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REVIEW ARTICLE

PHOSPHATIDYLETHANOLAMINE AND LYSOPHOSPHATIDYLETHANOLAMINE

By D. C. Robins, M.Pharm.

From the Welsh School of Pharmacy, Welsh College of Advanced Technology, Cathays Park, Cardiff

Phosphatidylethanolamine and Lysophosphatidylethanolamine belong to a group of phospholipids generally termed cephalins. The phospholipids are universally distributed among living organisms and are thought to be essential components of cell membranes. From the quantities of lipoidal and protein material found in cell membranes (Danielli and Stein, 1956) together with surface tension and related studies (Danielli and Harvey, 1935; Danielli and Davson, 1935), the membranes are considered to consist of a bimolecular leaflet of lipids with a unimolecular layer of protein adsorbed onto the polar surface.

The course of investigation of the cephalin fraction has not been reviewed in recent years, and here is discussed the general scope of the work in this field, under the headings of preparation, structure, synthesis and physical properties.

Preparation

Phosphatidylethanolamine

The cephalin fraction was first isolated and recognised as a separate entity apart from lecithins by Thudichum in 1884. He extracted it from brain (hence the name) and used its sparing solubility in warm ethanol as a method for separating it from lecithin.

Trier, in 1912, made the significant observation that when phospholipids were treated with cadmium chloride the lecithins formed a relatively insoluble complex whilst the cephalins formed a soluble complex in ether. Maclean (1915) utilised this knowledge and obtained a greatly improved separation of lecithins and cephalin.

Obata, Nukata and Sasa (1950) in attempting to extract the cephalin fraction from soybean, showed that the cadmium chloride method was not applicable to plant material. It was necessary to treat an emulsion of the soybean phospholipids with lead acetate and to centrifuge and boil the precipitate with methanol, before adding the cadmium chloride. The cephalin was released from the complex by adding hydrochloric acid.

Kirk (1938) introduced a method of separation in which the phospholipid mixture was first precipitated with acetone and magnesium chloride, and then extracted with moist ether. Kirk claimed that moist ether dissolved lecithins and cephalins but not sphingomyelin. This method was criticised by Sinclair and Dolan (1942) who indicated that the residue left from the extraction with moist ether was not primarily sphingomyelin, but merely a portion of the mixture of phospholipids, since the amount precipitated was proportional to the concentration of magnesium chloride added with the acetone.

The Reinecke salt was suggested by Thannhauser and Setz (1936) as a specific precipitating agent for sphingomyelin. Despite claims of complete precipitation of the sphingomyelin, the evidence did not preclude the possibility of other phospholipids being precipitated.

Folch and Schneider, in 1941, showed that cephalin was not a single substance, as the methods of preparation yielded substances having not all of the base as ethanolamine, some 40–70 per cent being serine.

Later, Folch and Woolley (1942) demonstrated the existence of a phospholipid in the cephalin fraction containing inositol.

Folch (1942a) then proved the existence of three fractions in the cephalin portion. By adding successive quantities of ethanol to a solution of cephalin in chloroform, precipitates of inositol-, serine-, and (by the addition of acetone) ethanolamine-containing fractions respectively were obtained. The significant point was that phosphatidylethanolamine, unlike the cephalin portion as a whole, was freely soluble in ethanol.

A similar separation was obtained by Lovern (1949), but he found that solvent fractionation still gave compounds that were far from pure, and suggested that chromatographic procedures would give better separations.

Chromatographic Separations

Adsorption chromatography was applied to the separation of mixed lipids by Trappe in 1940. Using magnesia as adsorbent, he showed that lipids were more strongly adsorbed as their polarity increased.

Other workers (Taurog, Entenmann, Fries and Chaikoff, 1944; Fauré, 1950; Hanahan, Turner and Jayko, 1951) used magnesia and alumina as adsorbents and successfully separated choline-containing from amino-containing lipids. However, they did not recover the cephalin fraction.

Rhodes (1956) obtained a separation and recovery of lecithin and cephalin fractions of egg yolk phospholipids by chromatography on alumina. By eluting with a solvent of methanol-chloroform (1 : 1 v/v) the lecithin travelled as a sharp band with the solvent front, and on changing to a solvent of ethanol-chloroform-water (5 : 2 : 2 v/v) the cephalins were eluted in 92–98 per cent yield. He suggested that by the use of less powerful eluents at the second stage, fractionation of the cephalins would be obtained.

Paper chromatography was first used to separate phospholipids by Chargaff, Levine and Green (1948). They worked with minute quantities and after hydrolysing the lipids by boiling with hydrochloric acid, they identified the liberated bases by paper chromatography. Choline was detected by reaction with phosphomolybdic acid, followed by reduction to molybdenum blue with stannous chloride. Amino-containing bases were detected by staining with ninhydrin. They later (1951) used these staining techniques for quantitative determination of the bases.

Hecht and Mink (1952) employed a filter paper method described earlier for the separation of amino-acids (Consden, Gordon and Martin, 1944). This involved the successive use of various solvents (phenol: water, phenol : ammonia, butanol : water, butanol : pyridine and butyric acid : water) and a fractionation of the cephalins was obtained. Since

the various products all gave the same ninhydrin-reacting bases on hydrolysis, it is difficult to interpret their results.

In 1951 Bevan, Gregory, Malkin and Poole claimed that it was possible to separate choline-containing phospholipids from those containing serine and ethanolamine using filter paper or cellulose columns. This was apparently confirmed by Hack (1953) who stated that amino-containing phospholipids were firmly bound to cellulose, whilst those containing choline ran with the solvent front. Lea and Rhodes (1953) were unable to confirm this separation. They noted that amino-acids were strongly bound to cellulose, while the phospholipids moved together in a solvent of chloroform : ethanol : water (800 : 200 : 25 v/v). This was confirmed in the subsequent removal of eleven free amino-acids from the column.

The cellulose method, therefore, offers a convenient means of removing amino-acids from lipid mixtures.

The use of silicic acid as an adsorbent for chromatography of lipids was first made by Kauffman in 1939, and in the following year by Trappe. In 1944, Rathman found that silicic acid was to be preferred to other adsorbents for column chromatography of phospholipids.

Diemair and Poetsch in 1949 extracted the phospholipids from yeast, and separated them by adsorption on silica gel. They stated that the various lipids could be recognised by their luminescence under ultra-violet light. This was criticised by Lea, Rhodes and Stoll (1955) who suggested that the fluorescence was contributed by deterioration products.

Fillerup and Mead (1953) defined the conditions of column preparation, loading, diameter and length, for the separation of lipid mixtures. By employing solvents of different polarity, fractionation of the main classes of lipids into their constituents was obtained.

Lea and Rhodes (1954) obtained a satisfactory separation of cephalins from lecithins of egg yolk (amino-acids having previously been removed with cellulose) on silica impregnated papers, prepared by the method of Kirchner and Keller (1950). Using a solvent of methanol-chloroform (1 : 3 v/v), the amino-containing phospholipids were fast moving, while the choline-containing substances were slower. In later publications (Lea, Rhodes and Stoll, 1955; Lea and Rhodes, 1955) a full list of R_F values was given and these values, which were observed to alter slightly from paper to paper, were subsequently shown to be due to variation in the water content of the paper. They also prepared columns of commercial sodium silicate which had been previously washed with 10N hydrochloric acid to remove iron and aluminium impurities, and filtered to remove the finely divided material that blocked the column. After drying, the silicic acid was "activated" by heating overnight at 110° (Mallinkrodt AR silicic acid worked well with or without activation). This was slurried with a solvent of methanol-chloroform (1 : 3 v/v), poured into the column and allowed to pack under a slight pressure of nitrogen. A similar separation was obtained to that with the impregnated paper, except that lysophosphatidylethanolamine could not be separated from lecithin on the column.

Lea (1955) continued this work and found no difficulty in obtaining phosphatidylethanolamine free from choline-containing lipids and containing only traces of substances with amino-acid nitrogen. Running just ahead of the phosphatidylethanolamine was a group of yellow-brown pigments which required care to separate cleanly without the use of a second column. In view of the tendency of unsaturated fatty material to autoxidise when adsorbed on to solid surfaces, the silicic acid was heated to 100° under high vacuum to remove adsorbed oxygen, and then nitrogen was admitted. De-aerated solvents were used, and in one case, Lea even passed a previously prepared sample of phosphatidylethanolamine down the column to adsorb any remaining oxygen, before making a separation on the column, but results showed that this procedure was unnecessary.

Hanahan, Dittmer and Warashina (1957) used columns of silicic acid to separate the mixed phospholipids from rat liver, beef liver and yeast. By varying the solvent proportion (chloroform-methanol, 4:1, then 3:2, then 1:4 v/v) a satisfactory separation of phosphatidylethanolamine, phosphatidylserine, phosphoinositides and lecithins was obtained.

Hirsch and Ahrens in 1958 gave meticulous details for the pre-treatment of silicic acid before its use for phospholipid separations. Since some workers had found that separations could be unreliable, a systematic study of the variables was reported; these were, particle size of adsorbent, adsorption values, water content, column preparation, loading capacities and solvents. They indicated that abrupt changes of solvent gave sharper separations of the phospholipids than was obtainable by gradient elution, thus confirming studies by Hanahan and others (1957). In direct contradiction was a report by Wren (1959) who stated that, in dealing with lipid extracts, the need was for a continuous gradient elution. He described a simple apparatus for obtaining this with chloroform-methanol mixtures, and by reference to blood lipids, indicated that peaks were sharper, and less trailing than with discontinuous gradient elution. This view was supported later by Bader and Morgan (1962).

Different solvent systems were employed by other workers: Marinetti and Stotz (1956), Marinetti, Erbland and Kochen (1957) used di-isobutylketone: acetic acid: water (40:20:3 v/v) successfully with one and two dimensional silica-impregnated papers. Rapport and Alonzo (1955) used 30 per cent ethanol in hexane to elute amino-containing lipids from a silicic acid column, whereas 40 per cent methanol in hexane was required to elute lecithin. Dieckert and Reiser (1955) used methanol: ether (1:1 v/v) with an adsorbent of glass fibre impregnated with silicic acid, and obtained satisfactory separation of tissue phospholipids. Similar use of glass paper was reported by Agranoff, Bradley and Brady (1958), and Muldrey, Miller and Hamilton (1959).

Thin-layer chromatography was used by Wagner (1960) employing silica-gel suspensions as adsorbent. A good separation was obtained of a synthetic mixture of lecithin, lysolecithin, sphingomyelin, phosphatidylethanolamine, cerebroside and cardiolipin, using chloroform: methanol:

water (65 : 25 : 4 v/v) as a solvent. The advantages of thin layer chromatography were (*a*) rapidity, (*b*) good separations, and (*c*) emulation of conditions of column chromatography more realistically than with paper-chromatography. Wagner (1961) was able to determine the concentrations of phosphatidylethanolamine and lecithin in extracts of soybean oil, egg yolk, heart muscle and brain.

Jatzkewitz and Mehl (1960) determined the R_F values of over 30 test substances from brain lipids, by thin-layer chromatography, using eleven different solvents, with clean separations of phospholipids.

Vogel, Doizaki and Zieve (1962) used Mallinkrodt silicic acid with plaster of Paris as the binding agent for thin-layer chromatography of serum phospholipids, and obtained a clean separation of phosphatidylethanolamine, lecithin, sphingomyelin and lysolecithin. Cornatzer, Sandstrom and Reiter (1962), and Skidmore and Entenmann (1962) obtained good separations of mixtures of naturally occuring phospholipids.

Bungenberg de Jong and Hoogeveen (1960) described the use of silicon tetrafluoride for impregnating papers. Using a solvent of di-isobutylketone : acetic acid : water (50 : 25 : 5 v/v) a separation of phospholipids was obtained, and in a later report Bungenburg de Jong (1961) gave details of the mechanism of the separation of phosphatidylethanolamine and lecithin on such papers.

