7. It is concluded that *trans-trans*-muconic acid is probably a true metabolite of benzene and is not produced *in vivo* from the *cis-cis*-acid. It is suggested that the *cis-cis*-acid need not be an intermediate in the formation of the *trans-trans*-acid in the animal body.

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The Separation of Esters of Choline by Filter-paper Chromatography

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The technique of paper chromatography (Consden, Gordon & Martin, 1944) has been applied in recent years to the separation of numerous classes of substances. It has now been applied to esters of choline in connexion with work on the identification of choline esters in tissue extracts and on the mechanism of enzymic reactions involving choline esters. Preliminary accounts have been given by Whittaker (1951) and Whittaker & Wijesundera (1951).

METHODS

Chromatographic procedure

Whatman no. 4 filter paper was used throughout. For preliminary screening of solvents, tall inverted beakers or wide-mouthed bottles with screw caps were employed as containers. Solutions of esters in water (10–50 μg. in 10 μl.) were delivered, 3 cm. from the lower edge, on to a rectangle of filter paper of suitable dimensions by means of an 'Agla' micropipette (Burroughs Wellcome Ltd.). The paper was rolled into a cylinder whose axis was parallel to the intended direction of solvent flow, and the apposed ends of the paper secured by wire staples. The cylinders were then placed upright in a pool of solvent at the bottom of the container and irrigated by capillary ascent, a 10 cm. run taking 2–4 hr. Promising solvents were then tried with longer runs, using both upward and downward irrigation in containers of conventional design.

Reading of chromatograms

Esters were rendered visible on the paper by three methods, one specific for carboxylic esters and certain other compounds containing an acyl linkage (e.g. lactones, acyl phosphates), the other two relatively non-specific.

Carboxylic reagent

The first method employed a reaction first used by Feigl, Anger & Frehden (1934, cf. Feigl, 1939) as the basis of a spot test, and later applied to the quantitative estimation of acyl phosphates (Lipmann, 1946) and acetylcholine (Hestrin, 1949). The reaction involves the conversion of the carboxylic ester into a hydroxamic acid with alkaline hydroxylamine, followed by the formation of a purple

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complex between the hydroxamic acid and Fe^{+++} in acid solution:

$$R.CO.OR' + NH_2OH \rightarrow R.CO.NHOH + R'OH$$

$$R.CO.NHOH + \tfrac{1}{3}Fe^{+++} \rightarrow R{-}C(=O)(\rightarrow Fe)(NH{-}O)$$

In preliminary studies (by V.P.W.) the reagents of Hestrin (1949) were used. The papers were hung in a box inside a fume cupboard, and sprayed first with alkaline hydroxylamine, then, after 1 min., with acid $FeCl_3$. An initial brown colour (due to $Fe(OH)_3$) was replaced by yellow when sufficient acid had been delivered, the esters showing as purple spots. This method suffers from the following disadvantages: the spots are fugitive and must be outlined at once in pencil; the esters are soluble in the aqueous reagents and tend to diffuse away from their original positions; the reagents, being strongly alkaline and acid, are unpleasant to use. These disadvantages have been largely overcome, and the sensitivity considerably increased, by the use of non-aqueous reagents, which are also more stable.

Stock reagents. The following stock solutions are employed:

(*A*) Aqueous ethanolic hydroxylamine hydrochloride. Hydroxylamine hydrochloride (20 g.) is dissolved in water (50 ml.) and diluted to 200 ml. with 95% (v/v) ethanol. Stable when stored in the cold.

(*B*) Ethanolic KOH. The hydroxide (50 g.) is dissolved in the least quantity of water and diluted to 500 ml. with 95% (v/v) ethanol.

(*C*) Ethereal acid $FeCl_3$. Well powdered $FeCl_3.6H_2O$ (10 g.) is dissolved in 20 ml. 10N-HCl and shaken with 300 ml. ether until a homogeneous solution results. Stable indefinitely in well stoppered bottles.

