RESEARCH PAPERS

THE PHOTOMETRIC DETERMINATION OF CHOLINE AND CHOLINE DERIVATIVES

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Choline and its derivatives can be determined photometrically as reineckates which are soluble in acetone giving solutions having a reddish colour.[1,2,3,4,5] In 1943 Ackermann and Mauer[6] found that 2:4:6-hexanitrodiphenylamine formed well defined compounds with choline, acetylcholine and some other organic bases. These compounds are soluble in acetone forming deep yellow solutions, and the authors suggest the use of this reaction for the photometric determination of choline and acetylcholine.

2:4:6-hexanitrodiphenylamine (dipicrylamine) is slightly soluble in water, but the sodium and magnesium salts are very soluble. The compound is usually used as the magnesium salt. The potassium salt, on the contrary, is slightly soluble in water and potassium can be determined photometrically with hexanitrodiphenylamine.[7] Other organic bases and several alkaloids are also precipitated by hexanitrodiphenylamine.[6,8,9] Langhans[8,9] suggests the name *hexylate* for these salts, and that name will be used in this paper.

Experimental

Preparation of the Hexylates

Magnesium hexylate reagent (*Kolthoff and Bendix*[7]). 12 g. of hexanitrodiphenylamine are mixed with 5 g. of magnesium oxide and the mixture is transferred with the aid of 400 ml. of water to a 500-ml. Erlenmeyer flask. The solution is well stirred, allowed to stand for 15 to 20 hours and filtered. The solution contains about 3 per cent. of magnesium hexylate, and the concentration can be determined by evaporating 5·00 ml. of the reagent to dryness and weighing the residue.

The reagent is stable, but if it becomes turbid it should be filtered before use.

Hexylates of choline, acetylcholine, carbamylcholine and OO-*succinyldicholine.* These were prepared according to Ackermann and Mauer[6] in the following manner. To an ice-cold, slightly alkaline, aqueous solution of the halogen salt of the derivatives magnesium hexylate reagent was added. The precipitate formed was washed with water and recrystallised from hot water. The succinyldicholine hexylate is very slightly soluble even in boiling water. This hexylate was therefore dissolved in dry acetone and precipitated by the addition of petrol ether. The hexylates were recrystallised until constant melting points were obtained. The yields and the melting points are given in Table I.

TABLE I

MELTING POINTS AND YIELDS OF HEXYLATES

Compound	g.	Magnesium hexylate (3·3 per cent. w/v) ml.	Yield of the hexylate per cent.	Melting point of the hexylate (corr.) ° C.
Choline chloride	1	135	82	235° to 236°
Acetylcholine chloride	1	125	81	125° to 126°*
Carbamylcholine chloride	1·5	175	87	134° to 135°
OO-succinyldicholine diiodide	2	140	83	141° to 142°

* According to Ackermann and Mauer (*loc. cit.*) the melting point of this hexylate should be 187° C., but this value could not be verified. The melting point given in the table was obtained with two different samples of acetylcholine chloride.

Analysis of the Hexylates

The content of hexanitrodiphenylamine in the hexylates was determined by reduction with titanium trichloride in a citrate buffer according to Kolthoff.[10] About 0·02500 g. of hexylate was dissolved in 25 ml. of acetone and to the solution 30 ml. of an aqueous solution of sodium citrate (20 per cent. w/v) was added. Nitrogen was bubbled through the mixture for 10 minutes. 25·00 ml. of 0·1N titanium trichloride was added. After 5 minutes 7 ml. of an aqueous solution of hydrofluoric acid (40 per cent.) and 25 ml. of hydrochloric acid (d. 1·19) were added. The excess of titanium trichloride was determined with 0·1N ferric ammonium sulphate. A blank was run on the reagents. The results, which are the average of at least two determinations, are given in Table II. Accuracy: ±2 per cent.

TABLE II

PERCENTAGE OF HEXANITRODIPHENYLAMINE IN HEXYLATES OF CHOLINE AND DERIVATIVES

Hexylate of	Hexanitrodiphenylamine	
	Calculated per cent.	Found per cent.
Choline ($C_{17}H_{18}N_8O_{13}$ = 542·37)	80·98	81·5
Acetylcholine ($C_{19}H_{20}N_8O_{14}$ = 584·41)	75·15	76·8
Carbamylcholine ($C_{18}H_{19}N_9O_{14}$ = 585·40)	75·03	75·1
OO-succinyldicholine ($C_{38}H_{38}N_{16}O_{28}$ = 1166·81)	75·29	74·6

Determination of the Solubility in Water at 0° C.