Horhammer, Wagner and Richter (1959) were able to obtain reproducible separations of phospholipids by using specially prepared formaldehyde papers, and a mixture of butanol, acetic acid, and water as the solvent. They also reported the use of Malachite Green as a specific reagent for detecting lysophospholipids.

Mumma and Benson (1961) reported the use of anion exchange papers in the separation of lipid mixtures. Reproducibility, freedom from oxidative adsorption and commercial availability of the papers were the advantages. However, the order of the R_F values of phosphatidylethanolamine and lecithin were reversed and were rather too close together for general preparative work.

Counter-current Separations

Scholfield, Dutton, Tanner and Cowan (1948) and Scholfield, Dutton and McGuire (1950) were the first to apply this technique to phospholipids, using a metal apparatus of the same design as Craig's (1944). Later Kies and Davis (1951) extended the method to involve a series of liquid-liquid extractions in which one phase continually flows over the other, termed the "cascade" technique. This greatly extended the scope of the method and was used by Lovern (1952) to obtain a useful degree of separation of lecithin from amino-containing lipids from ox brain. The separation of phosphatidylethanolamine from phosphatidylserine was poor.

Cole, Lathe and Ruthven (1953) employed a solvent system of water : methanol : carbon tetrachloride to overcome the difficulty of emulsification

during counter-current distribution. Working with fractions from brain lipids, they obtained a good separation of lecithin but, like Lovern, they found that phosphatidylethanolamine and phosphatidylserine were inseparable.

Scholfield and Dutton (1954) have claimed that after the precipitation of inositol-containing substances by the addition of lead acetate, a solvent system of hexanol-methanol gave phosphatidylethanolamine in 90–95 per cent purity.

However, Rhodes (1956) commented that as the solubility of phospholipids is so influenced by the constituent fatty acids, and coupled with the emulsification difficulties, the use of counter-current distribution may well be precluded.

Ion Exchange

Lea, Rhodes and Stoll (1955) considered the possibility of separating lecithin from phosphatidylethanolamine on ion-exchange resins, since the former substance is approximately neutral, whilst the latter is acidic. Using a strong cationic resin, and a solvent of water : ethanol : ether (5 : 3 : 2 v/v) or 95 per cent ethanol, lecithin was readily separated from phosphatidylethanolamine, but the capacity of the resin for the relatively large phospholipid molecule was so small (1–3 per cent) as to render the method useless for preparative work. Furthermore much hydrolytic decomposition took place.

Perrin and Sanders (1960) used Dowex ion-exchange resins in the bicarbonate form, and successfully removed the amino-containing lipids in mixed egg phosphatides in the preparation of lecithins. No recovery of the phosphatidylethanolamine was made.

Paper Electrophoresis

Employing a solvent of methoxyethanol : tetrachlorethane : water (47 : 47 : 6 v/v), Garvin (1956) obtained a satisfactory separation of lecithin, phosphatidylethanolamine and phosphatidylserine in a synthetic mixture. In subsequent work (1958) Garvin and Wallach gave the rates of migration on paper under precise conditions.

Zipper and Glantz (1958) studied the effects of various solvent systems, voltages and time periods. They confirmed that a satisfactory separation of lecithin, phosphatidylethanolamine, phosphatidylserine and diphosphoinositide could be obtained.

Lysophosphatidylethanolamine

Delezenne and Fourneau in 1914, prepared lysocephalin by treating an emulsion of egg yolk with cobra venom for three days. The acetone insoluble material was fractionated into lysolecithin and lysocephalin by crystallisation from ethanol.

Levene and Rolf (1923) added cobra venom to a suspension of egg yolk in phosphate buffer at 40°. The lysophospholipids were precipitated with cadmium chloride and the cadmium salts were decomposed and the liquid concentrated until a precipitate began to form which contained 75 per

cent lysocephalin. Since the lysocephalin was almost insoluble in chloroform compared with lysolecithin, it was purified by dissolving in chloroform and re-precipitating with ether. The product was purer than previously prepared.

King and Dolan, in 1933, used a similar method, but separated the mixture by fractional precipitation from glacial acetic acid, through the addition of ether and acetone. The lysocephalin was concentrated in the first fractions. These early methods in aqueous media undoubtedly resulted in poor yields and impure products.

In 1939, Chargaff and Cohen initially separated lecithin and cephalin by way of their cadmium chloride complexes but found it impossible to prepare lysocephalin by the action of snake venom upon the isolated cephalin fraction. However it had been reported (Van Leeuwen and Szent-Gyorgi, 1923; Dunn, 1934) that snake venom acted upon isolated cephalin to give lysocephalin, but was much less reactive than with lecithin.

This difficulty was encountered by Lea and others (1955) who were able to obtain a satisfactory preparation of lysolecithin when reacting venom with egg lecithin, but could not repeat this for phosphatidylethanolamine. They therefore prepared a lysolecithin-lysophosphatidylethanolamine mixture by the direct action of venom on egg yolk, and obtained the individual lyso compounds by chromatographic separation on silicic acid. Using a solvent of chloroform:methanol (80:20 v/v) the lysophosphatidylethanolamine was well separated from the lysolecithin.

Davidson, Long and Penny (1955) noted a lag period in the reaction of venom on lecithin in the presence of phosphatidylethanolamine, and concluded that phosphatidylethanolamine has a higher affinity for the enzyme, but is degraded more slowly.

Long and Penny (1957) showed that phospholipase A hydrolyses phosphatidylethanolamine more slowly than lecithin, requiring 24 hr. compared with 1–2 hr. for lecithin. However, the addition of a small amount of ammonia accelerated the reaction, degradation being complete in 6 hr.

De Haas and van Deenen (1961a) showed that nearly one molar equivalent of fatty acid was liberated from synthetic phosphatidylethanolamines by reacting with venom for 20 hr. at 30°.

STRUCTURE

Phosphatidylethanolamine

Thudichum (1884) was the first to investigate the structure of cephalin, and from hydrolysis studies he proposed two structures; one where the fatty acid and glycerol radicals were attached to the phosphorus by substitution of the hydroxyl groups in phosphoric acid, and the other was analagous to that already indicated for lecithin:

CH_2—fatty acid
|
CH—fatty acid
|
CH_2—phosphoric acid-base.

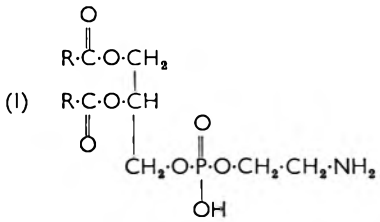
This second structure was found to be more correct after the finding of glycerophosphoric acid in the hydrolysis products (Frankel and Dimitz, 1909). The acid was optically active indicating the α rather than the β form. It had been shown by Bailly and Gaumé (1934) and Chargaff (1942) that chemical hydrolysis of methyl glycerophosphate caused the phosphoric acid moiety to migrate reversibly from the α to the β position, and an equilibrium mixture was obtained, the proportions of which depended on the pH. Folch (1942b) indicated the significance of this in analytical procedures involving hydrolysis of cephalins. Later comparative work with synthetic compounds (Baer, 1956; Long and Maguire, 1953; Baer, Stancer and Korman, 1953; Thannhauser, Boncoddo and Schmidt, 1951) indicated that natural phospholipids occured in the L-α-form.

Much controversy arose over the nature of the base in cephalin. Parnas (1909), Baumann (1913), and Renall (1913) showed that ethanolamine was the base, and found no evidence of any other nitrogen containing base, and that all the nitrogen could be estimated by the method of Van Slyke (1912). MacArthur (1914) showed that ethanolamine did not account for all the nitrogen, and from the large proportion of amino-acid present, he suggested that two cephalins existed; one having nitrogen in the ethanolamine form, and the other in the amino-acid form. This was later confirmed (Thierfelder and Schulze, 1915). Various suggestions came forward (MacLean and MacLean, 1927; Gray, 1940) to explain the divergence between the expected calculated elemental percentages and the observed results, but were not entirely satisfactory.

MacArthur, Norbury and Karr (1917) observed that the ethanol-soluble fraction of phospholipids contained its nitrogen equally divided between choline and ethanolamine, showing that the separation of lecithin from cephalin on ethanol solubility was entirely unsatisfactory. It was not until 1930, that Rudy and Page isolated from the ethanol-soluble fraction, a substance containing ethanolamine, which gave the correct analytical figures for the structure that had been ascribed to cephalin.

Folch (1942a) suggested that the ethanolamine ester of diacylglycerophosphoric acid is actually ethanol-soluble, and is not the material generally termed cephalin, which is characterised by its ethanol-insolubility. He proposed that this material be termed "Phosphatidylethanolamine" rather than cephalin. Together with Schneider (1941) he also isolated cephalin from the ethanol-insoluble fraction, and found it reacted with ninhydrin to give carbon dioxide in a manner similar to α-amino-acids. Analyses of various samples of the ethanol-insoluble fraction indicated that 40–70 per cent of the nitrogen was present in the amino-acid form, and it was suggested that the amino-acid was serine. Thus phosphatidylserine was isolated and explained the original analytical anomaly.

This lead to the assigning of the structure (I) to L-α-phosphatidyl-ethanolamine



where R represents the fatty acids.

Positionally, the fatty acids of phosphatidylethanolamine were assumed to be identical with those of lecithin. It was known for some time that the fatty acids split off from lecithin by phospholipase A were mainly unsaturated, and it had been shown (Hanahan, 1954a; Long and Penny, 1954) that these acids were removed from the α-position. Furthermore, Hanahan (1954b) claimed that the fatty acids are not distributed randomly in lecithin, but that the α-position is always occupied by the unsaturated, and the β-position by the saturated fatty acids, since reaction with phospholipase A removed all the unsaturation from the molecule.

However, Lea and Rhodes (1956) suggested that the situation is more complex than that found by Hanahan, since a small proportion (0·15 double bond per molecule) of unsaturation remains in the lyso compound of both lecithin and phosphatidylethanolamine indicating the presence of some unsaturated fatty acids in the β- as well as in the α-position. After hydrogenation, the released acids were found to be 47 per cent stearic and 53 per cent palmitic acids, proving the presence of C_{18} and C_{16} unsaturated acids in the original molecule.

Hawke (1959) found marked differences in the fatty acid composition of lecithin and phosphatidylethanolamine from egg yolk. Of the total fatty acids in phosphatidylethanolamine, 39·5 per cent were saturated C_{18} and 20·1 per cent unsaturated C_{18} acids, whereas in lecithin they were 14·2 and 44·7 per cent respectively. About 60 per cent of the total acids of phosphatidylethanolamine were saturated. His work showed that most of the acids attached to the α-carbon atom were unsaturated, whilst most of those attached to the β carbon atom were saturated.

Subsequent studies, however (Hanahan, Brockerhoff and Barron, 1960; de Haas and Van Deenen, 1961a), have shown that the distribution of unsaturated and saturated acids is the reverse of this, since phospholipase A liberates the fatty acids from the β-position.

Lysophosphatidylethanolamine

Lysophosphatidylethanolamine is obtained by the action of the enzyme phospholipase A upon phosphatidylethanolamine (Levene and Rolf, 1923; Fairbairn, 1945; Long and Penny 1957; de Haas and Van Deenen, 1961a; Robins and Thomas, 1963b). Some controversy has arisen over the position of attack of the enzyme. Ludecke (1906) found that the fatty acids released after enzymatic degradation of lecithin were invariably unsaturated, whereas the acid remaining was saturated. Despite a criticism of this by Latzer (1927) the finding was confirmed by Delezenne

and Fourneau (1914) and Belfanti, Contardi and Ercoli (1936), and this led to the thought that the enzyme specifically released the unsaturated acids.