Spray reagents. These were made from the stock solutions as follows:

(I) Ethanolic alkaline hydroxylamine. Stock solution *A* is mixed with twice its volume of solution *B* and filtered from precipitated KCl. Stable for over 2 weeks in the cold.

(II) Stock solution *C* used directly.

Procedure. The dry chromatograms were clamped in a box, reagent I applied thinly and evenly with an atomizer supplied with compressed N_2, dried briefly and sprayed with reagent II. The esters showed up almost at once as purplish spots on a yellow ground.

Sensitivity. As little as 0·5 μg. acetylcholine/cm.^{2} gave a faint fugitive spot and 2 μg./cm.^{2} gave distinct spots which remained visible for several weeks. With weaker reagents, larger volumes have to be delivered on to the papers, thus making them wet and increasing the risk of diffusion. Ethereal ethanolic hydroxylamine in place of reagent *A* gave fugitive spots.

Iodine method

Brante (1949) found that nitrogenous bases could be separated by paper chromatography and visualized as brown spots by immersing the paper in I_2 vapour. As pointed out by Marini-Bettolo-Marconi & Guarino (1950), this method is non-specific and can be applied to a wide variety of compounds. On exposing the treated paper to the air, the colour fades and the paper may then be treated with another reagent or assayed pharmacologically. We have found the iodine method particularly useful in conjunction with other methods, and exact coincidence was obtained in areas developed by the iodine and carboxylic methods.

Phosphotungstic acid-stannous chloride method

The method of Chargaff, Levine & Green (1948) for choline was found to be applicable to choline esters. It was found to be less convenient for our purposes than the other two methods, being less specific and less sensitive; moreover the washing-out stages were attended with a risk of damage to the chromatograms.

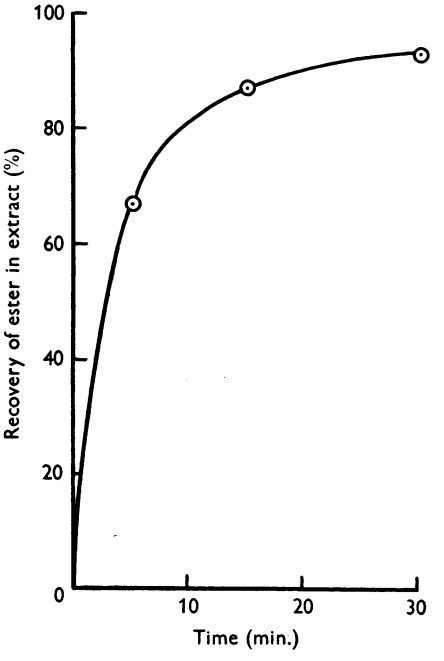

Fig. 1. Time required for extraction of acetylcholine from filter paper.

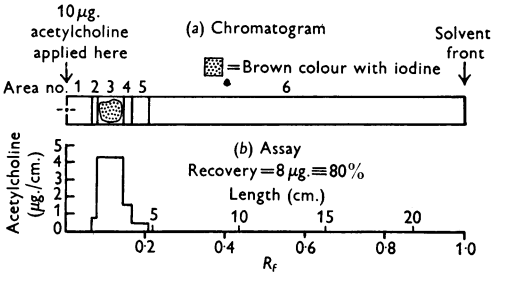

Fig. 2. (*a*) Chromatogram of acetylcholine. Solvent, *n*-butanol saturated with water (ascending). (*b*) Distribution of acetylcholine on chromatogram, as revealed by pharmacological assay (guinea pig ileum).

