Observations on the periodide method for the estimation of choline

According to BEST AND LUCAS[1], who have reviewed the methods available for the quantitative determination of choline, the most sensitive microchemical method depends upon precipitation of choline as choline periodide. APPLETON, LA DU, LEVY, STEELE AND BRODIE[2] have recently described a procedure, based on this principle, for the determination of choline in plasma, by which 0.05–0.5 μmole can be accurately estimated. In this method the precipitate of choline periodide is dissolved in ethylene dichloride and the optical density of the solution compared at 365 mμ with that of known amounts of choline similarly treated. At this wavelength choline periodide shows a strong absorption band, while interference by traces of iodine contaminating the precipitate is minimal. Possible losses of choline periodide entailed in washing the precipitate as a necessary preliminary to the chemical estimation of the iodine present in it are thus avoided, and the method has the advantages of speed and simplicity. APPLETON *et al.*[2] point out however that their procedure, as described for plasma, cannot be applied directly to the determination of choline in tissue extracts. Nevertheless HAYAISHI AND KORNBERG[3] have adapted a similar technique to the estimation of choline released from lecithin by the action of a bacterial enzyme.

The present communication deals with a brief investigation into the method, with a view to its use for the measurement of choline released enzymically from choline-containing phospholipids by tissue preparations.

In preliminary experiments it was found that precipitation as choline periodide gave satisfactory recovery of choline from aqueous 5% trichloroacetic acid solutions. Furthermore, chloroform (Hopkin and Williams, Analar) was found to be a satisfactory solvent for the precipitate, and has the advantage over ethylene dichloride that it can easily be obtained pure. In the technique finally adopted, 0.5 ml samples of 5% (w/v) trichloroacetic acid containing suitable amounts of choline were placed in a finely pointed centrifuge tube and 0.2 ml of KI_3 reagent, consisting of 15.7 g of iodine (B.D.H., Analar) and 20 g potassium iodide (B.D.H., Analar) made up to 100 ml in distilled water, was added. After stirring, cooling in ice, centrifuging and removing the supernatant fluid as described by APPLETON *et al.*[2], the precipitate was dissolved in 10 ml chloroform and the optical density read in an ultraviolet Spectrophotometer at 365 mμ. Stable readings were obtained over a period of several hours. Experiments showed that the optical densities of such solutions do not obey Beer's Law. Molar extinction coefficients ($\pm$ coefficients of variation) calculated from the optical densities obtained for standards of 10, 20 and 40 μg of choline chloride in 16 replicate determinations were $1.8 \cdot 10^4$ ($\pm$ 4.4%), $2.3 \cdot 10^4$ ($\pm$ 1.8%), and $2.6 \cdot 10^4$ ($\pm$ 1.3%) respectively. The optical density of blanks averaged only 0.007. These values agree well with the data given by APPLETON *et al.*[2] and by HAYAISHI AND KORNBERG[3].

With regard to the specificity of the method, it was found that the acetyl, propionyl, butyryl, acetyl-β-methyl and benzoyl esters of choline, and also dimethylaminoethyl acetate gave precipitates with the iodine reagent under the above conditions. Solutions of these precipitates in chloroform showed in each case an absorption peak at 365 mμ. The molar extinction coefficients of the choline derivatives did not differ significantly from that of the iodine complex of choline. Impure preparations of ovolecithin and human brain sphingomyelin also formed insoluble complexes with iodine which, when dissolved in chloroform, absorbed maximally at 365 mμ. HAYAISHI AND KORNBERG[3] reported similar findings for lecithin. Several other substances are known to give precipitates with the triiodide reagent[1]. In agreement with APPLETON *et al.*[2], pure solutions of phosphoryl choline, prepared by the method of RILEY[4], and glycerophosphoryl choline gave no precipitate.

Quantitative recovery of added choline was obtained from brain homogenates, and from emulsions of ovolecithin, after addition of an equal volume of 10% trichloroacetic acid, suggesting that precipitation of phospholipids by this concentration of trichloroacetic acid was essentially complete.

