, and Bergmann(2) at pH 5.0-5.1, and 0.01 M cysteine was used for maximum activity.

Work in this laboratory has demonstrated the existence in a wide variety of tissues of both types of carboxypeptidase. We have designated as carboxypeptidase *a* (CP*a*) that enzyme which requires cysteine for activity and which has optimal activity at pH 5.5-6.5 and 8.0-8.5. This enzyme has not previously been detected in many tissues because, first, it is much less active in the neighborhood of pH 7.5, and second, because most tissues also contain a cellular inhibitor of this enzyme. We have designated as carboxypeptidase *b* (CP*b*) that enzyme which has pH optima of 5.5 and 7.5 and is active in the absence of cysteine; in fact, the cysteine concentration used in the assay of CP*a* completely inhibits CP*b*. The natural tissue inhibitor of CP*a* is without effect on CP*b*. The inhibitor is apparently protein in nature, is generally found with the particulate matter in centrifuged homogenates, and inhibits the enzyme in a non-competitive fashion.

Experimental. Animals used were normal, Sprague-Dawley or Maguran rats and Carworth Farm mice. In general, CP assays were carried out by incubating a given tissue fraction with chloracetyl-L-tyrosine, with or without cysteine, for 10 minutes at 37°C. The chloracetyl-L-tyrosine was present in a final concentration of 0.0088 M. Tissue preparation, substrate, and cysteine were each brought to pH 5.5 before the assay. Aliquots were removed immediately after mixing, and again exactly ten minutes later, into 10 volumes of absolute alcohol. The 2 alcoholic solutions were then titrated with alcoholic KOH, approximately 0.01 N, using thymolphthalein as indicator. The titration thus resembles that of Grassmann and Heyde(5). That the titration increment is directly proportional to the amount of enzyme source added is demonstrated by the data of Fig. 1. Protein was determined by the method of Robinson and Hogden(6), as modified by Keyser and Vaughn(7).

Results. Efforts were first directed at the detection of CP in a variety of tissues. When whole homogenates (9 ml H_2O per g wet tissue) were used with 0.0088 M cysteine present, enzyme activity was detected in rat kidney but not in liver or intestine. The

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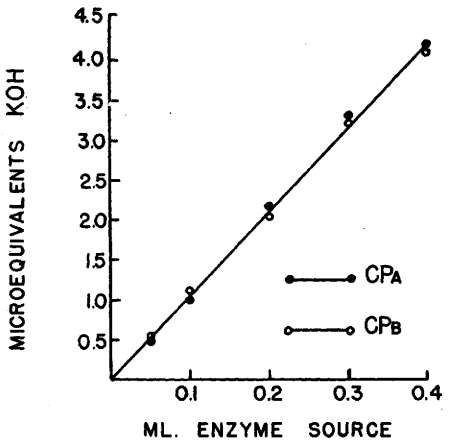

FIG. 1. Linearity of titration increment with respect to amount of enzyme source. Ordinate represents difference in titration of aliquots taken immediately after mixing, and 10 min. later. Abscissa gives volume added of approximately 40% rat kidney homogenate. The unusually concentrated enzyme source was used in order to permit a wider variety of aliquot sizes. pH = 5.5; temp.= 37.0°C.

possibility was investigated that some "inactive" tissues might actually contain CP, and also a CP inhibitor (CPI), which would prevent the demonstration of CP activity. This consideration was encouraged by the finding that the CP activity of whole rat kidney homogenate was increased 23% by shaking with $CHCl_3$, with a simultaneous reduction in protein content of 53%. There was thus obtained an increase of 180% in CP activity per mg protein.

If the $CHCl_3$ phase is evaporated to dryness at room temperature and the residue resuspended in water, it has no effect on the CP activity of the aqueous phase; it thus appears likely that treatment with $CHCl_3$ destroys the inhibitor, probably by denaturation.

In order to obtain the inhibitor in assayable form, an attempt was made to separate it by differential centrifugation after water homogenization.

