Studies on Phospholipids

3. DETERMINATION OF CHOLINE*

By L. W. WHEELDON AND F. D. COLLINS

*Department of Biochemistry, John Curtin School of Medical Research,
The Australian National University, Canberra, A.C.T. Australia*

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Micromethods for the determination of choline have been described by Beattie (1936) and Glick (1944). Both procedures involved the use of Reinecke salt as a precipitant, required 20–150 μ moles of choline and depended on measurement of the pink colour of an acetone solution of the precipitate. More sensitive modifications of the reineckate method depend upon measurement of the chromium content of the precipitate (Marenzi & Cardini, 1943) or the absorption at 328 m μ (Winzler & Meserve, 1945). The use of potassium periodide as a choline precipitant was described first by Stanek (1905) and has since been modified by Erickson, Avrin, Teague & Williams (1940) and by Appleton, La Du, Levy, Steele & Brodie (1953).

Levine & Chargaff (1951) have described a semi-quantitative method involving chromatography on paper and subsequent treatment with phosphomolybdic acid. Choline shows up as a yellow spot which may be reduced with stannous chloride to give a blue colour.

The reaction between choline and phosphomolybdic acid has been investigated and this paper presents the results obtained and provides details of a method for the microdetermination of choline.

MATERIALS

Choline chloride. This material was recrystallized from isobutanol and dried over P_2O_5 .

Choline phosphomolybdate. To 10 ml. of an aqueous solution containing 50 mg. of choline chloride was added 1 g. of phosphomolybdic acid dissolved in 5 ml. of water. The mixture was kept for 30 min. at 5° and the precipitated choline phosphomolybdate was removed by centrifuging. The precipitate was washed twice with isobutanol to remove excess of phosphomolybdic acid, and the solvent was removed by centrifuging and the choline phosphomolybdate dried *in vacuo*.

The solubility of choline phosphomolybdate in 0.1N-HCl corresponded to 0.035 μ mole of choline/0.5 ml. at 20° and 0.008 μ mole of choline/0.5 ml. at 5°; in the presence of excess of phosphomolybdic acid the solubility would be still lower. Phosphomolybdic acid is freely soluble in isobutanol whereas the solubility of choline phosphomolybdate is less than 0.001 μ mole/ml.; this solvent was therefore

used for washing the precipitated choline salt. Choline phosphomolybdate contained 3 moles of choline/g. atom of P.

N-Dinitrophenylethanolamine and **N-dinitrophenylserine** were prepared as described by Wheeldon & Collins (1957).

Lecithin. This was prepared from rat-liver acetone-insoluble lipids by chromatography on alumina as described by Hanahan, Turner & Jayko (1951). The sample contained 110 μ moles of P, 3 μ moles of amino N and 105 μ moles of choline per 100 mg.

Kephalin. This was prepared from ox-brain lipids as described by Folch (1942) and corresponded with the fraction I of Folch. The sample contained 104 μ moles of P, 43 μ moles of amino N and less than 1 μ mole of choline per 100 mg.

The rat-liver and brain lipids and the dinitrophenylated lipids were prepared as described by Wheeldon & Collins (1957).

Phosphomolybdic acid. A.R. grade was used. A saturated solution in water was clarified by centrifuging.

Stannous chloride solution. This consisted of a 5% (w/v) solution of $SnCl_4 \cdot H_2O$ in 10N-HCl.

METHODS

Phosphorus and amino N determinations were carried out as described by Wheeldon & Collins (1957).

Hydrolysis of samples. A sample (4–24 mg.) of fat (containing 2–12 μ moles of choline) was hydrolysed by heating at 100° with a mixture of 2 ml. of 6N-HCl and 2 ml. of ethanol. The mixture was placed in a flask with an air condenser so that for the first hour there was enough ethanol to maintain a homogeneous mixture, but over the course of the hydrolysis (18 hr.) the ethanol was gradually lost and the reaction was completed in an aqueous system. The hydrolysate was then evaporated to dryness *in vacuo* to remove the excess of HCl, 2 ml. of water was added and the pH adjusted to 6–7 by the addition of N-NaOH. The fatty acids were removed by extraction with diethyl ether; the aqueous solution was filtered, the filter washed with 5 ml. of water and the filtrate evaporated to dryness and the residue was dissolved in 2 ml. of water. Choline was determined on this solution.

