A spectrophotometric microdetermination of choline*

The reaction of choline with iodine to form an insoluble periodide has been used by several workers in choline determinations (see [1] and [2] for review). In these methods, the periodide precipitate is isolated by filtration or centrifugation and the iodine in the complex measured chemically. Recently, APPLETON *et al.*[3,4] introduced a spectrophotometric method in which the periodide precipitate, after centrifuging and removal of the supernatant by aspiration, is dissolved in ethylene dichloride and the amount of choline determined from the optical density of the ethylene dichloride solution at a wave length of 365 mμ. They reported that amounts of choline as small as 5 μg can be measured.

The method reported here is a modification and simplification of that of APPLETON *et al.* The latter used specially made, finely tapered centrifuge tubes. With either ordinary conical centrifuge tubes or such tubes drawn out to a fine tip, however, it has been difficult to obtain accurate, reproducible results, there being always danger either of removing too little of the iodine-containing supernatant or of sucking off some of the minute, finely-packed precipitate. Periodide precipitates formed in the presence of certain biological media, moreover, were sometimes difficult to redissolve.

These sources of error and the need for special equipment can be eliminated by omitting the centrifuging altogether. APPLETON *et al.* have pointed out that in ethylene dichloride the free iodine has a lower extinction coefficient at 365 mμ than does choline periodide. It was found here that the addition of choline to an iodine solution leads to an increase in the *total* optical density at 365 mμ of the ethylene dichloride-soluble material. The increase in optical density is proportional, over a wide range, to the choline concentration, and choline may be determined by measuring this increase. The blank reading due to dissolved iodine may be reduced by lowering the amount of iodine present, although, as the increase in optical density is also affected by the iodine concentration, the blank reading cannot be decreased below about 0.050 without unduly reducing the sensitivity.

The optical density of iodine in ethylene dichloride has been observed to increase with time, sometimes as much as 100% in 30 minutes. The cause of this increase is not known. Shaking in air appears to accelerate the process, and it occurs even with ethylene dichloride purified by redistillation. Variation due to this increase can be minimized by using anaerobic conditions and by standardizing the time lapse between adding ethylene dichloride to the aqueous solution and reading the optical density of the ethylene dichloride layer. Chloroform, benzene, toluene, ether, and petroleum ether have been tested as solvents for this method but have proven unsuitable.

The following method of choline determination is recommended:

Reagents

Choline: Choline chloride (Merck or Nutritional Biochemicals) is dried over silica gel to constant weight, dissolved in water in a concentration corresponding to 1 mg choline per ml, stored in the cold, and diluted as required.

Iodine reagent: 2.5 g I_2 and 3.1 g KI are dissolved in water and made up to a volume of 250 ml.

Ethylene dichloride: The solvent (Fisher or B.D.H.) is refluxed for one hour over zinc dust and NaOH and redistilled.

Procedure

The sample, containing up to 50 μg choline, is placed in a test tube of about 20 ml capacity and made up to 2.0 ml with water. One ml of the iodine reagent is added, the test tube shaken, and placed in an ice bath for 20 minutes. Ten ml ethylene dichloride are added and the two layers mixed by bubbling a fine stream of nitrogen through them for 30 seconds. The upper aqueous layer is sucked off, and, exactly two minutes after adding the ethylene dichloride, the optical density of the ethylene dichloride layer at 365 mμ is measured using a Beckman Model DU spectrophotometer. Choline standards and a water blank are treated with iodine and run through the same procedure, and the blank value (about 0.100 under the above conditions) is subtracted from each of the other readings.

The combined results of four choline concentration-series presented in Fig. 1 show that the increase in optical density over the blank value is proportional to the amount of choline, up to 50 μg. The mean increase in optical density produced by 10 μg choline is 0.171 ± 0.014, and that produced by 50 μg is 0.842 ± 0.016. Thus, this method appears at least as accurate as that of APPLETON *et al.*

The following compounds in 100 μg amounts gave no reaction for choline: DL-alanine,

* Contribution No. 287, Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Ontario, Canada.

L-arginine, L-aspartic acid, L-cysteine, L-cystine, glycine, L-histidine, DL-*iso*leucine, DL-leucine, DL-lysine, DL-methionine, L-phenylalanine, L-proline, DL-serine, DL-threonine, L-tyrosine, DL-valine, ethanolamine, histamine, D-glucosamine, diphenylamine, adenosine, L-asparagine, L-glutamine, and glycyl glycine. Acetylcholine, acetyl β-methylcholine, and tryptophan react with iodine to yield precipitates dissolving in ethylene dichloride and absorbing light at 365 mμ. On a molar basis, the three are respectively 80%, 40%, and 10% as active as choline. Phosphorylcholine gives no reaction by this method.

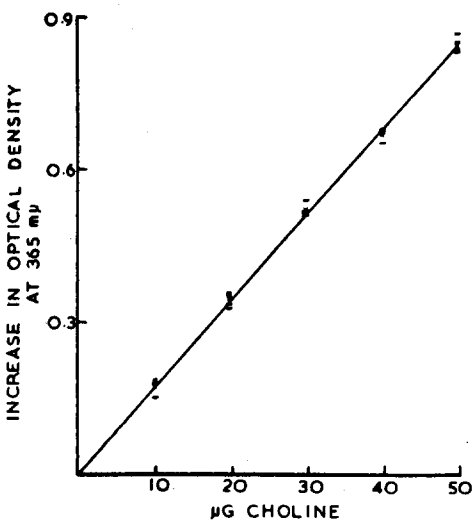

Fig. 1. Choline standard curve. - = observed readings; × = average values.

Since neither serine nor ethanolamine give a reaction for choline, this method should be useful in determining the choline content in hydrolysates of mixed phospholipids. In Table I duplicate figures are given for choline determinations by this method in an alkaline hydrolysate of purified egg lecithin and for the recoveries (97–98%) of added choline. Analysis of a larger amount of the hydrolysate by the reineckate method[5] gave a value of 22.4 μg choline in 180 μg lecithin, as compared to 22.6 μg by the iodine method. In this hydrolysate, then, both the iodine and the reineckate methods give virtually the same result, but the former method is more than 200 times as sensitive. Quantitative recovery of added choline is also observed in the presence of nutrient broth.

TABLE I

ASSAY OF CHOLINE IN A LECITHIN HYDROLYSATE

Material assayed	*Choline present (μg)*			*% Recovery*
	Sample 1	*Sample 2*	*Average*	
Hydrolysate	21.4	23.8	22.6	
Hydrolysate + 10 μg choline	32.6	32.1	32.4	98
Hydrolysate + 20 μg choline	42.3	41.7	42.0	97

45 mg purified lecithin were hydrolyzed with 6.0 ml *N* KOH for 24 hours at 37° C, acidified with glacial acetic acid, and free fatty acids were removed with petroleum ether extraction before diluting for assay. The amounts taken correspond to 180 μg lecithin.

This method is currently being used to measure the enzymic hydrolysis of phosphorylcholine by a crude seminal phosphatase[6].

The recovery of choline added to the phosphatase and measured after deproteinization is only 80–90%. The method of APPLETON *et al.* has also been reported to give low recoveries in crude tissue extracts[6]. In measuring the production of choline in the presence of the phosphatase, it is necessary, therefore, to apply appropriate corrections.

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Forest Insect Laboratory, Sault Ste. Marie, Ontario (Canada) DONN J. KUSHNER

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