Experiments were carried out in which phosphoryl choline was hydrolysed by an acetone-dried preparation of human cerebral cortex at pH 8.9 under the conditions described by STRICKLAND, THOMPSON AND WEBSTER[5], followed by estimation of both free choline and inorganic phosphate in trichloroacetic acid filtrates of the incubation media. The free choline:phosphate ratio found in these experiments averaged 1.30 instead of the theoretical value of 1.0. In this system, however, the molar concentration of unhydrolysed phosphoryl choline at the end of the experiment was 6–7 times greater than that of the free choline. When experiments were carried out using standards containing choline and phosphoryl choline in these proportions, the free choline:phosphate ratio was reduced to 1.05. It appears, therefore, that although phosphoryl choline itself does not form an insoluble complex with iodine, possibly because of its acidic nature, its presence influences the degree of precipitation of the iodine complex of choline. The presence of a relative excess of glycerophosphoryl choline also caused a slight increase in the apparent amount of choline estimated.

It was concluded from these investigations that provided care is taken to use standards

containing amounts of phosphoryl choline and glycerophosphoryl choline similar to those in the solutions to be assayed, and appropriate blanks and controls, the technique outlined above offers a means of estimating, chemically, amounts of choline between 0.05–0.5 μmole which could be applied to the study of the enzymic breakdown of choline-containing phospholipids by tissue preparations.

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[1] C. H. BEST AND C. C. LUCAS, *Vitamins and Hormones*, 1 (1943) 1.
[2] H. D. APPLETON, B. N. LA DU, Jr., B. B. LEVY, J. M. STEELE AND B. B. BRODIE, *J. Biol. Chem.* 205 (1953) 803.
[3] O. HAYAISHI AND A. KORNBERG, *J. Biol. Chem.*, 206 (1954) 647.
[4] R. F. RILEY, *J. Am. Chem. Soc.*, 66 (1944) 512.
[5] K. P. STRICKLAND, R. H. S. THOMPSON AND G. R. WEBSTER, *J. Neurol. Neurosurg. Psychiat.*, (1956) (in press).

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Preliminary Notes

The action of enterokinase on trypsinogen

Trypsinogen is activated to trypsin either by trypsin itself or by enterokinase[1]. In the former case, a peptide is split off from the amino-end of trypsinogen, the amount of peptide split off being proportional to the tryptic activity developed as suggested by ROVERY, FABRE AND DESNUELLE[2] and recently confirmed by DAVIE AND NEURATH[3]. However, no studies have been made of the chemical changes taking place during the activation by enterokinase which seems to be highly specific for trypsinogen.

In this communication, it is shown that *iso*leucine is the N-terminal amino acid of trypsin developed from trypsinogen by enterokinase activation.

Trypsinogen was prepared from beef pancreas according to the method of NORTHROP AND KUNITZ[4] through crystallization and further purification by trichloracetic acid. Enterokinase of a high degree of purity was prepared from swine duodenal fluid contents according to the author's method[5,6].

The activation was carried out at 0° C and pH 5.6 using 0.1 *M* triethylammonium acetate buffer, 5 mg of trypsinogen and 0.8 mg of enterokinase containing 2000 units per mg being dissolved in 12.5 ml of the activation mixture. Under these conditions, 50% activation could be obtained within 8 minutes. The reaction was essentially of first order kinetics throughout the whole activation without any appreciable signs of autoactivation or autodigestion due to trypsin. The activation was stopped by the addition of 1.0 ml of 1 *N* HCl and aliquots were taken for the measurement of tryptic activity (hemoglobin method) and the rest was submitted to the determination on N-terminal amino acids. EDMAN's method[7] was used for this purpose. Thus, an equal volume of pyridine containing phenyl*iso*thiocyanate in a concentration of 50 μl per ml was added to the activation mixture and the pH was adjusted to 9.0 by the addition of a few drops of triethylamine. The activities of enterokinase and of trypsin were almost instantaneously blocked during this treatment. After the completion of the coupling, the mixture was washed by benzene and the aqueous solution was then lyophilized.

The dried material was hydrolyzed by 1 *N* HCl at 100° C for one hour and the phenylthiohydantoin derivatives of amino acids were extracted by ethyl acetate. The aliquots were