However, Zeller (1925) found that the enzyme would attack a sample of L-α-dimyristoyl lecithin. Furthermore, King (1934) showed that hydrolecithin prepared by catalytic reduction of natural lecithin was attacked as rapidly as the original compound. This clearly indicated that the ester groupings of the unsaturated acids were not exclusively hydrolysed.

Chargaff and Cohen (1939) investigated the possibility that the enzyme preferentially attacked the more highly unsaturated acids, but found that this was not so.

Zeller (1925) postulated that the enzyme exclusively attacked at either the α- or β-position, irrespective of the state of unsaturation of the fatty acids at that ester linkage.

Hanahan (1954a) oxidised lysolecithin and subsequent acid hydrolysis yielded phosphoglyceric acid as the only phosphorus containing compound, indicating that the enzyme had attacked at the α-position. This was confirmed for lecithin and phosphatidylethanolamine in a similar investigation by Gray (1958).

Long and Penny (1954) worked on the hypothesis that if the fatty acid split off was from the α-position, then a free primary alcoholic grouping would be exposed: if from the β-position, then a secondary alcohol would result. From the quantity of potassium dichromate required to oxidise lysolecithin, it appeared that a primary alcoholic grouping had been exposed, and thus it was confirmed that the enzyme had liberated the fatty acid from the α-position.

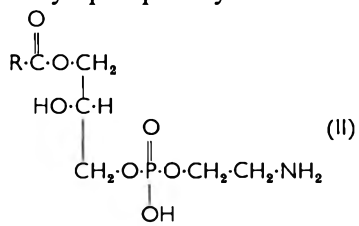
This was criticised by Marinetti, Erbland and Stotz (1959) who concluded from their results that the enzyme attacked indiscriminately at the α- or β-position. They later (1960) indicated that phospholipase A showed chain length specificity with respect to the hydrolysis of the fatty acids of lecithin, the C_{22} and C_{20} acids being hydrolysed in preference to the C_{18} and C_{16} acids.

Tattrie (1961), in investigating the positional distribution of fatty acids in lecithin, employed pancreatic lipase as a specific liberator of the fatty acids in the α-position. He showed that the saturated acids occupied the α-position and the unsaturated β-position. Since phospholipase A liberates the unsaturated fatty acids, then the site of attack must be at the β-position.

Hanahan and others (1950) re-investigated the problem and were able to confirm Tattrie's findings.

The difficulty has been conclusively settled for both lecithin and phosphatidylethanolamine by de Haas and van Deenen (1961a). They reacted phospholipase A with synthetic α-stearoyl β-oleoyl phosphatidylethanolamine and α-oleoyl β-stearoyl phosphatidylethanolamine and the former yielded oleic acid only, and the latter stearic acid only after enzymatic hydrolysis, thus proving that the enzyme has β-ester specificity. In view of these findings they suggested that the name "β-phosphatidase" be given to the enzyme.

Hence the structure of lysophosphatidylethanolamine must be (II).



(II)

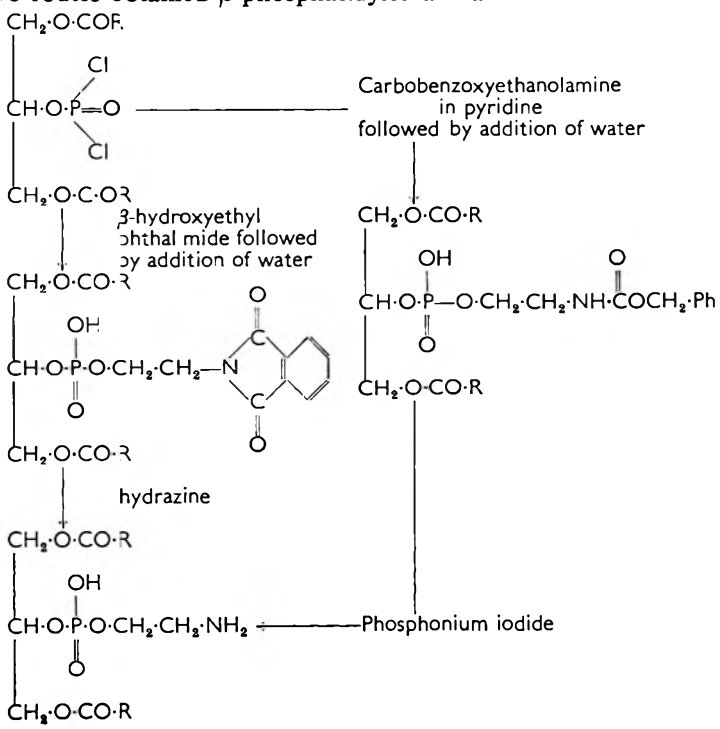
SYNTHESIS

Phosphatidylethanolamine

The first reference to an attempt to synthesise phosphatidylethanolamine was by Grün and Limpacher in 1927. They heated an $\alpha\beta$-diglyceride with phosphorous pentoxide and reacted this with the bicarbonate of amino-ethanol. It is very doubtful whether they obtained phosphatidylethanolamine, since the $\alpha\beta$-diglyceride had probably rearranged to the more stable $\alpha\gamma$-form, and the bicarbonate could not have completely prevented the phosphoric acid group from forming a salt with the amino-group of the ethanolamine.

Kabashima (1938) prepared an α-phosphatidylethanolamine by reacting an acetal of glycerol with phosphorous oxychloride, then stearoyl chloride, then introducing the ethanolamine moiety with bromoethylamine picrate.

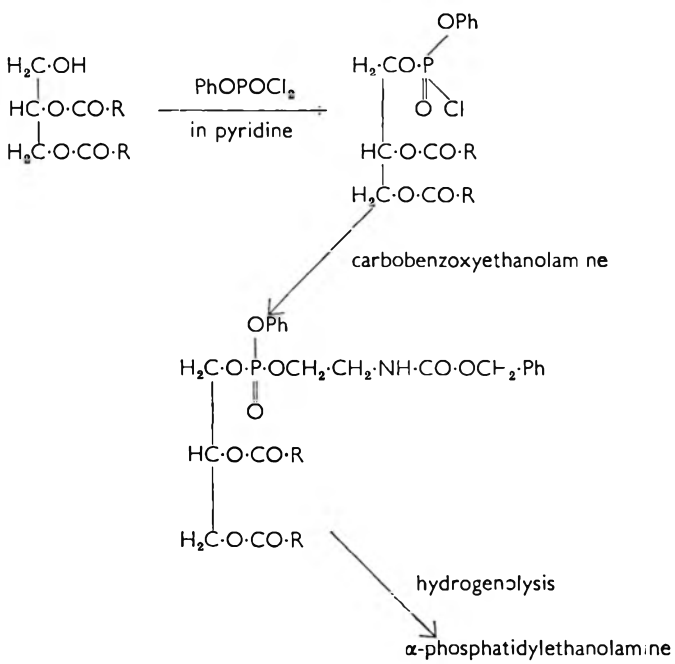
Rose (1947) commenced with an $\alpha\gamma$-dipalmitoyl ester of glycerol and by two routes obtained β-phosphatidylethanolamine.



This product was insoluble in ether, soluble in ethanol, sintered at 180° and melted at 194°. By a similar method, Hunter, Roberts and Kester (1948) prepared phosphatidylethanolamine containing two myristic acid groups, which had a melting-point of 174°. They did not isolate the intermediate phthalimide derivative, thus avoiding emulsification and crystallisation losses, so that the yield was greatly increased.

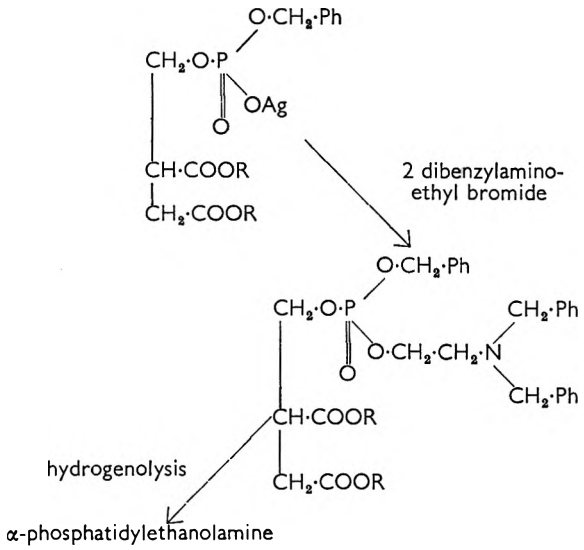
Baer, Maurukas and Russell, in 1951, claimed that no previous attempt to synthesize α-phosphatidylethanolamine could be considered successful, and reported the synthesis of three enantiomorphic compounds, the distearoyl, dipalmitoyl, and dimyristoyl L-α-phosphatidylethanolamines.

They commenced with a diglyceride which was phosphorylated with phenylphosphoryl chloride and then esterified with carbobenzoxyethanolamine in pyridine and then the protective groups removed by hydrogenolysis:



The products were insoluble in acetone and ether, but soluble in ethanol, benzene, pyridine and chloroform.

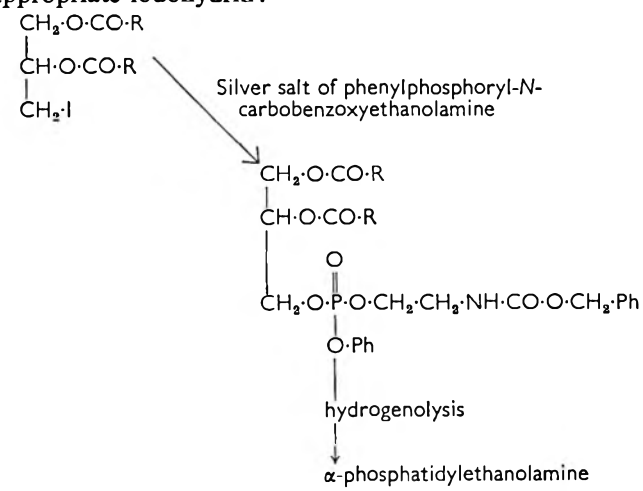
Hoefnagel, Stegerhoek and Verkade (1960) published a method of synthesizing the same α-phosphatidylethanolamines, by commencing with a substituted phosphate derivative of a diglyceride:



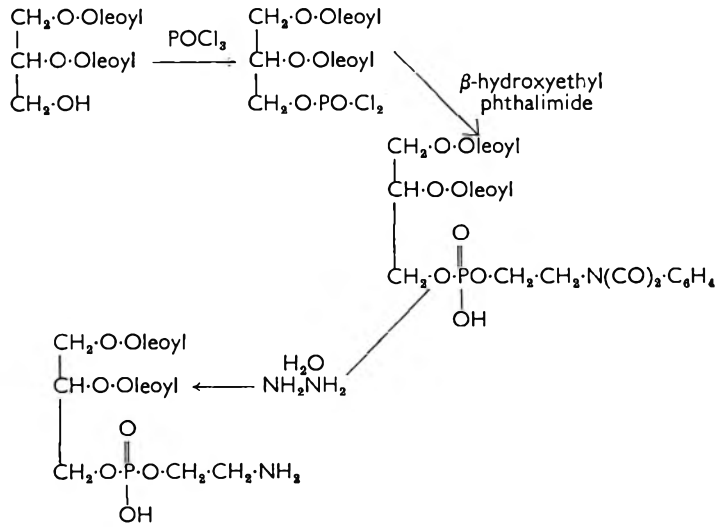
Baylis, Bevan and Malkin (1955) pointed out the difficulties that beset the routes of synthesis.