Determination of choline as choline phosphomolybdate. A sample (0.5 ml.) of hydrolysate containing 0.3–3.0 μ moles of choline was mixed in a 15 ml. centrifuge tube with 0.1 ml. of a saturated aqueous solution of phosphomolybdic acid. The mixture was kept for 30 min. at 5° and then centrifuged; the supernatant was decanted and the tube was inverted and allowed to drain for 15 min. The precipitate

* Part 2: Collins & Wheeldon (1957).

was then suspended in 1 ml. of *isobutanol* and centrifuged, the supernatant was discarded and the process was repeated. The washed precipitate was then dissolved in 3 ml. of acetone. To this solution was added 1 ml. of 10N-H₂SO₄ and 0.05 ml. of SnCl₂ solution and the mixture made up to 10 ml. with ethanol. The optical density of the blue solution was measured in a Hilger Spekker absorptiometer with an Ilford 608 filter. The term 'molecular-extinction coefficient' as used in this paper refers to the value obtained with the optical density thus measured and is approximately 87% of the true value at 630 m μ . The plot of concentration (0.3–3.0 μ moles/0.5 ml.) against the optical density was linear and passed through the origin. The value of the 'molecular-extinction coefficient' was 4810 ± 170 (mean of 24 determinations).

RESULTS

Interfering substances. Serine and ethanolamine did not interfere provided that their concentrations did not exceed 20 μ moles/0.5 ml. This amount of serine or ethanolamine was equivalent to 16 mg. of phosphatidylserine or phosphatidylethanolamine and the sample for choline determination should not contain more than this amount of kephalin. Ammonia interfered with the method but was not present in lipid hydrolysates prepared as described by Wheeldon & Collins (1957). The presence of a reducing sugar, such as glucose, did not cause any reduction of phosphomolybdic acid and thus did not interfere. If the lipid was hydrolysed in an alkaline medium the presence of certain cations complicated the procedure. Na⁺ ions produced no precipitate but K⁺ (if more than 20 μ equiv./0.5 ml.), Ba²⁺ and Ca²⁺ ions formed insoluble salts with phosphomolybdic acid, which, however, differed from choline phosphomolybdate in that they were insoluble in acetone.

Application to lipid hydrolysates. The recovery of choline added to lipid hydrolysates was quantitative, as is shown in Table 1. As a further check on the recovery of choline a sample of kephalin was mixed in varying proportions with a sample of lecithin, the total weight of the mixture being kept

constant at 2.61 mg. The plot of the percentage of lecithin (100, 80, 50, 20 and 0) against the amount of choline found was linear, showing that the determination of choline was unaffected by the presence of kephalin. In the determination of choline in lipid hydrolysates the agreement between duplicates was 2.4% (fiducial limits for $P=0.95$).

Comparison with reineckate procedure. A comparison with the results obtained by precipitation as choline phosphomolybdate and as choline reineckate, by the procedure of Glick (1944), showed close agreement. The ratio of the choline content of three samples of rat-brain lipids and three of rat-liver lipids (each in duplicate) as determined by the reineckate to that determined by the phosphomolybdate method was 1.06 ± 0.034 . The 'molecular-extinction coefficients' used in the phosphomolybdate and reineckate methods were 4810 ± 170 and 108 ± 3 respectively. There is thus an uncertainty in this ratio of 4.5% due to the uncertainty in the extinction coefficients alone.

Application to dinitrophenylated lipids. Dinitrophenylethanolamine and dinitrophenylserine were not precipitated by phosphomolybdic acid at any concentration, but when they were heated with 6N-HCl for 18 hr. there was some production of a reducing material which resulted in the formation of molybdenum blue on the addition of phosphomolybdic acid. The application of the unmodified method to dinitrophenylated lipids led to high results for choline, probably due to the adsorption of the molybdenum blue onto the precipitated choline phosphomolybdate. When the period of heating was 3 hr. there was no interference. Hydrolysis with N-HCl in ethanol was found to split off the dinitrophenylated bases quantitatively without any decomposition, and the following procedure for the determination of choline in dinitrophenylated lipids was developed.