In early experiments, 4 fractions were separated, representing cellular debris and nuclei (700 g), mitochondria (5,000 g), a high-speed fraction (18,000 g), and the residual supernatant. The bulk of the CP activity is found in the high-speed supernatant. If we arbitrarily assign the value of 100 CPU/mg protein to this fraction, then the activities of the other fractions of a rat kidney homogenate in a typical experiment were: whole homogenate, 13; nuclear fraction, 0; mitochondria, 15; and high-speed precipitate, 0. Because of this distribution, our routine technic has been to homogenize the tissue with nine volumes of cold distilled water, then centrifuge for 30 minutes at 18,000 g in the cold. The supernatant is assayed for CP activity, and the precipitate is re-suspended by homogenization with the original volume of cold distilled water and assayed for inhibitor.

It has been found that the amount of inhibition is linearly proportional to the volume of well-suspended inhibitor source added, and so we have defined our enzyme and inhibitor units as follows: a) Carboxypeptidase unit (CPU): that amount of enzyme which causes

TABLE I. Distribution of Carboxypeptidase *a* and *b* and Carboxypeptidase *a* and *b* Inhibitor in Normal Rat Tissue.

Tissue	Whole homogenate	Supernatant (enzyme fraction)	Precipitate (inhibitor fraction)
	CP*a*U	CP*a*U	CP*a*IU
Blood	—	4	130
Kidney	60	132	72
Spleen	0	59	89
Liver	3	59	59
Testis	17	59	104
Intestine	4	93	97
Heart	0	131	118
Brain	63	143	101
Lung	0	56	46
Stomach	66	69	0
Skeletal muscle	0	6	16
	CP*b*U	CP*b*U	CP*b*IU
Blood	—	3	0
Kidney	162	171	3
Spleen	85	56	3
Liver	74	59	0
Testis	8	59	0
Intestine	84	84	13
Heart	80	72	0
Brain	80	76	0
Lung	6	16	7
Stomach	56	49	3
Skeletal muscle	3	0	0

Blood divided into cells (here "precipitate") and plasma (here "supernatant") by low speed centrifugation. Other tissues homogenized in H_2O, and a portion centrifuged at 18000 g for 30 min. Whole homogenate and supernatant assayed for carboxypeptidase *a* (CP*a*) and carboxypeptidase *b* (CP*b*); precipitate returned to original volume with water and assayed for CP*a* inhibitor (CP*a*I) and CP*b* inhibitor (CP*b*I). All figures based on 1 g (or in case of blood, 1 ml) original wet tissue.

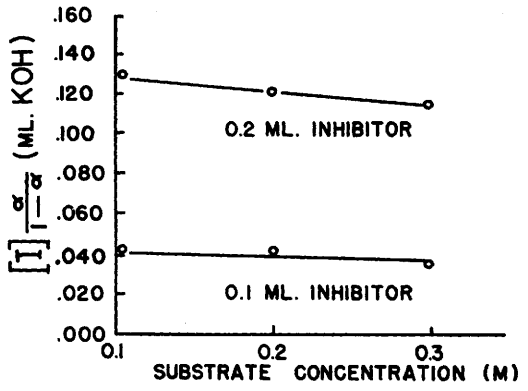

FIG. 2. Non-competitive nature of carboxypeptidase inhibition by rat kidney residue fraction. I = conc. of inhibitor; V and V_1 = velocity in absence or in presence of inhibitor, respectively; $\alpha = \frac{V_1}{V}$.

an increase of one microequivalent of alkali titration under our standardized conditions, and b) Carboxypeptidase inhibitor unit (CPIU): that amount of inhibitor which reduces the enzyme activity of any CP preparation by one CPU.

By the use of this centrifugation technic, we have found that CP is more widely distributed than generally recognized, but its presence is masked in whole tissue preparations by the simultaneous presence of inhibitor. Table I indicates the extent of this distribution. In Table I, 20 units of enzyme or inhibitor is considered the approximate limit of accuracy of the method. This high value is due to a large dilution factor. The actual titration is reproducible to approximately 0.1 microequivalent of alkali.

That the inhibitor is non-competitive in its action is shown by Fig. 2, the results of an experiment based on the suggestion of Massart(8) for determining the type of inhibition. That it is probably protein in nature is indicated by the data of Table II where it is seen that the inhibitor is heat-labile and non-dialyzable. The non-dialyzability, however, may mean only that the inhibitor is in suspension and not in true solution. Because of the possibility that the blood content of the various tissues may be responsible for all inhibitor noted, hemoglobin was tested for inhibitor activity and found negative. This question is further discussed below. Urine also was found to be inhibitor-free.