(1) Syntheses commence with $\alpha\beta$-diglycerides, which readily transform to the $\alpha\gamma$-form. (2) The action of reagents such as phenylphosphoryl dichloride on diglycerides tend to give rise to mixtures of esters which are difficult to separate. (3) To obtain unsaturated acids in the final product, it is necessary to prepare unsaturated $\alpha\beta$-diglycerides and to remove the protecting groups by some other method than catalytic hydrogenation.

They were able to overcome the first two difficulties by commencing with the appropriate iodohydrin:



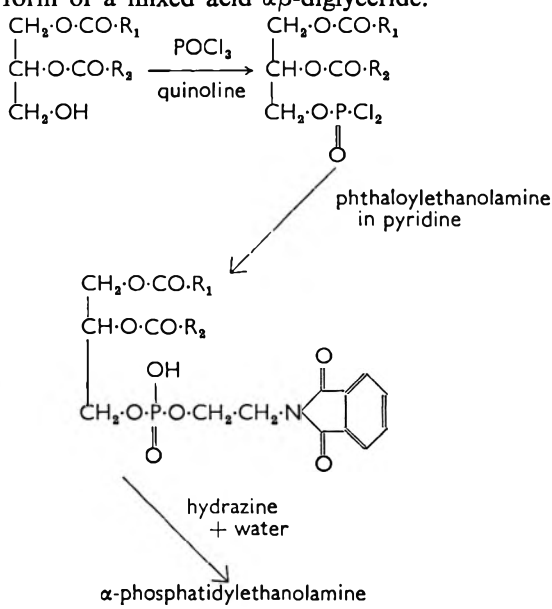
Later Baer and Buchnea (1959) were able to overcome the third difficulty and prepared dioleoyl phosphatidylethanolamine commencing with a 1 : 2-diolein:



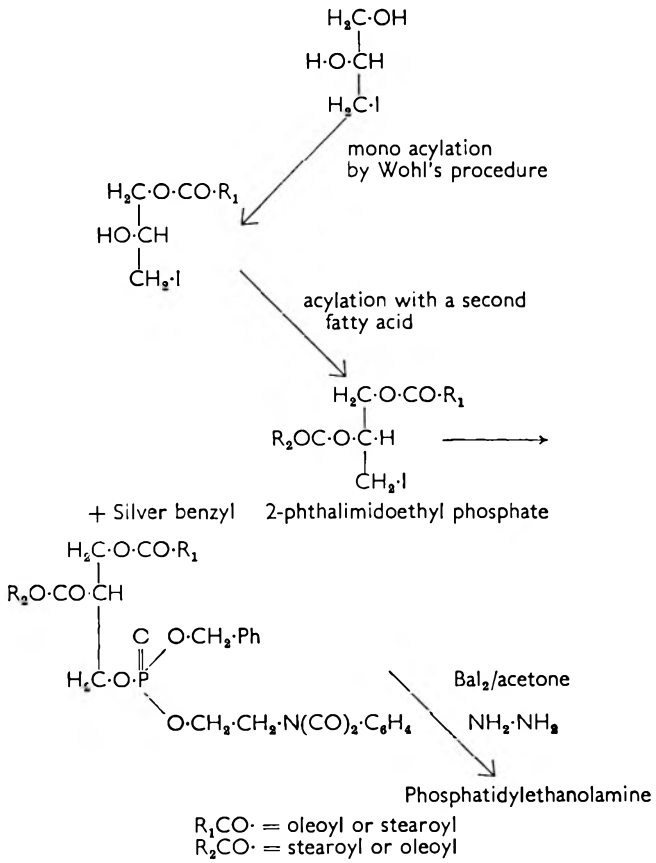
Bevan and Counsell (1961) synthesized DL-dioleoyl phosphatidylethanolamine by a method involving the use of an iodohydrin rather than the somewhat inaccessible 1,2-diolein.

Since, naturally occurring phospholipids appear to possess at least two dissimilar fatty acid substituents, one of which is unsaturated, this led various workers to attempt to prepare phosphatidylethanolamine containing one saturated and one unsaturated fatty acid.

Baer and Buchnea (1961) prepared α-stearoyl-β-oleoyl- and α-oleoyl-β-stearoyl-phosphatidylethanolamines by commencing with the correct enantiomorphic form of a mixed acid αβ-diglyceride.



De Haas, van Deenen and Daemen (1962) commenced with an iodohydrin and substituted the required fatty acids in separate steps:



Kennedy and Weiss (1956) demonstrated the function of cytidine coenzymes in the biosynthesis of phospholipids, by following the incorporation of radioactive phosphorus into the lipids. Two steps were involved, the first being catalysed by a cytidyl transferase enzyme, and the second by a glyceride transferase enzyme:

1. Cytidine triphosphate + phosphorylethanolamine ⇌
Cytidine diphosphate ethanolamine

2. ⟋ diacyl glycerol
phosphatidylethanolamine

Later work (Kennedy, Borkenhagen and Smith, 1959) with de-oxy cytidine precursors gave slower reactions and poorer yields.

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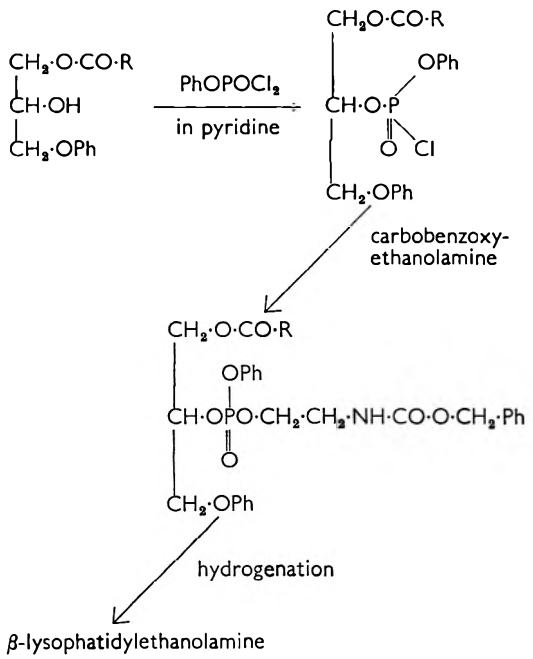
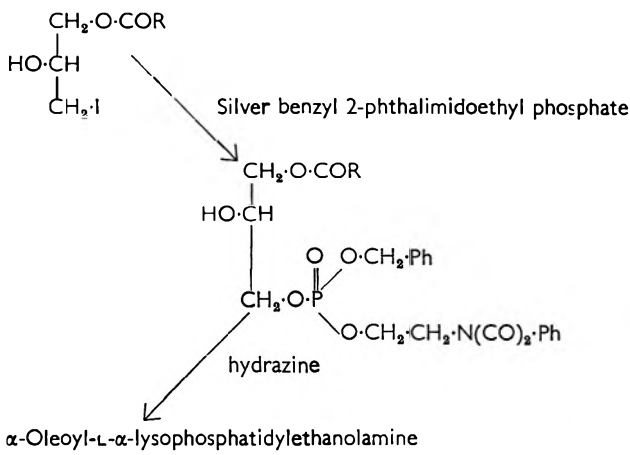
Lysophosphatidylethanolamine

Bayliss and others (1955) first synthesized a lysophosphatidylethanolamine by commencing with a monoglyceride, and following the method of Baer and others (1951) for phosphatidylethanolamine.



Following the conclusion that naturally occurring lysophatidylethanolamine has the fatty acid in the α-position, de Haas, van Deenen and Daemen (1962) prepared α-lysophatidylethanolamine commencing with γ-oleoylglycerol-L-α-iodohydrin:



However, the product contained a mixture of other compounds. Pure lysophosphatidylethanolamine was obtained by chromatographic separation of the final mixture.

PHYSICAL PROPERTIES

General

Phosphatidylethanolamine is a white hygroscopic powder that rapidly darkens on exposure to air to give a dark brown sticky mass, due to autoxidation (Lea, 1955). It is soluble in methanol, chloroform, ethanol, glacial acetic acid, ether and benzene, but insoluble in acetone. It is optically active, the specific rotation in chloroform at 20° being $+6{\cdot}1°$ (de Haas and van Deenen, 1961b). Lysophosphatidylethanolamine is a gleaming white, non-hygroscopic powder, and does not change colour on storage (Levene, Rolf and Simms, 1924). It is less soluble in organic solvents than phosphatidylethanolamine (Robins and Thomas, 1963b). The specific rotation of α-oleoyl glycerol-β-phosphorylethanolamine in chloroform at 20° was $-2{\cdot}5°$ (de Haas, van Deenen and Daemen, 1962).

Aqueous Solutions

Both substances form stable hydrophilic sols with water, whose turbidity increases with concentration. The sols are precipitated by small concentrations of potassium and calcium chlorides (Robins and Thomas, 1963a,b). Spiegel-Adolf (1935) indicated that phosphatidylethanolamine acted as a protective colloid for cholesterol against precipitation by salts and proteins, and protected colloidal gold from flocculation by neutral salts.

Phosphatidylethanolamine exists in water as a colloidal electrolyte and forms micelles in which the molecules are arranged in double layers with the polar groups orientated towards the aqueous medium and the hydrocarbon chains away from the micelle surface. Such bimolecular leaflets have a thickness of 30–60Å depending on hydrocarbon chain length, temperature and hydration (Engstrom and Finean, 1958). Electron microscopic examination of phosphatidylethanolamine in 0·01M ammonium carbonate solution, showed particles that were round or irregular flat discs having a width of 50–70Å (Wallach, Maurice, Steele and Surgenor, 1959).

By means of X-ray diffraction studies, Schmidt and Palmer (1940) found that the spacings between the leaflets in phosphatidylethanolamine sols were considerably larger than those in the dry substance, indicating the presence of water molecules between the leaflets.

Low angle diffraction studies by Finean and Millington (1955) showed that the spacing of phosphatidylethanolamine in the bimolecular leaflet decreased, usually in well defined steps, with rise in temperature. They concluded that this was due to tilting of the long chain molecules, as a result of the change of intermolecular Van der Waals and ionic forces.

Robins and Thomas (1963a, b) showed that both substances have marked surface-active properties, 0·01 per cent w/v aqueous sols reducing

the surface tension of water to 30 dynes/cm. The critical micelle concentration of phosphatidylethanolamine was in the range 0·002 to 0·01 per cent w/v, while that of lysophosphatidylethanolamine was in the range 0·001 to 0·002 per cent w/v. Surface tension studies indicated that both substances showed a large surface-ageing effect. These large changes of surface tension of lysophosphatidylethanolamine with time have been related to an intramolecular migration of the phosphate-ethanolamine moiety from the γ- to the β-position.

Surface Films

Phosphatidylethanolamine will form monomolecular films on water, and from force: area measurements the molecular area of distearoyl phosphatidylethanolamine was found to be 36Å^2 (van Deenen, Houtsmuller, de Haas and Mulder, 1962). Monomolecular films have been employed to study lipid-protein complexes with a view to investigating the nature of cell membranes. Apparently the protein molecules are spread out along the polar interface of the phosphatidylethanolamine monolayer, the basic groupings of the protein binding with the phosphoric acid groupings of phosphatidylethanolamine (Doty and Schulman, 1949; Payens, 1960). Many factors such as pH, salt concentration, dielectric constant, affect these associations, which alter the hydrogen bonding, electrostatic and Van der Waals forces between the protein and the lipid (Fraser, 1957).

Alexander, Teorell and Arborg (1939) studied the effect of various salts on monolayers of phosphatidylethanolamine. At the air: water interface there was little effect, but at the benzene: water interface calcium ions greatly stabilised the lipid in the interface. Rosano, Schiff and Schulman (1962) state that calcium ions link with phosphatidylethanolamine at the water: oil interface to form calcium cephalinate which prevents the transport of sodium and potassium ions across the interface. The calcium ion breaks up the phosphoric acid-amine polar group intramolecular association, and prevents intermolecular association between neighbouring molecules. However, no such interaction was detected with magnesium ions.

