CARBOXYPEPTIDASE

III. THE ESTIMATION OF CARBOXYPEPTIDASE AND PRO-CARBOXYPEPTIDASE

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This paper describes the estimation of carboxypeptidase, CP, by formol titration, with chloracetyl tyrosine or a peptic digest of edestin as substrate. To estimate the inactive precursor of carboxypeptidase, pro-carboxypeptidase [PCP], the precursor is activated with trypsin and the resulting carboxypeptidase is estimated.

Peptic digests have not hitherto been used as substrates of carboxypeptidase because they are digested not only by carboxypeptidase but also by other proteolytic enzymes of the pancreas. I have found, however, that carboxypeptidase activity is not stopped by formaldehyde and that the digestion of a peptic digest by a crude pancreatic extract is due entirely to the carboxypeptidase in the extract, if the digestion is carried out in the presence of formaldehyde. If a crude extract of pancreas and a given solution of crystalline carboxypeptidase have the same activity as measured with chloracetyl-tyrosine, they likewise have the same activity as measured with a formolized peptic digest of edestin.

The peptic digest is much cheaper than chloracetyl-tyrosine, which is important when many thousands of analyses have to be made. Furthermore, the peptic digest is stable at the pH used for estimation, whereas a fresh chloracetyl-tyrosine substrate solution has to be made every day.

Preparation of Chloracetyl-Tyrosine Substrate.—To 2 gm. of chloracetyl-tyrosine (Hoffman-La Roche) are added 50 ml. of 0.02 M di-potassium phosphate, 15 ml. of 36–38 per cent formaldehyde solution, 2 ml. of 0.5 per cent phenolphthalein in 50 per cent alcohol, enough 0.5 N sodium hydroxide to dissolve the
chloracetyl-tyrosine and make the solution just detectably pink, and finally enough water to make the total volume 100 ml.

**Preparation of Formalized Peptic Digest of Edestin.**—To 100 gm. of edestin (Pfanstiehl) are added 200 ml. 1 N hydrochloric acid, 500 ml. of water, 4 gm. pepsin (Parke, Davis), and a little toluol. The solution is kept at 37°C. overnight and is then filtered. To each 100 ml. of filtrate are added 3 gm. of decolorizing charcoal (Norit) and 1 gm. of the filter aid Standard Super-Cel (Johns-Manville), and the suspension is filtered on a Buchner funnel through a bed of Standard Super-Cel. 5 N sodium hydroxide is added to the filtrate until the solution is alkaline to thymolphthalein and the resulting precipitate is filtered off with the aid of Standard Super-Cel. The further addition of sodium hydroxide should cause no further precipitation. It might be thought that the three steps which have just been described could better be combined but the charcoal removes more color if the residue not dissolved by pepsin is first removed and if the solution is kept acid. To each 50 ml. of the clear alkaline solution are added 15 ml. of formaldehyde, 2 ml. of 0.5 per cent phenolphthalein, enough 5.0 N sodium hydroxide to make the solution just detectably pink, and enough water to make the total volume 100 ml. The solution is stored at room temperature and is not used until it has stood overnight. If the solution ceases to be pink more sodium hydroxide is added.

**Preparation of Standards.**—The standard pink solutions are kept in tightly stoppered, lipless 50 ml. centrifuge tubes. They contain roughly 0.0007 per cent phenolphthalein completely converted into the colored form by an excess of sodium hydroxide. The total volume is roughly 7.5 ml., which is about the volume of the titration mixture. The edestin standard contains 2.5 ml. of the peptic digest which has been made alkaline and filtered but to which the water, formaldehyde, and indicator have not yet been added. Thus, both the pink standard and the digestion mixture contain the same amount of brown peptic digest in the same volume and a good match is obtained at the end point of the titration. When the colorless chloracetyl-tyrosine is used as a substrate, the standard is simply an alkaline solution of phenolphthalein.