Preliminary attempts to determine choline derivatives as hexylates indicated that the solubilities of the hexylates in water at room temperature were too great to permit accurate work at this temperature. At 0°, however, they seemed to be much less soluble and the solubilities were therefore determined at this temperature in the following way. Unsaturated and supersaturated aqueous solutions of the hexylates were shaken in a test tube of resistant glass, closed with a rubber stopper. To maintain the temperature as constant as possible, the test tube was enclosed in a vacuum flask filled with ice. After shaking for 60 hours the solution was

filtered off with the aid of a filter stick immersed into the mixture while the test tube remained in the vacuum flask. 10·00 ml. of the filtrate were evaporated to dryness on the water bath, and the residue was dissolved in enough acetone to produce 25·00 ml. The content of hexylate in the acetonic solution was determined photometrically at 415 mμ with a Beckman spectrophotometer Model DU, using acetone as a blank. The standard curve was prepared with known amounts of hexylate dissolved in acetone. The results are given in Table III.

TABLE III

SOLUBILITIES OF HEXYLATES OF CHOLINE AND DERIVATIVES AT 0° C.

Hexylate of	Dissolved hexylate in 100 ml. of solution. Solution supersaturated before shaking mg.	Dissolved hexylate in 100 ml. of solution. Solution unsaturated before shaking mg.	Average
Choline	2·1	1·9	2·0
Acetylcholine	2·2	2·0	2·1
Carbamylcholine	3·2	2·9	3·1
OO-succinyldicholine	0	0	0

As seen in Table III the solubilities of the hexylates of choline and acetylcholine are about the same, while the hexylate of carbamylcholine is more soluble. The succinyldicholine dihexylate, however, is practically insoluble at this temperature. The accuracy of the determinations is considered good enough for practical use, though the values obtained with solutions supersaturated before shaking are somewhat higher than those obtained with unsaturated solutions indicating that the shaking time was too short to reach equilibrium.

Absorption Data of the Hexylates

The absorption of the pure hexylates in acetone solutions was determined on a Beckman spectrophotometer Model DU, using acetone as a blank. The four absorption curves were identical with maxima at 415 mμ (Fig. 1). The molar extinction coefficients, ϵ, were calculated and the results are given in Table IV.

TABLE IV

MOLAR EXTINCTION COEFFICIENTS AT 415 mμ OF HEXYLATES OF CHOLINE AND DERIVATIVES IN ACETONE AT 20° C.

Hexylate of	Mg. of hexylate dissolved in 100 ml. of acetone	ϵ	ϵ average
Choline	1·058	$3·02 \times 10^4$	$2·99 \times 10^4$
	0·782	$2·97 \times 10^4$	
Acetylcholine	1·042	$3·01 \times 10^4$	$2·99 \times 10^4$
	0·598	$2·97 \times 10^4$	
Carbamylcholine	1·029	$2·97 \times 10^4$	$2·98 \times 10^4$
	1·169	$2·99 \times 10^4$	
OO-succinyldicholine	1·256	$5·88 \times 10^4$	$5·90 \times 10^4$
	1·118	$5·92 \times 10^4$	

The molar extinction coefficient for the hexylates of choline, acetylcholine and carbamylcholine is the same in each case, but it is twice as large for *OO*-succinyldicholine dihexylate, which verifies the assumption that the colour depends exclusively on the hexylate moiety.

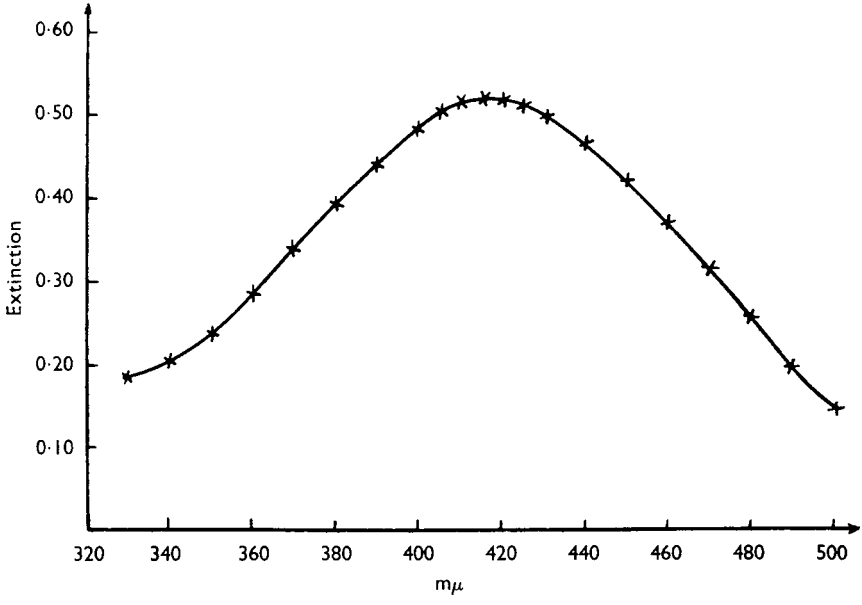

FIG. 1. Absorption curve of carbamylcholine hexylate in acetone. Beckman spectrophotometer model DU.