Acid-Base Relationships

Since the two substances contain a relatively weak basic group (ethanolamine) and a strong acidic group (phosphoric acid) they form zwitterionic structures in water. Various values for the iso-electric points have been estimated (Bull and Frampton, 1936; Garvin and Wallach, 1958; Wallach, Maurice, Steele and Surgenor, 1959; Payens, 1960) and have been found by surface tension measurements (Robins and Thomas, 1963a, b) to be at pH 3·1 for phosphatidylethanolamine, and at pH 3·25 for lysophosphatidylethanolamine.

Phosphatidylethanolamine has been shown to form zwitterions in water, whereas in solvents of low dielectric constant it exists as ions or neutral molecules (Fischgold and Chain, 1935). Jukes (1934) showed from electrometric titrations that phosphatidylethanolamine had two pK

values, at pH 1·1 and 8·9. Unlike lecithin, it bound alkali and exhibited a buffering capacity. The two pKa values for lysophosphatidylethanolamine have been quoted as 3·9 and 9·5 (Levene, Rolf and Simms, 1924). Phosphatidylethanolamine has been shown to bind inorganic cations over a range of pH, which suggested its possible role in biological electrolyte equilibria (Christensen and Hastings, 1940).

Blood Coagulation

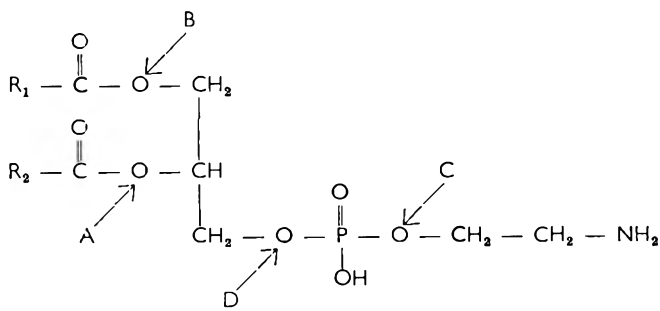
The cephalin group of phospholipids has been identified with the mechanism of blood coagulation. Thrombin formation was thought to be due to an enzymic mobilisation of cephalin and calcium at the colloidal surface of prothrombin (Wadsworth, Maltaner and Maltaner, 1931; Ferguson and Erikson, 1939).

Chargaff (1944) studied numerous phospholipid fractions from heart and brain tissue, and found that phosphatidylethanolamine showed only a slight thromboplastic activity. He concluded that thromboplastic active lipids could not be identified with any of the known phospholipids.

Poole and Robinson (1956) showed that phosphatidylethanolamine has a direct role in blood clotting. This activity was not due to lysophosphatidylethanolamine or contaminants since synthetic phosphatidylethanolamines had identical activity to phosphatidylethanolamine prepared from natural sources. This was later confirmed (Poole, Robinson and Harris, 1957). O'Brien (1956) indicated that phosphatidylethanolamine was contained in the platelets and is liberated when blood is shed.

Enzymatic Hydrolysis

There are four enzymes that can hydrolyse the phospholipids, and their points of attack are shown below.



The source and site of action of phospholipase A has already been discussed.

Phospholipase B removes the α-acyl grouping (Hanahan, 1955) but only from lysophosphatidylethanolamine: it has no action on phosphatidylethanolamine (Fairbairn, 1948).

Phospholipase C yields ethanolamine on hydrolysis of egg phosphatidylethanolamine and synthetic dimyristoyl DL-α-phosphatidylethanolamine, whereas synthetic β-phosphatidylethanolamine is only slightly attacked (Davidson and others, 1955).

A fourth enzyme, phospholipase D, hydrolyses phosphatidylethanolamine at the glycerol-phosphoric acid ester linkage, yielding phosphorylethanolamine. Until recently, the enzyme was considered to have no reaction upon cephalins (MacFarlane, 1948), but it has now been shown (de Gier, de Haas and van Deenen, 1961) that phosphatidylethanolamine emulsified with lecithins is susceptible to attack by the enzyme obtained from *Clostridium welchii*. These conditions of emulsification probably favour the requirements in charge and orientation of the molecules necessary for enzyme-substrate interaction. However, the phospholipase obtained from *Bacillus cereus* appeared to be active upon isolated and synthetic phosphatidylethanolamines. Since the mode of action of both bacterial enzymes were identical, the enzymes must differ in their distribution of charge.

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RESEARCH PAPERS

EFFECT OF PRONETHALOL ON SOME INHIBITORY ACTIONS OF CATECHOLAMINES

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On the blood pressure of the pithed rat isoprenaline produced a vasodepressor, noradrenaline a pressor, and adrenaline a biphasic response consisting of a rise followed by a fall in the pressure. The magnitude of the responses to increasing doses of adrenaline was not proportional to the doses. An intravenous injection of pronethalol (nethalide) hydrochloride (1 mg./kg.) changed the responses to adrenaline into purely pressor, well-graded responses; they were also increased. The responses to isoprenaline after pronethalol were reduced, but those to noradrenaline remained usually unaltered. Similar results were obtained in anaesthetised rats, rabbits and guinea-pigs, but the augmentation of the pressor effect of adrenaline was not so marked as in pithed rats. The vasodepressor action of dopamine in rabbits and guinea-pigs was not blocked by pronethalol (2·5 to 10 mg./kg.). The inhibitory actions of adrenaline, noradrenaline and isoprenaline on the isolated rat uterus and rabbit colon were not consistently antagonised by concentrations of pronethalol up to 1·0 μg./ml. In experiments on the rabbit duodenum and ileum, pronethalol, 0·2 to 1·0 μg./ml., reduced the relaxations produced by all three catecholamines. These results are discussed in relation to Ahlquist's theory of a dual adrenergic receptor mechanism.

The report by Powell and Slater (1958) on the pharmacology of dichloroisoprenaline (DCI) aroused considerable interest in the possibility of blocking those actions of catecholamines, notably the cardiac acceleration, vasodepression and inhibition of smooth muscle, which were not effectively antagonised by the classical anti-adrenaline drugs. Recently, Black and Stephenson (1962) have reported that the compound 2-isopropylamino-1-(2-naphthyl) ethanol hydrochloride (pronethalol, nethalide, Alderlin) possessed similar pharmacological properties to those of dichloroisoprenaline, but was superior in lacking intrinsic sympathomimetic activity.

The classical anti-adrenaline drugs such as the ergot alkaloids, and yohimbine and phenoxybenzamine, can block the pressor effect of noradrenaline and reverse the pressor effect of adrenaline into a vasodepressor effect. This phenomenon of "adrenaline-vasomotor-reversal" was interpreted as consequence of blockage of the excitatory (alpha) effect of adrenaline and an unmasking of the vasodilatation (beta-effect), which is overshadowed by the stronger pressor component under normal circumstances.

The present study was made to see the effect of pronethalol on the responses of the blood pressure of rats, rabbits and guinea-pigs to adrenaline, noradrenaline and isoprenaline. Special attention was given to experiments with atropine-treated, pithed rats, since they are particularly

suitable for studying the peripheral action of drugs. In addition, the influence of pronethalol on the vasodepressor effect of dopamine in rabbits and guinea-pigs (Burn and Rand, 1958) was also investigated. Experiments were also made on isolated smooth muscle preparations of rat uterus and rabbit intestine in an attempt to determine whether pronethalol blocks the inhibitory action of catecholamines on these tissues.

METHODS

Albino rats of either sex, 150–300 g. weight, were pretreated with atropine sulphate (2 mg. per rat s.c.), anaesthetised with ether and pithed as described by Shipley and Tilden (1947).

In some experiments rats were anaesthetised with urethane, 0·7 ml. of a 25 per cent solution per 100 g. weight, subcutaneously. The blood pressure was recorded from a carotid artery. A cannula was inserted into the trachea to respire the pithed rats artificially, or to aid respiration in the urethane-anaesthetised animals. Drugs were injected into a cannulated femoral vein and washed in with 0·9 per cent solution of sodium chloride. The total volume injected at one time was 0·3 ml.

Rabbits and guinea-pigs were anaesthetised with 25 per cent solution of urethane; the former were injected with urethane intravenously at a slow rate until pain reflexes were abolished; for guinea-pigs the dose of urethane, injected intraperitoneally, was the same as in rats. The carotid blood pressure was recorded with a conventional mercury manometer. Drugs were administered through a cannulated femoral or jugular vein.

Single horns of the uterus from non-oestrus rats were suspended in De Jalon's solution in a 2 ml. bath. Contractions were induced at regular intervals with carbachol (1 μg./ml.), as described by Gaddum and Lembeck (1949). The inhibitory actions of adrenaline, noradrenaline and isoprenaline were tested by adding each amine to the bath 60 sec. before carbachol. Pronethalol hydrochloride in concentrations of 0·01 to 10·0 μg./ml. was added to the bath 30–60 sec. before catecholamines. In some experiments pronethalol was added to a reservoir of De Jalon's solution, so that the organ could be exposed longer to the action of the drug.

Segments of rabbit duodenum, ileum and terminal colon were suspended in Krebs' solution in a 25 ml. organ bath and gassed with a mixture of 95 per cent O_2 and 5 per cent CO_2. Records were made with a gimbal lever exerting a tension of 0·5 g. and magnifying five times. Pronethalol hydrochloride in concentrations of 0·1 to 10·0 μg./ml. was either added directly to the bath 2–3 min. before adding catecholamines, or was incorporated into the Krebs' solution in the reservoir and allowed to act continuously.

Drugs were dissolved in 0·9 per cent solution of sodium chloride. Stock solutions of (−)-adrenaline base, (−)-noradrenaline bitartrate, (±)-isoprenaline sulphate and dopamine hydrochloride were prepared in 0·01N hydrochloric acid in a concentration of 1 mg./ml. of the base. From these, dilutions were made with 0·9 per cent sodium chloride

solution, containing 10 mg. ascorbic acid per 100 ml. For pithed rats diluted solutions of adrenaline, noradrenaline and isoprenaline as low as 100 ng./ml were prepared, whereas for anaesthetised animals dilutions of 1 μg./ml. or 10 μg./ml. were used. Dopamine was used in concentrations of 100 to 200 μg./ml. The amounts of catecholamines are expressed in terms of their bases. The amounts of pronethalol are given in terms of the hydrochloride.

RESULTS

Observations on Blood Pressure

In pithed rats small doses of adrenaline (1 to 10 ng.) produced usually biphasic responses containing a rapid rise and then a fall in blood pressure (Fig. 1). Occasionally only a fall was observed. The pressor component of the response to adrenaline was not consistently increased with increasing doses. Paradoxically, sometimes a smaller dose provoked a higher rise in blood pressure than did a larger dose (Fig. 1). In contrast, noradrena-

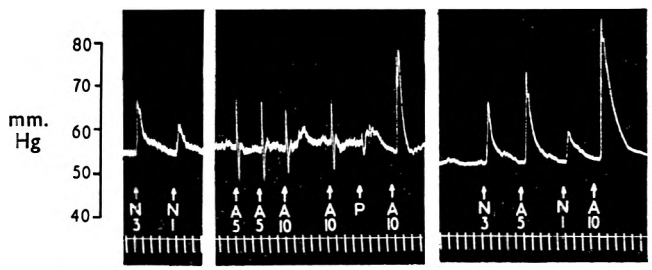

FIG. 1. Carotid blood pressure in mm. Hg of a pithed rat (220 g.) A, adrenaline; N, noradrenaline; doses in ng. P, pronethalol hydrochloride, 1 mg/kg. Between the second and the third record 30 min. elapsed. Time in min.

line always produced a purely pressor response with a good dose-response curve. After repeated injections of adrenaline and noradrenaline, especially after giving larger doses (20 to 30 ng.) the sensitivity of the rat to both amines increased, but the responses to adrenaline remained biphasic and poorly graded.