**Technique of Formol Titration.**—In the estimation of proteolytic enzymes by formol titration the usual procedure is to add enzyme to a substrate solution, to stop the digestion after a given time by the addition of formaldehyde, and finally to add phenolphthalein and find out how much sodium hydroxide is needed to make the solution as pink as a standard pink solution. A blank titration is done on a solution to which formaldehyde has been added before the addition of enzyme; i.e., on a solution in which no digestion has taken place. The increase in titration due to digestion is a measure of the number of amide linkages split. This usual procedure has to be slightly modified for the estimation of carboxypeptidase because carboxypeptidase activity, unlike tryptic activity, is not stopped by formaldehyde. A slightly alkaline substrate solution is used which already contains formaldehyde and phenolphthalein. The blank is obtained by titrating immediately after the addition of enzyme. The digestion is then measured by
titrating 10 minutes after the addition of enzyme. Since the formaldehyde and the indicator are not added separately and since the acid groups "liberated" from the peptic digest are already neutralized in the substrate solution, the modified procedure for formol titration is even simpler than the ordinary procedure.

The Blank Titration.—1 ml. of enzyme solution is added to 5 ml. of substrate solution in a tube like that in which the standard is kept, the tube is whirled to mix the solutions, and the mixture immediately titrated with 0.02 N sodium hydroxide until its color matches that of the standard. It is important that the titration be carried out rapidly because digestion goes on and carbon dioxide is absorbed during the titration.

The Digestion Titration.—If the blank titration with enzyme added is more than 0.2 ml. more than the blank titration with water added, then, in order that the digestion mixture should have the proper pH, an amount of 0.02 N sodium hydroxide is added to the substrate solution sufficient to make the blank with enzyme plus sodium hydroxide the same as that with water alone without enzyme or added sodium hydroxide. After the enzyme is added the tube is stoppered and titrated after 10 minutes. The substrate solution is brought to 25°C. before the enzyme is added and the digestion mixture is kept in a 25°C. water bath during the digestion.

If the increase in formol titration is more than 1.8 ml. with chloracetyl-tyrosine as substrate or 0.8 ml. with the peptic digest as substrate, then the digestion is repeated with less enzyme or with a shorter digestion time. If the increase in titration with chloracetyl-tyrosine as substrate is more than 1.2 ml. but less than 1.8 ml. then after 6 minutes of digestion 0.7 ml. of sodium hydroxide is added to the digestion mixture and the titration is completed after 10 minutes as usual.

Estimation of Pro-Carboxypeptidase.—The pro-carboxypeptidase is activated with crystalline trypsin (Kunitz and Northrop (1935-36)) and the resulting carboxypeptidase is estimated as already described. The concentration of pro-carboxypeptidase to be estimated should be such that 1 ml. of the activated solution can be used directly for the estimation of carboxypeptidase.

The trypsin is dissolved in 0.02 N hydrochloric acid to give a solution having $2 \times 10^{-5}$ hemoglobin units (Anson and Mirsky (1933-34)) per ml. 1 drop of this solution is used for each milliliter of pro-carboxypeptidase solution. First the trypsin is put in a test tube, then an equal volume of 1 M di-potassium phosphate, then the pro-carboxypeptidase. Usually this solution is red to phenol red; if it is not, 0.1 N sodium hydroxide is added until the solution is red to phenol red and the volume of sodium hydroxide added is recorded. The test tube is left for 5 minutes in a water bath kept at 37°C.

The final solution is slightly alkaline because trypsin acts most rapidly in slightly alkaline solution. The alkali is added after the trypsin and before the enzyme in order to protect the enzyme from the acid in the trypsin solution.

Doubling the amount of trypsin or the activation time does not affect the results.
The Activity Units.—A solution of carboxypeptidase by definition has 1 carboxypeptidase unit per ml. ([CP.u.] mL.) when it causes an increase in formol titration under standard conditions at the initial rate of 1 milliequivalent of sodium hydroxide per minute (cf. Northrop (1932–33)). An amount of crystalline carboxypeptidase which has 1 mg. nitrogen has 0.081 [CP.u.] CT when the substrate is chloracetyl-tyrosine and 0.103 [CP.u.] PDE when the substrate is a peptic digest of edestin. Thus 1 [CP.u.] CT is equivalent to 1.27 [CP.u.] PDE. Initially the peptic digest is digested by a given amount of carboxypeptidase 1.27 times more rapidly than is chloracetyl-tyrosine.