All of the above results were obtained with experimental systems containing 0.0088 M cysteine at pH 5.5. The possibility was next considered that two different enzymes may be present. This has proven to be the case.

Until such time as 2 distinct enzymes are actually isolated, the demonstration that 2 separate entities are involved, and that no overlapping of activities occurs, must be an indirect one. Data of a typical experiment are presented in Table III. These data, it seems to us, are very suggestive of one particular set of interpretations. Since the inhibitor fraction inhibits all CP*a* activity (*i.e.*, in presence of cysteine), but has *no inhibitory effect whatsoever in the absence of cysteine,* then no part of the CP*b* activity (*i.e.* in absence of cysteine) can be attributed to the CP*a*. On the other hand, since it is seen that the cellular inhibitor has no effect on CP*b*, yet permits *no residual activity whatsoever in the presence of cysteine,* then no part of the CP*a*

TABLE II. Evidence for the Protein Nature of Carboxypeptidase Inhibitor.

Addition to standard enzyme	CP*a*U/ml	CP*a*IU/ml
None	32	—
I,* freshly prepared	20	12
I, heated 1 hr at 37°C	20	12
I, " 3 min. at 100°C	32	0
None	28	—
I, 24 hr in cold	17	11
I, dialyzed 24 hr in cold	18	10

* I = Inhibitor fraction.

Normal rat kidney homogenized in H_2O and centrifuged at 18000 g 30 min. Supernatant assayed for carboxypeptidase *a* (CP*a*); precipitate returned to original volume with H_2O and assayed for carboxypeptidase *a* inhibitor (CP*a*I). All units based on 1 g original wet tissue.

TABLE III. Evidence for Co-Existence of Two Carboxypeptidases.

Components present	Titration increase, meq.
Enzyme + cysteine	.54
" + cysteine + inhibitor	.00
" alone	.30
" + inhibitor	.31

"Enzyme" and "inhibitor" were supernatant and precipitate fractions, respectively, of homogenized, centrifuged rat kidney. Cysteine was present, where indicated, at 0.0088 M.

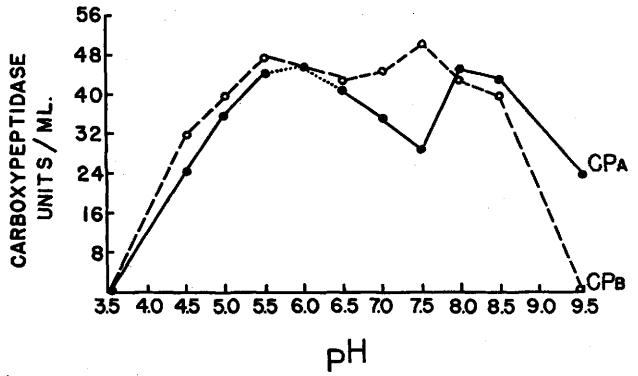

FIG. 3. Effect of pH on activity of carboxypeptidase *a* and *b*. Technical difficulties made it necessary to determine the pH 6 point in a separate experiment, at which time the pH 5.5 and 6.5 points were repeated for purposes of curve-fitting. This is the reason for the dotted area of the curve.

activity can be attributed to the CP*b*. This question is further discussed below.

Fig. 3 shows pH activity curves for CP*a* and CP*b* in freshly prepared kidney homogenate supernatant. It will be noted that pH optima for CP*a* are at pH 5.5-6.5 and 8.0-8.5, and for CP*b*, pH 5.5 and 7.5. It is of particular interest that at pH 7.5, where the cysteine-inhibited carboxypeptidase is ordinarily assayed, the cysteine-requiring enzyme has minimal activity. In some preparations we have actually failed to detect any CP*a* activity whatsoever at pH 7.5, while considerable activity is shown at pH 5.5. From the fact that each enzyme apparently has two pH optima, the possibility must be considered that we actually are dealing not with 2, but with 4, enzymes. However, no other evidence has been observed to bolster this possibility.