Isoprenaline caused fall in blood pressure (Fig. 2). The extent and duration of responses were proportional to the doses used.

In anaesthetised rats, rabbits and guinea-pigs, isoprenaline had a depressor effect. Noradrenaline and adrenaline had pressor effects, but with the latter amine, a secondary small fall in the pressure was often seen.

In rabbits and guinea-pigs, dopamine in doses of 10 to 250 μg. always produced depression in blood pressure. Its duration and extent were proportional to the doses used (Fig. 4).

A single injection of pronethalol, 1 mg./kg., in pithed rats caused a transitory small rise (about 10 to 20 mm. Hg) in blood pressure. However, in anaesthetised animals pronethalol always produced a fall of the pressure associated with bradycardia. Pronethalol was administered usually in a single injection following a series of control injections of

adrenaline, noradrenaline, isoprenaline or dopamine. Five or 10 min. after the injection of pronethalol, as soon as the pressure returned to the initial level, the amines were reinjected in the same doses and their effects compared with the controls.

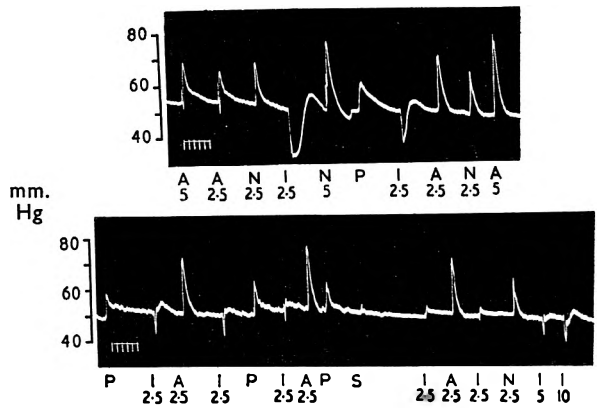

FIG. 2. Carotid blood pressure in mm. Hg. of a pithed rat (245 g.) A, adrenaline; N, noradrenaline; I, isoprenaline; doses in ng. P, pronethalol hydrochloride 1 mg./kg. S, 0·3 ml. of 0·9 per cent solution of sodium chloride. Time in min.

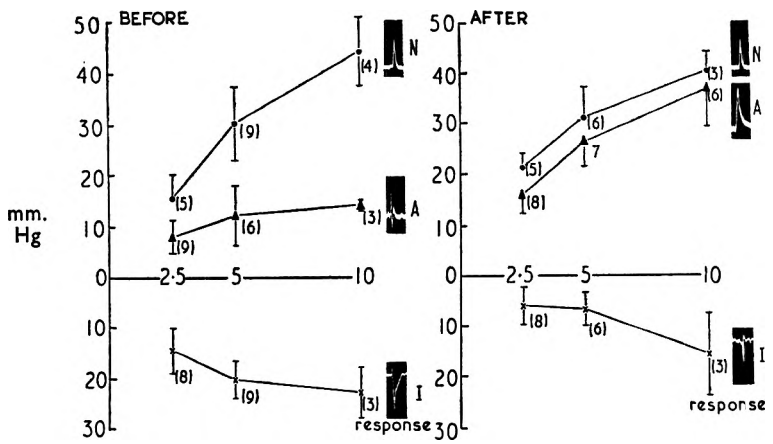

FIG. 3. Dose-response curves of adrenaline (—▲—), noradrenaline (—●—) and isoprenaline (—×—) before and after administration of pronethalol hydrochloride (1 mg./kg.) in 5 rats. Doses on the abscissae (2·5, 5 and 10 ng.); changes in blood pressure are given on the ordinates. Zero-line: initial blood pressure. The type of response to each amine (A, adrenaline, N, noradrenaline, I, isoprenaline) before and after pronethalol is shown on the right hand side of each graph. Note that the flat dose-response curve of adrenaline, on the left, was converted after pronethalol (right) into a steeper curve, parallel with that of noradrenaline. The figures in brackets refer to the number of observations. Each point represents the mean of the observed responses to a given dose. The standard deviations are shown by vertical bars.

Responses after pronethalol. In pithed rats adrenaline produced purely pressor responses, which were much larger than the pressor components of the controls (Fig. 1). A good dose-response relationship was

established and this, together with the increased sensitivity, allowed discrimination of doses differing by 1 or 0·5 ng. The smallest dose of adrenaline to produce a detectable response was often as low as 0·5 ng. This selective potentiation of adrenaline was sometimes so marked that adrenaline became more pressor than an equal dose of noradrenaline.

The responses to noradrenaline were usually unaltered (Fig. 1). In only occasional experiments a slight increase in the responses to this amine was observed. This change, however, may be attributed to the spontaneous increase in the sensitivity of the animal, unrelated to the action of pronethalol.

Pronethalol reduced the depressor action of isoprenaline. For a complete blockade of isoprenaline, however, higher doses of pronethalol than those which potentiated adrenaline, were required. Fig. 2 illustrates an experiment in which the augmentation of the effect of adrenaline was observed after 1 mg./kg. of pronethalol, which was sufficient only to

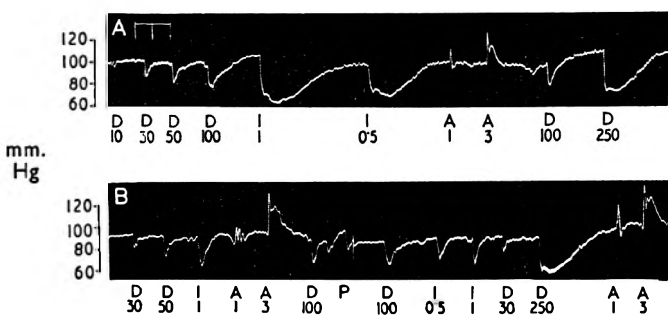

FIG. 4. Carotid blood pressure of a rabbit (2·25 kg.) under urethane-aneasthesia. A, adrenaline; D, dopamine; I, isoprenaline. The numbers denote doses in μg. Between A and B, pronethalol hydrochloride, 2·5 mg./kg., i.v. B taken 10 min. after the injection of pronethalol. At P, an additional dose of pronethalol hydrochloride, 2·5 mg./kg.,i.v. was given very slowly. At the vertical line the kymograph was stopped for 10 min. Time in min.

reduce the effect of 2·5 ng. of isoprenaline. To block the response to isoprenaline it was necessary to administer a total of 4 mg./kg. of pronethalol. Eventually the effect of isoprenaline was reversed into a small, but definite, increase in blood pressure. Larger doses of isoprenaline (5 and 10 ng.) still caused a slight fall in blood pressure.

Fig. 3 illustrates graphically the responses to adrenaline, noradrenaline and isoprenaline before and after administration of 1 mg./kg. of pronethalol in 5 rats. The dose-response curve and the type of response to adrenaline after pronethalol were changed and became similar to those of noradrenaline. These changes were associated with reduction of the responses to isoprenaline.

Similar observations to those described above were made in anaesthetised animals. Here again, pronethalol in doses of 2·5 to 10 mg./kg. antagonized the effect of isoprenaline. The pressor effect of adrenaline

was increased, although not so much as in pithed rats. Slight enhancement of the responses to noradrenaline was often observed.

The depressor response to dopamine in rabbits and guinea-pigs was not affected by pronethalol in doses of 2·5 to 10 mg./kg. (Fig. 4). The latter dose, however, was always sufficient to reduce or even abolish the responses to equipotent doses of isoprenaline.

Experiments on Isolated Organs

Of eleven uterine horns tested, in only one did pronethalol, in a concentration of 0·1 μg./ml. added to the reservoir, block the effect of adrenaline and isoprenaline (Fig. 5). This block was partially reversible after washing. In all other experiments the concentrations of 0·1 μg./ml. of pronethalol were not sufficient to block adrenaline and isoprenaline. Concentrations of pronethalol above 0·1 μg./ml. caused deterioration of the preparation and the drug itself exerted an inhibitory effect on the carbachol-induced contractions.

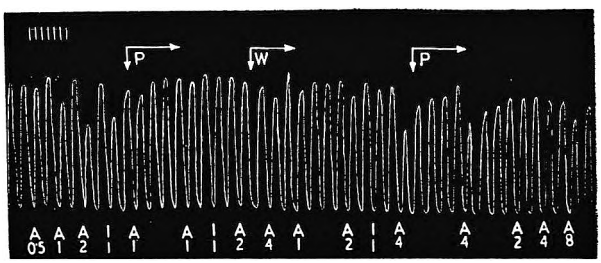

FIG. 5. Contractions of the rat uterus elicited by carbachol (1 μg./ml.). A, adrenaline; I, isoprenaline; the doses are given in ng. P, pronethalol hydrochloride 0·1 μg./ml. from a reservoir with De Jalon's solution. W, washing, change to normal De Jalon's solution. Time in min.

Concentrations up to 1 μg./ml. of pronethalol were usually ineffective in blocking the relaxations of the rabbit terminal colon produced by adrenaline, noradrenaline and isoprenaline. Concentrations of 1 μg./ml. or more usually caused depression of the tone and reduced the spontaneous contractions of the colon. In only one case pronethalol, added to the bath in a concentration of 0·4 μg./ml. and allowed to act for 3 min., reduced the relaxations produced by adrenaline (0·1 μg./ml.) or isoprenaline (0·1 μg./ml.). In another experiment pronethalol (0·1 μg./ml.), together with phenoxybenzamine (1 μg./ml.), partially blocked the relaxations of the colon induced by adrenaline, noradrenaline or isoprenaline (0·1 μg./ml.).

In the experiments on rabbit duodenum and ileum, pronethalol in concentrations of 0·2 to 1·0 μg./ml. only partially blocked the relaxations induced by adrenaline, noradrenaline or isoprenaline (Fig. 6). Higher concentrations than 1 μg./ml. caused depression of the tone and reduced the frequency of the rhythmic movements of the intestine.

Discussion

The present experiments show that pronethalol selectively potentiates the pressor response to adrenaline presumably by blocking the vasodilator component of its action. Powell and Slater (1958) working with cats, and Outschoorn and Jacob (1960) working with rats, reported blockade of the vasodilator action of adrenaline by dichloroisoprenaline, a drug which has similar pharmacological properties to those of pronethalol. In both species, the pressor activities of adrenaline and noradrenaline after dichloroisoprenaline were enhanced, but in neither a selective potentiation for adrenaline was observed. In experiments on man, Bharadway and Shanks (1962) found that dichloroisoprenaline blocked the vasodilatation in the arm caused by adrenaline. Larger doses of dichloroisoprenaline abolished not only the dilator, but also the constrictor action of adrenaline.

The characteristic effect of pronethalol observed in pithed rats, seems to be explicable in terms of the dual receptor mechanism, proposed by Ahlquist (1948). According to this theory the sympathomimetic amines

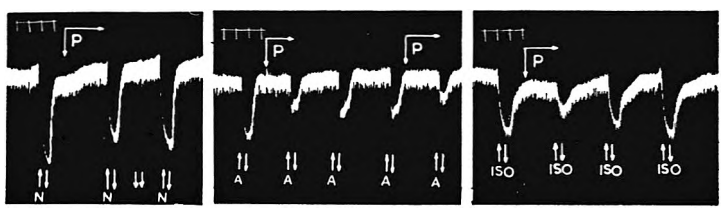

FIG. 6. Rabbit duodenum. First record: effect of pronethalol (P, 0·2 μg./ml.) on the relaxations caused by noradrenaline (N, 0·1 μg./ml.) Middle record: effect of pronethalol (P) on the relaxations caused by adrenaline (A, 0·04 μg./ml.) Left P, pronethalol 0·2 μg./ml.; Right P, pronethalol 0·4 μg./ml. Last record: effect of pronethalol (P, 1 μg./ml.) on the relaxations caused by isoprenaline (ISO, 0·04 μg./ml.) Upward arrows indicate the addition of the amines to the bath; downward arrows indicate washings. Time in min.

exert their action by combining either with alpha- or with beta-receptors, or with both. In agreement with this conception, the purely pressor response to noradrenaline, due to a predominant vasoconstriction, is an alpha-effect, while the depressor action of isoprenaline, a result of vasodilatation, is a beta-effect. Adrenaline appears to possess both effects. If pronethalol blocked the beta-receptors and consequently abolished the vasodilatation, this in turn would enhance the constrictor action, since it is no longer counteracted by the dilator action. Therefore with pronethalol we have another type of "adrenaline-reversal", which is the opposite of that obtained with the classical anti-adrenaline drugs.