A solution of pro-carboxypeptidase has 1 pro-carboxypeptidase unit [PCP.u.] if after activation with trypsin it has 1 carboxypeptidase unit.

Activity Curve.—Fig. 1 shows the extent to which chloracetyl-tyrosine and a peptic digest of edestin are digested by increasing amounts of crystalline carboxypeptidase in 10 minutes under the standard conditions described. The digestion of chloracetyl-tyrosine is proportional to the amount of enzyme until the increase in formol titration is 1.6 ml. of 0.02 N sodium hydroxide. In the case of the digestion of a peptic digest of edestin the deviation from proportionality begins with very small amounts of digestion and the peptic digests of other easily available proteins are even less satisfactory than the peptic digest of edestin. If the substrate concentration is increased then the extent of digestion is proportional to the amount of enzyme for greater extents of digestion. This advantage, however, is more than outweighed by the disadvantage that with higher concentrations of substrate the substrate solutions are more highly buffered and hence the titration is less sensitive. With the concentrations of substrate used the titrations are sensitive to 0.05 ml. of 0.02 N sodium hydroxide.

Calculations.—If the digestion is carried out with 1 ml. of enzyme solution, then the carboxypeptidase units of the carboxypeptidase in 1 ml. of solution are read directly from Fig. 1. If the digestion time is 5 minutes instead of 10 minutes, then the carboxypeptidase units values must be doubled. If the digestion time is 20 minutes, then the carboxypeptidase units values must be halved.

Specificity of Formalized Peptic Digest as Substrate.—The evidence that in the presence of formaldehyde carboxypeptidase alone is re-
sponsible for the digestion of a peptic digest of edestin by a crude extract of pancreas is that the same values for the carboxypeptidase activity of a crude extract are obtained whether chloracetyl-tyrosine or the peptic digest is used as a substrate. It may be that there are proteolytic enzymes other than carboxypeptidase which attack peptic digests in the presence of formaldehyde. If there are, their quantitative importance in crude pancreatic extracts is small compared with that of carboxypeptidase. None of the known proteolytic enzymes of the pancreas other than carboxypeptidase has been shown to be active in the presence of formaldehyde.

Reproducibility of Formalized Peptic Digest as Substrate.—Trypsin digests different proteins, even samples of edestin prepared in different ways, at quite different rates. In order to obtain reliable absolute values for trypsin activity it is necessary to use a highly reproducible substrate such as hemoglobin. Carboxypeptidase in contrast attacks the peptic digests of widely different proteins at about the same initial rate, and in the case of edestin small differences in the preparation of the peptic digest do not cause any change in the rate at which the digest is attacked by carboxypeptidase. Since, however, the reproducibility of peptic digests is always suspect, every batch is checked
by means of an enzyme solution whose activity has been measured with chloracetyl-tyrosine as a substrate.

*Stability of Substrates.*—The chloracetyl-tyrosine solution changes slowly. It should be kept cold and used the day it is prepared. The formalized peptic digest of edestin does not change in a month when kept at room temperature.

*Effect of Tyrosine and Glycerine.*—The digestion of chloracetyl-tyrosine or a peptic digest of edestin by crystalline carboxypeptidase is not changed by the addition to the digestion mixture of 1 ml. of 4 per cent glycerine or 1 ml. of 0.02 M tyrosine.

*Effect of Acid.*—Since acid slows digestion by carboxypeptidase any acid introduced with the enzyme solution is neutralized before digestion is begun. The amounts of acid produced during the digestion of the peptic digest which causes an increase in formol titration of less than 0.8 ml. of 0.02 N sodium hydroxide do not affect the rate of digestion. In the digestion of chloracetyl-tyrosine the acid formed is important if it causes an increase in formol titration of more than 1.2 ml. of sodium hydroxide. If the final increase in formol titration is to be more than 1.2 ml. of sodium hydroxide part of the acid formed by the digestion should therefore be neutralized with sodium hydroxide during the digestion, as has been described.

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