Discussion. The possibility that all observed inhibitor of tissues may be due to the blood cells present in the tissue is further discussed in the succeeding paper. This possibility is fortified by the data of Table I, in which the relatively enormous concentration of inhibitor in blood cells is apparent. The possibility has been suggested that the entire phenomenon of inhibition may be merely one of adsorption of the enzyme onto particulate matter, since the inhibitor has not yet been separated from a particulate fraction. This seems to us unlikely, for two reasons: First, if it were so, the amount of inhibition should be critically dependent upon the fineness of re-suspension of the precipitate fraction; we have never detected such dependence. Second, if this were so, it seems unlikely that we could ever separate enzyme and inhibitor by simple centrifugation. The critical question yet remains, however, as to just how the inhibitor does function, since we have thus far failed to obtain the inhibitor in true solution.

The evidence given in Table III for the co-existence of two carboxypeptidases cannot be considered absolute proof. Other, though less likely, possibilities may be suggested. For example, it is conceivable that the cysteine activates not only carboxypeptidase *a*, but the inhibitor as well; if this is so, then the line of reasoning presented is flawed.

The possibility has been considered that the enzyme we have chosen to call carboxypeptidase *b* is actually identical with the cathepsin I of Fruton *et al.*(2). Definitive proof is difficult to obtain, yet one piece of evidence appears to favor the non-identity of CP*b* with cathepsin I. Fruton *et al.* refer to the thermolability of cathepsin I as a distinguishing feature; their data show a 52% decrease in activity of cathepsin I in beef kidney, and a 24% decrease in swine kidney, after heating for 15 minutes at 50°C. Under these circumstances, we have found the CP*b* of rat kidney to be decreased only 8%, while the CP*a* in the same preparation was decreased 26%. Although suggestive this cannot be considered firm proof, however, because of the possibility of species variation in this respect.

The possibility was also considered that our

CP*a* and CP*b* may be, respectively, the pro-carboxypeptidase and carboxypeptidase of Anson(9). To test this, a portion of kidney supernatant was treated with trypsin according to Anson's method for assay of the enzyme and zymogen. The treated and untreated portions were then assayed for CP*a* and CP*b*. The only effect of the trypsin treatment was an overall diminution in both enzymes; the ratio of the two was unaffected. It is thus apparent that CP*a* is not a trypsin-activatable precursor of CP*b* and so is not identical with Anson's pro-carboxypeptidase.

Preliminary experiments indicate that if a kidney homogenate is permitted to stand at room temperature for several days under a layer of toluene, the amount of CP*b* increases at the expense of CP*a*, which decreases to an approximately equivalent degree. This interconversion is apparently enzymatic in nature and occurs only at certain pHs, of which 8.5 appears to be optimum. The reverse reaction has not been detected.

Summary. Evidence is presented that the enzyme carboxypeptidase is more widely distributed throughout mammalian tissues than is commonly realized. The reasons for previous failure to detect the enzyme are: a) the existence of a natural cellular inhibitor, the effect of which is to mask the enzymatic activity, and b) the fact that two carboxypeptidases probably co-exist in many tissues; the pH optima of these two are different, and one is activated, the other inhibited, by cysteine. The natural cellular inhibitor affects only one of the carboxypeptidases, and does so in a non-competitive fashion.

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Development of Sarcomas in Marsh-Albino Mice Following Injection of Desoxycorticosterone Acetate in Sesame Oil.* (20250)

E. A. MIRAND, M. C. REINHARD, AND H. L. GOLTZ. (Introduced by G. E. Moore.)
From the Roswell Park Memorial Institute, Buffalo, N. Y.

While investigating strain differences in mice, an incidental observation was made in the Marsh strain, namely the development of tumors following prolonged injections of a mineral corticoid, desoxycorticosterone acetate (DCA; Organon's Doca Acetate) in sesame oil. This development has interest in view of the numerous reports that the administration of DCA in various forms has no tumorigenic effect [Kuhlman *et al.*(1), Carnes *et al.*(2), Ludden *et al.*(3), Shimkin and Grady(4), Shimkin and White(5), Burrill and Greene(6), Forbes(7), Rakoff *et al.*(8), Rodard and Freed(9), Gineste *et al.*(10), Sarason(11), Selye and Hall(12), Selye *et al.*(13), Bruzzone *et al.*(14), Segaloff(15), and Lipschutz(16)]. Shimkin and Wyman(17), however, implanted DCA pellets in C3H mice, and mammary tumors developed. These authors considered the results to be insignificant in view of the small number of animals involved; furthermore, the C3H strain is known to produce spontaneous mammary adenocarcinomas at an early age. Woolley

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