The sample of dinitrophenylated lipid (5–25 mg.) was hydrolysed by heating with 5 ml. of N-HCl in ethanol under reflux for 3 hr. The mixture was evaporated to dryness *in vacuo* to remove excess of HCl, 1 ml. of water was added and the aqueous mixture was extracted three times with 5 ml. of light petroleum (b.p. 40–60°). The aqueous layer was then extracted with three 5 ml. portions of diethyl ether and the ethereal extracts were discarded. The light-petroleum extract after removal of solvent was combined with the aqueous solution, 1 ml. of 10N-HCl was added to bring the normality to approximately 6 and the hydrolysis was continued by heating at 100° for 10 hr. From this point the procedure was the same as in the normal method.

There was no loss of P (see Table 2) involved when the ethereal solution containing the dinitrophenylated bases was discarded, although there was

Table 1. *Recovery of choline added to a lipid hydrolysate*

Known amounts of choline were added to a 0.5 ml. sample of a rat-liver lipid hydrolysate containing 1.06 μ -moles of choline and the total choline was estimated by the phosphomolybdate method.

Sample	Choline added (μ moles)	Total choline found (μ moles)	Recovery (%)
1	0.28	1.34	100
2	0.83	1.88	99
3	1.11	2.13	97
4	1.39	2.44	99
5	1.67	2.68	97

Table 2. *Distribution of phosphorus between light petroleum, diethyl ether and water after hydrolysis of a dinitrophenylated lipid*

Four samples of a dinitrophenylated lipid from rat liver were hydrolysed and extracted as described in the text. P was determined in each of the three fractions.

Wt. of lipid (mg.)	Distribution of P (%)			
	3.4	4.6	4.2	5.8
Light-petroleum extract	3	13	16	15
Diethyl ether extract	<1	1	4	2
Aqueous solution	97	86	80	83

still unhydrolysed phospholipid in the light-petroleum extract. The application of the method to samples of fat before and after treatment with 1-fluoro-2:4-dinitrobenzene showed that the ratio of the choline content of dinitrophenylated fat to that of the native fat was 0.99 ± 0.06 (seven samples).

Choline contents of rat-liver lipids. The choline content of 18 samples of rat-liver lipids was 20.4 ± 3.6 (S.D.) $\mu\text{moles/g.}$ of fresh tissue. Artom (1945) found 23.5 ± 0.8 (S.D.) $\mu\text{moles/g.}$ of fresh tissue. Assuming a molecular weight of 800 these figures correspond to 1.6 and 1.9% of choline phospholipids in the moist liver respectively. Artom & Fishman (1943) and Cardini & Serantes (1942) respectively obtained values of 1.9 and 2.2% of choline phospholipids in moist liver.

DISCUSSION

The method for the determination of choline described in this paper has several advantages and is as sensitive as alternative methods. The reagent is light-stable, unlike ammonium reineckate solutions, and the precipitate is stable, in contrast with choline enneaiodide. With choline phosphomolybdate the final colour absorbs maximally at $630\text{ m}\mu$ and is not subject to interference from impurities which interfere with measurements in the ultra-violet region. Levine & Chargaff (1951) showed that the only material from lipid hydrolysates which reacted with phosphomolybdic acid was choline and therefore the specificity of the method as applied to phospholipids is equal to that of other methods involving a precipitation reaction. For a given amount of choline the precipitate obtained

with phosphomolybdic acid was the largest; for example $15\text{ }\mu\text{g.}$ of choline corresponds to $60\text{ }\mu\text{g.}$ of choline reineckate, $160\text{ }\mu\text{g.}$ of choline enneaiodide and $260\text{ }\mu\text{g.}$ of choline phosphomolybdate. The procedure described for the determination of choline has been applied to a large number of lipid samples and has given results that agree with those obtained by other methods.

SUMMARY

1. A colorimetric method for the determination of choline in lipids has been developed in which the choline in a hydrolysate is precipitated by phosphomolybdic acid.
2. Choline added to a lipid hydrolysate was quantitatively recovered, as was also the choline in mixtures of lecithin and kephalin.
3. The method agreed with the reineckate method but was not affected by light and is simpler to use.

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