Pharmacological assay

Esters which are pharmacologically active can be localized by bioassay. Fig. 1 shows that elution of acetylcholine from filter paper with water is 93% complete in 30 min. In this experiment a series of spots, each containing 150 μg. acetylcholine chloride placed on filter paper, were run in *n*-butanol saturated with water. The papers were dried and the positions of the spots located by developing

control spots with iodine. Areas corresponding to the spots were then cut up and extracted in 3 ml. water each for various lengths of time. The acetylcholine content of samples withdrawn at 5, 15 and 30 min. was determined by the method of Hestrin (1949). Fig. 2 (*a*) is the tracing of a chromatogram of 10 μg. acetylcholine run in *n*-butanol saturated with water. The chromatogram was cut into numbered areas; each area was cut up and eluted for 30 min. in 2 ml. of a saline solution acidified to pH 4, the pH at which acetylcholine has maximum stability. Each extract was assayed in the usual manner using a guinea pig ileum preparation. The result of the assay is shown in the lower half of the figure (Fig. 2*b*). It will be seen that good localization and recovery of the acetylcholine are obtained.

RESULTS

The R_F values of a number of esters in different solvents are presented in Table 1. Essentially the same values were obtained for an ester whether run alone or as a component in a mixture, but the values obtained in any one experiment were found to be influenced by the usual factors and rigid control of temperature, length of run and composition of solvent were necessary to secure uniform results.

0·13–0·16 above those of the chlorides. Increasing water content raised R_F values and reduced separation; with ethanol and acetone it also reduced the 'tailing' which occurred with the pure solvents. Pyridine-, collidine-, lutidine-, phenol-, benzyl alcohol-, furfuryl alcohol- or dioxan-water mixtures proved either unsatisfactory or inferior in resolving power to those included in the table. Solvents containing ammonia destroyed choline esters, which are markedly alkali-labile.

DISCUSSION

The results presented above show that it is possible to separate esters of choline using paper chromatography, the best solvents being the less freely water-soluble alcohols, either alone or in combination. Three methods of chemical identification have been described; one, using alkaline hydroxylamine and acid ferric chloride, is specific for compounds containing the acyl linkage and should be applicable to other esters of low volatility, and possibly also to acyl phosphates and lactones, which can be estimated with the reagents (Kent, 1951).

Table 1. *Mean R_F values of choline esters and related compounds*

(Length of run: 25 cm. (upward); 35 cm. (downward); (*a*) denotes upward; (*d*) downward irrigation. Composition of solvents given as proportions by volume of constituents. Whatman no. 4 filter paper.)

Compound	*n*-Butanol saturated with water		*n*-Butanol-*n*-propanol-water (4:2:1)	*n*-Propanol-water (9:1)	*n*-Propanol-formic acid-water (8:1:1)	*n*-Propanol-benzyl alcohol-water (5:2:2)	
	(*d*)	(*a*)	(*a*)	(*a*)	(*a*)	(*d*)	(*a*)
			Chlorides				
Acetylcholine	0·09	0·14	0·17	0·24	0·46	—	0·33
Acetyl-β-methylcholine	0·15	0·19	0·23	0·35	0·56	—	0·37
Propionylcholine	0·17	0·22	0·27	0·35	0·59	—	—
Butyrylcholine	0·22	0·28	0·29	0·43	0·66	—	0·46
Benzoylcholine	0·23	0·28	0·30	0·43	0·65	—	0·49
Lactylcholine	—	0·18	—	—	—	—	—
Valerylcholine	—	—	0·55	—	—	—	—
Choline	0·06	0·09	0·13	0·24	0·38	—	0·25
			Perchlorates				
Acetylcholine	0·22	0·30	0·34	0·40	0·59	—	0·48
Acetyl-β-methylcholine	0·31	—	0·38	0·52	—	—	—
Propionylcholine	0·32	0·42	0·39	0·49	0·67	—	0·54
Butyryl-β-methylcholine	0·49	—	0·59	0·70	0·75	—	—
Lactylcholine	—	0·34	—	—	—	—	—
Succinylmonocholine	Unsatisfactory		0·04	Unsatisfactory	0·62	0·10	0·17
Succinyldicholine	Unsatisfactory		0·12	Unsatisfactory	0·30	0·19	0·30
Choline	0·21	0·24	0·31	0·39	0·60	0·50	0·46
β-Methylcholine	0·29	0·45	0·40	0·48	0·56	—	—