The depressor action of dopamine in rabbits and guinea-pigs (Burn and Rand, 1958) does not appear to be a beta-effect, since it was not blocked by pronethalol.

The question of the blockade of the actions of catecholamines on the rabbit intestine and rat uterus is more complex. According to Ahlquist (1962) the rat uterus contains beta-receptors, whereas in the intestine

there are both alpha- and beta-receptors. Furchgott (1959), however, suggested that the receptors in the intestinal smooth muscle were neither alpha nor beta. The present inconsistent and mostly negative results on the isolated preparations of the rat uterus and rabbit intestine with pronethalol, neither support nor contradict the above-mentioned conceptions. One of the difficulties in the assessment of the blocking activity of pronethalol was the fact that it itself exerted an inhibitory action on the uterine and intestinal tissues. Whether it was a genuine sympathomimetic beta-effect or only papaverine-like action, it was not determined. Therefore, for practical reasons, pronethalol is not a suitable agent for blocking the actions of catecholamines on the intestine and uterus.

However, pronethalol appears to be a useful pharmacological tool in the study of other effects of sympathomimetic amines. Vanov and Vogt (1963) have found it useful in the bioassay of adrenaline on pithed rat blood pressure. Black and Stephenson (1962) have considered even the possibility for the therapeutic use of pronethalol in some heart diseases.

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THE SOLUBILITY OF IODINE IN AQUEOUS SOLUTIONS OF NON-IONIC SURFACE-ACTIVE AGENTS

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The solubility of iodine in aqueous solutions of Cetomacrogol 1000 B.P.C., polyethylene glycol 1540 and cetyl and lauryl ethers of polyoxyethylene glycol has been determined. In the region of the critical micelle concentration of cetomacrogol the solubility is below that in water. Well above the CMC, the solubility increases linearly with the concentration of cetomacrogol. Over the temperature range 20–50° the solubility of iodine in aqueous cetomacrogol solution increases with rise of temperature. When a solution is heated and cooled the amount of iodine in the cooled solution is greater than can be obtained by equilibration at that temperature alone. Not all the iodine added to the surface-active agent systems is available for titration with sodium thiosulphate; the unavailable iodine can be accounted for as hydrogen iodide. For 200 g./litre solutions of polyoxyethylene glycol cetyl and lauryl ethers, the available iodine in solution decreases with the number of ethylene oxide units of the ether, but the molecular ratio of iodine to ether increases with the number of ethylene oxide units. The evidence indicates that formation of a complex between iodine and the surface-active agents is in some way involved in the mechanism of the solubilisation process.

THE use of iodine as an antiseptic began soon after Courtois discovered the element in 1811 and the history of its use is described by Kelly (1961). The reviews of Gershenfeld and Witlin (1950a) and Gershenfeld (1957) describe the wide range of the applications of iodine's antiseptic properties and also its action.

Problems involved in formulating antiseptic iodine preparations are presented by its low water solubility (0·285 g./litre at 20° (Seidell, 1953), the irritant properties of certain solutions and the instability of some formulations. Solvents used in official formulae have included ethanol alone or with an alkali iodide, alkali iodides themselves, chloroform, ether, phenol, castor oil, oleic acid, liquid paraffin, glycerol and collodion. Of these, the ethanolic, ethanol/iodide and iodide solutions are in common use today, but their irritant and staining properties leave them open to criticism. MacDonald and Peck (1928) suggested the use of isopropanol as a solvent when iodine was to be used as a skin antiseptic. Gershenfeld and Witlin (1950b) found that propylene glycol was not entirely satisfactory, while Osol and Pines (1952) found that high proportions of glycols were required to include useful amounts of iodine. Cantor and Shelanski (1951) and Terry and Shelanski (1952a, b) reported that iodine dissolved in surface-active agents had antibacterial properties whilst Allawala and Riegelman (1953a, b) provided studies of iodine/non-ionic surface-active agent complexes. The complexes which form between iodine and surface-active agents have been termed "iodophors" and

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Terry and Shelanski (1952b) defined them as "a mixture of iodine and a carrier which greatly increases the solubility of and tends to stabilise iodine in aqueous systems to reactants other than micro-organisms." The term "carrier" is used to include polymeric materials which can also be used to form iodophors.

Reviews of the properties of iodophors have been published by Wetzler (1959), Wilson, Mizuno and Bloomberg (1960, 1961), Connor (1961) and Davies (1962). From these it would appear that iodophors possess over conventional formulations certain advantages, such as increased stability, especially in dilute solutions; reduction in staining, irritant properties and corrosion of metals; low oral toxicity and a reduction in general chemical reactivity without loss of antibacterial activity. Unfortunately many of the published works lack experimental evidence for the claims made and there are few comparisons between iodophors and conventional formulae.

By analogy with the ability of various non-ionic surface active agents to produce iodophors (Allawala and Riegelman, 1953a, b; Bartlett and Schmidt, 1957; Brost and Krupin, 1957), the possible use of Cetomacrogol 1000 B.P.C. and the properties of such a system have been investigated.

Experimental

Materials and Methods

The iodine used was of analytical reagent grade. Three batches of Cetomacrogol 1000, hereafter referred to as cetomacrogol were supplied by Boots Pure Drug Company. The specifications as required by the B.P.C. of these three samples, designated A, B and C, are in Table I,

TABLE I

Analytical data of samples of cetomacrogol

	B.P.C. requirements	Samples		
		A	B	C
Identification		+	+	+
Acetyl value	40·0–52·5	50·9*	49·3*	52·2†
Acid value	Not more than 0·5	0·0	0·0	0·0
Alkalinity	Neutral	0·0	0·0	0·0
Melting-point	Not lower than 38°	40·5°	39·5°	39·5°
Refractive index at 60°	1·448–1·452	1·4520	1·4517	1·4570
Saponification value	Not more than 0·5	0·0	0·4	0·5
Water content	Not more than 1·0 per cent	0·6	1·1 per cent	0·7

* By method of B.P.C. 1954. † By method of B.P.C. 1959.

from which it may be seen that neither B, nor C, comply with the B.P.C. requirements. The samples of polyethylene 1540 were kindly supplied by Union Carbide Company, whilst those of the polyoxyethylene monoalkyl ethers by Glovers Chemicals Ltd.

The critical micelle concentration (CMC) of the three batches of cetomacrogol were determined in duplicate by measurements of surface tension, using a Du Nouy tensiometer, dye solubilisation, employing dimethylaminoazobenzene (Koltoff and Stricks, 1948) and iodine solubilisation (Ross and Olivier, 1959), the results being given in Table II.

The concentration at which aqueous solutions of the cetomacrogols gelled, was determined approximately by dissolving known weights of cetomacrogol in 25 ml. of distilled water and observing the lowest weight to form a gel at 20°. For batches A, B and C, the weights were 8·4 g., 11·4 g. and 10·1 g. respectively.

TABLE II

VALUES OF THE CMC OF THE THREE BATCHES OF CETOMACROGOL IN AQUEOUS SOLUTION

	Concentration of cetomacrogol in g./litre			
Batch of cetomacrogol	Surface tension	Dye solubilisation	Iodine solubilisation	Average value
A	0·0119 0·0133	0·0122 0·0180	0·0141 0·0127	0·0137
B	0·0085 0·0097	0·0160 0·0140	0·0086 0·0095	0·0111
C	0·0175 0·0160	0·0170 0·0170	0·0188 0·0192	0·0174

Solubility determinations were made by placing finely powdered iodine in wide neck ampoules and adding the required solution. The ampoules were sealed and then agitated in a water-bath at the required temperature for the required time. They were then placed upright for 24 hr. to allow

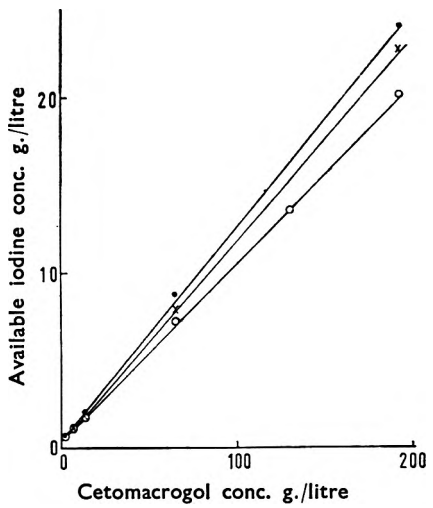

FIG. 1. The solubility of iodine in aqueous solutions of cetomacrogol at 20° C. X Batch A. ○ Batch B. • Batch C.

the solid iodine to settle before samples were removed. The iodine content of known weights or volumes of these solutions was determined with standard sodium thiosulphate solution, the end-point being determined by an amperometric method. For temperatures up to 40° solutions reached equilibrium within 24 hr. but above this temperature 72 hr. were required.

RESULTS

The Solubility of Iodine in Aqueous Solutions of Cetomacrogol

The solubility of iodine in aqueous solutions of the three batches of cetomacrogol at 20° is shown in Fig. 1. Dilutions of all these solutions remained clear. The effect of temperature on the solubility is shown in Fig. 2. In the region of the CMC, solutions did not clear on standing for

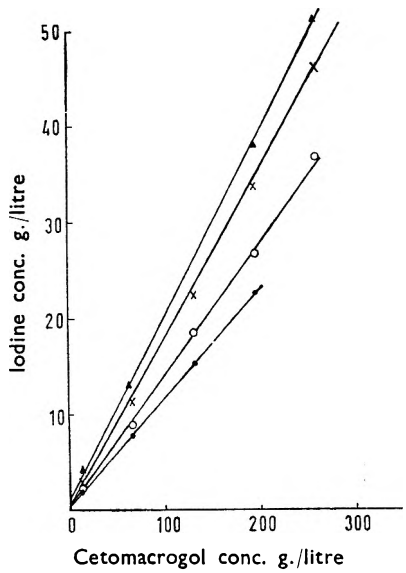

FIG. 2. The effect of temperature on the solubility of iodine in aqueous solutions of Batch A. cetomacrogol. • 20° C. ○ 30° C. × 40° C. ▲ 50° C.

24 hr., a fine suspension remaining. This suspension was however soluble on dilution with water and on estimation of such dilutions, Fig. 3a

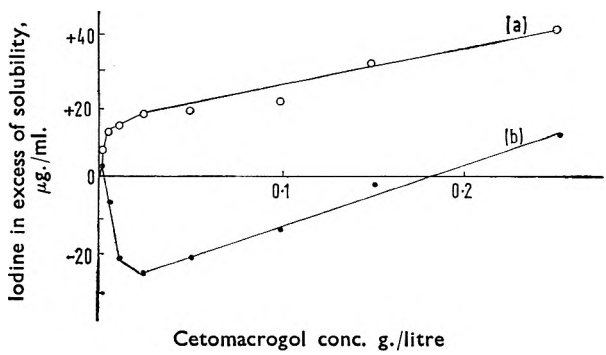

FIG. 3. The solubility of iodine in aqueous solutions of cetomacrogol Batch C, in the region of the CMC at 20° C. ○ Before centrifugation. • After centrifugation.

was obtained. A clear solution could be produced by centrifuging the ampoules in 50 ml. centrifuge buckets, containing water of the required temperature, for 10 min. at 4000 rev./min. When this was carried out the data presented in Fig. 3b was obtained for batch C, at 20°, the solubility curves for the three batches obtained by this method being shown in Fig. 4.