PROCEDURE FOR THE DETERMINATION OF CHOLINE AND CHOLINE DERIVATIVES

Reagents

Magnesium hexylate solution (about 3 per cent. w/v, see page 239).
Acetone of reagent purity.
Aqueous solution of the hexylate of the substance to be determined, saturated at 0° C.

Procedure

To an ice-cold, slightly alkaline, aqueous solution of the sample, equivalent to 0·2 to 0·4 mg. of choline, 1 ml. of the magnesium hexylate reagent is added, and the mixture is cooled in ice for 30 minutes. The test solution should not contain less than 0·015 per cent. of the substance. The precipitate is collected on a filter crucible (e.g. Jena G 4) and is washed with 2 × 5 ml. of the saturated hexylate solution. The precipitate is dried by suction and dissolved in acetone to 100·0 ml. The optical density of the acetone solution is determined at 415 mμ, using a 1 cm. cell, and with acetone as a blank.

Standard Curve

The standard curve is prepared in the same way with known amounts of the choline derivatives.* The standard curve for carbamylcholine chloride is given in Figure 2.

Discussion

The influence of the concentration of magnesium hexylate has been investigated and the results are given in Table V.

TABLE V

The influence of the volume of reagent added on the determination of carbamylcholine chloride

Hexylate reagent (3·3 per cent. w/v) ml.	Carbamylcholine chloride present mg.	Carbamylcholine chloride found mg.	Difference per cent.
0·1	0·504	0·455	−10
0·3	,,	0·485	−4
0·6	,,	0·500	−1
0·8	,,	0·505	0
1·0	,,	0·500	−1

1 ml. of a 3 per cent. w/v solution of the reagent is sufficient to secure a complete precipitation of the hexylates.

As the colour depends exclusively on the hexylate moiety it is essential that the precipitates are thoroughly washed. The recommended amount of wash liquid is sufficient in all cases except for the hexylate of *OO*-succinyldicholine which should be washed with 4 × 5 ml. of the saturated hexylate solution.

The colour is very stable. An acetonic solution of carbamylcholine hexylate did not change its optical density for 3 weeks.

The method is accurate within 2 per cent. The results are in good agreement with those obtained with the reineckate method.[5] In

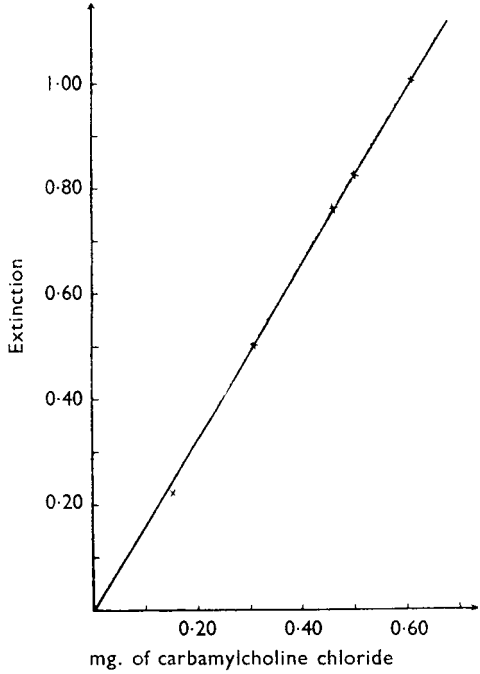

Fig. 2. Standard curve for carbamylcholine chloride determined as hexylate. Beckman spectrophotometer model B.

* Theoretically it is possible to prepare a standard curve by dissolving known amounts of the corresponding hexylate in acetone, but on account of the solubilities of the hexylates in water it is safer to use the described procedure in preparing the standard curve.

comparison with that procedure the recommended method has some advantages. Thus the magnesium hexylate reagent is stable and the method permits smaller amounts to be determined. A drawback, common to both methods, is that the reaction is unspecific.

Summary

1. Hexylates of choline, acetylcholine, carbamylcholine and *OO*-succinyldicholine have been prepared. Melting points, content of hexanitrodiphenylamine, and solubilities in water at 0° C. have been determined.

2. Absorption data of the 4 hexylates in acetone solutions have been recorded and the molar extinction coefficients determined.

3. A photometric method for the determination of choline and choline derivatives as hexylates has been worked out.

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