Increasing molecular complexity leads to increased R_F values. In *n*-butanol, for example, each additional CH_2 (starting with acetylcholine) increases the R_F by approximately 0·07; isomers, e.g. propionylcholine and acetyl-β-methylcholine ($[Me_3N^+.CH_2.CHMe.OAc]OH^-$), having closely similar values. The associated anion influences the R_F; in *n*-butanol, the R_F's of perchlorates are

SUMMARY

1. Choline esters can be separated from each other and from choline by the technique of paper chromatography. *n*-Propanol- and *n*-butanol-water mixtures, with or without the addition of a third component such as benzyl alcohol or formic acid, gave the best separations.

2. The esters were detected on the paper by an adaptation of the hydroxylamine-ferric chloride test for carboxylic esters, by the iodine method of Brante (1949), by the phosphotungstic acid-stannous chloride method of Chargaff *et al.* (1948) and, in the case of acetylcholine, by pharmacological assay.

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The Metabolism of ^{14}C-Labelled Bicarbonate in the Cat

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Studies on the metabolism of ^{14}C-labelled urea in mice (Leifer, Roth & Hempelmann, 1948; Skipper, Bennett *et al.* 1951) and cats (Kornberg, Davies & Wood, 1951*b*) have shown conclusively that urea is broken down in the mammalian body. Its rate of breakdown in these experiments has been measured from the rate of expiration of the ^{14}C-labelled carbon dioxide produced. In order to interpret the results of such studies on cats, it was necessary to have information on the rate of excretion from the blood of ^{14}C-labelled carbon dioxide, and on the rate of incorporation of ^{14}C into urea. The results of earlier studies on mice and rats injected with ^{14}C-labelled bicarbonate (Armstrong & Schubert, 1949; Armstrong & Zbarsky, 1949; Gould, Sinex, Rosenberg, Solomon & Hastings, 1949; Greenberg & Winnick, 1949; Schubert & Armstrong, 1949; Skipper, White & Bryan, 1949; Skipper, Nolan & Simpson, 1951; Skipper, Bennett *et al.* 1951) did not supply the required data, because of the great differences in body size between these animals and cats, and because the isotope in these experiments had been administered intraperitoneally.

Experiments are described in which ^{14}C-labelled sodium bicarbonate was injected intravenously into anaesthetized cats and the expired carbon dioxide continuously collected in a special respiration circuit. By this technique, information was obtained on the rate of expiration of labelled carbon dioxide from ^{14}C-labelled bicarbonate, and on the existence of exchange mechanisms between blood and tissue carbon dioxide in the animal. The results of these studies are very similar to those obtained independently in human subjects (Hellman, 1951). From determinations of blood urea and bicarbonate it was also possible to measure the rate of incorporation of ^{14}C into urea synthesized during the experiment, and to confirm that the urea carbon is derived from carbon dioxide.

Part of this work has been communicated to the Biochemical Society (Kornberg, Davies & Wood, 1951*a*).

EXPERIMENTAL

Treatment of cats. A weighed cat was anaesthetized with ether followed by chloralose (75 mg./kg. body weight) and was placed on an operating table covered by a large stainless steel tray to avoid contamination of the laboratory. Cannulae were inserted into the trachea for collection of expired CO_2, into the right external jugular vein for administration of intravenous injections, and into the right femoral artery for collection of blood samples during the course of the experiment. Both ureters were tied off. The animal was then connected through its tracheal cannula to the respiration circuit schematically represented in Fig. 1.

Respiration circuit. This system is so constructed that atmospheric pressure is maintained at the tracheal cannula, so that there is no resistance to free respiration. This is achieved by the two aquarium pumps P_1 blowing, and P_2 drawing air through the system at approximately 400 ml./min. Constancy of the pressure inside the circuit is controlled by three leaks L and a rubber balloon B which acts as a gas reservoir, these being placed at points which do not at any time come into contact with $^{14}CO_2$. Two soda-lime towers S remove atmospheric CO_2 from the incoming air, which can