Previous workers have reported that not all the iodine added to surface-active agents is available for titration with sodium thiosulphate. Allawala and Riegelman (1953b) claimed that half the iodine lost was organically bound, whilst the remaining half could be titrated with silver nitrate. Bartlett and Schmidt (1957) on unsubstantial evidence, claimed that the half not organically bound, appeared as hydrogen iodide. Brost and Krupin (1957), using non-ionic surface-active agents and Siggia (1957) using polyvinylpyrrolidone, reported that the sum of the iodine estimated with thiosulphate and iodide, estimated after removal of iodine with thiosulphate, could account for 99 per cent of the iodine added.

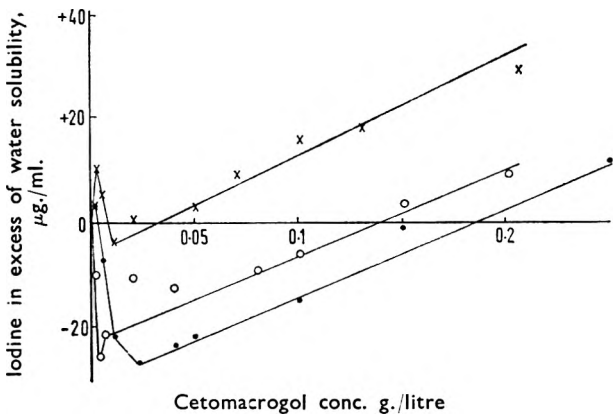

FIG. 4. The solubility of iodine in aqueous solutions of the three batches of cetomacrogol in the region of the CMC at 20° C. X Batch A. ○ Batch B. • Batch C.

To investigate this, the total amount of iodine present in the system was estimated for a series of aqueous solutions of iodine in cetomacrogol, by the sodium fusion method as described in the British Pharmacopoeia 1958 for Chinifon Sodium. On adding known amounts of iodine to cetomacrogol solutions, the percentage of iodine found by this method ranged from 99·2 to 102·6 per cent. Results for a series of cetomacrogol, batch C, solutions are shown in Fig. 5.

In the next stage of the investigation, analysis of an aqueous solution of cetomacrogol to which a known amount of iodine had been added was made. After determinations with sodium thiosulphate, methyl red was added, and the acid present was titrated with sodium hydroxide. The total iodine was determined by the sodium fusion method. The iodide content was determined with potassium iodate, with the cyanide stabilisation of the end-point and potentiometrically with silver nitrate, with

calomel and silver electrodes connected by an agar salt bridge. The results are shown in Table III indicating that the iodine not available for titration with thiosulphate can be accounted for by the hydrogen iodide content, as reported by Brost and Krupin (1957).

TABLE III

ANALYSIS OF AN IODINE/CETOMACROGOL/WATER SYSTEM

		Sum of available iodine and iodide g./litre
Iodine added g./litre	23·690	
Available iodine g./litre	20·320	
Total iodine g./litre (sodium fusion)	23·700	
Hydrogen iodide g./litre		
(*a*) Sodium hydroxide	3·365	23·685
(*b*) Potassium iodate	4·210	24·530
(*c*) Silver nitrate	3·320	23·640

The Interaction of Iodine and Cetomacrogol

Methods used industrially to produce iodophors have, as their first stage, a process of heating iodine with the carrier, e.g., U.S. Patent (1961). The effect of heating varying amounts of iodine and cetomacrogol together was investigated by placing known proportions of each into

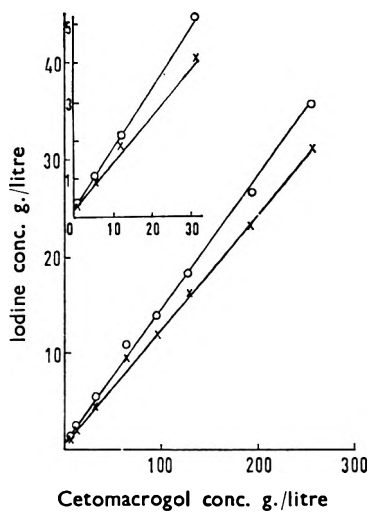

FIG. 5. The total and available iodine content of iodine/cetomacrogol/water systems at 20° C. Insert represents values at lower cetomacrogol concentrations. ○ Total iodine g./litre. × Available iodine g./litre.

stoppered containers and agitating for known times in a water-bath at either 50 or 60°. Samples containing no iodine crystals were removed and placed in a tared beaker, reweighed, and the available iodine content determined as before. The hydrogen iodide content was determined,

after titration of the iodine, with sodium hydroxide and methyl red as indicator. The results for the 40/60 per cent iodine cetomacrogol system at 60° showed an available iodine content varying from 0·03 to 0·02 g./g. mixture while the hydrogen iodide content varied between 0 and 0·009 g./g. For a 20/80 per cent mixture at 50 and 60°, the available iodine varied from 0·017–0·012 g./g. and the hydrogen iodide from 0–0·005 g./g. For a 10/90 per cent mixture the figures were 0·009–0·007 and 0·003–0·002 respectively.

The effect of heat on aqueous solutions of iodine in cetomacrogol was investigated by agitation of excess iodine in a 20 per cent solution cetomacrogol, batch C, for known times at 60°. The iodine was allowed to settle and the solution to cool at 20° for 48 hr. The available iodine and hydrogen iodide contents were determined as before. The available iodine concentration rose to 0·035 g./g. mixture in 10 hr. and then

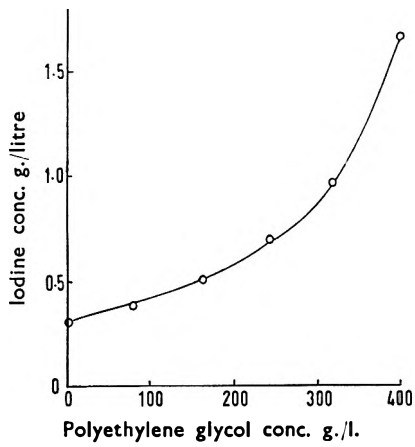

FIG. 6. The solubility of iodine in aqueous solutions of polyethylene glycol 1540 at 20° C.

increased very slowly to 0·04 g./g. after 60 hr. The hydrogen iodide content varied from 0·002–0·003 g./g. over the same period. The figures are below the iodine content determined at 60° (0·055 g./g.) but are above that determined at 20° (0·022 g./g.).

The Solubility of Iodine in Polyethylene Glycol and Polyoxyethylene Ethers

The solubility of iodine in aqueous solutions of polyethylene glycol 1540 at 20° is shown in Fig. 6. Solutions containing more than 300 g./litre polyethylene glycol gave a precipitate on dilution with water above 1 in 10, but cleared on dilutions above 1 in 100.

The solubility of iodine in 20 per cent aqueous solutions of cetyl and lauryl ethers of polyoxyethylene are shown in Fig. 7a and the molecular ratio of available iodine to ether, assuming the manufacturer's value for the number of ethylene oxide units are given in Table IV. The effect of preheating these systems for 72 hr. at 60° is shown in Fig. 7b and the molecular ratios given in Table IV.

Discussion

The Table of B.P.C. specifications, the gel point and the CMC values indicate that the three batches of cetomacrogol have different characteristics. It is interesting to note the surface tension/concentration curves

TABLE IV

RATIO OF MOLECULES OF IODINE TO MOLECULES OF POLYOXYETHYLENE GLYCOL ETHERS

Ether and number of ethylene oxide units	Ratio of molecules of available iodine to molecules of polyoxyethylene glycol ether	
	When dissolved at 20°	When preheated for 72 hr. at 60° and cooled to 20°
Cetomacrogol, batch C	0·627	1·055
Cetyl alcohol ether		
16	0·608	0·733
18	0·580	0·765
24	0·676	0·863
30	0·736	0·923
45	0·863	1·191
60	0·882	1·371
Lauryl alcohol ether		
9	0·263	0·383
16	0·355	0·769
23	0·401	0·854

gave no noticeable minima which characterise impure surface-active agents. Each batch of cetomacrogol also varied in its ability to dissolve iodine (Fig. 1), but the source of this variation cannot be stated from the information available.

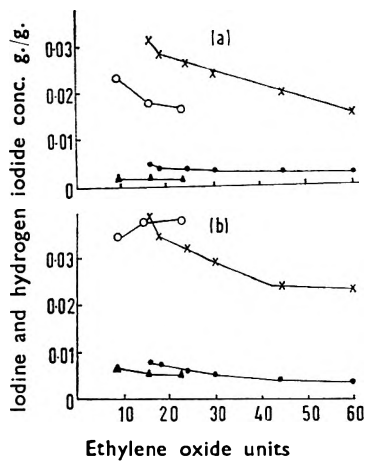

FIG. 7. The Solubility of iodine in 20 per cent aqueous solutions of polyoxyethylene cetyl and lauryl ethers (*a*) at 20° C. and (*b*) at 20° C., after preheating at 60° C. for 72 hr. X Available iodine content of cetyl ethers g./g. ○ Available iodine content of lauryl ethers g./g. • Hydrogen iodide content of cetyl ethers g./g. ▲ Hydrogen iodide content of lauryl ethers g./g.

Terry and Shelanski (1952a) and Allawala and Riegelman (1953a, b) considered the solubility of iodine in non-ionic surface-active agents to be due to the presence of micelles in solution, although Lazarus (1954) was

not fully convinced that this was so. The phenomenon of micellar solubilisation as described fully by McBain and Hutchinson (1954), was shown by McBain and McBain (1936) to be reproducible and independent of the system's previous history. The iodine/cetomacrogol complex described here is not reproducible in that, when heated in the presence of excess iodine, and then cooled to a lower temperature, the iodine content does not return to that of a saturated solution at the lower temperature. We have, therefore, evidence that micellar solubilisation cannot provide the complete answer. Considering the solubility curve in the region of the CMC, Fig. 3a shows that, where the water-soluble suspension was not removed, the solubility of iodine increased more rapidly with cetomacrogol concentration, below the CMC. When the suspension was removed (Fig. 4) there was an increase in the solubility of iodine, below the CMC of batches A and C, followed by a decrease in solubility in the region of the CMC. A similar decrease has been noted for benzene (McBain and Lissant, 1951; Mulley and Metcalf, 1962), chloroxylenol (Mulley and Metcalf, 1962) and possibly octanol (Mulley and Metcalf, 1962) in low concentrations of non-ionic surface-active agents. Above the CMC there was a linear relationship between the amount of available iodine and the cetomacrogol concentration (Fig. 1), the available iodine in solution at a given cetomacrogol concentration increasing with temperature (Fig. 2).

Further evidence against micellar solubilisation is the occurrence of a chemical interaction between iodine and the surface-active agent. That this reaction results in the production of an equivalent amount of hydrogen iodide is indicative of an oxidation/reduction reaction, a substitution reaction would yield half the amount of hydrogen iodide whilst in an additional reaction no hydrogen iodide would be produced. There does not appear to be any reports of such a reaction between iodine and polyoxyethylene ethers in the chemical literature, but Johnson and others (1951) report that halogens form loose addition compounds with ethers. With thioethers Hickinbottom (1957) reports that such an addition occurs readily, forming compounds of the R_2SBr_2 type, but these are unstable tending to lose the halogen in water to yield sulphoxides, e.g.,

$$R_2SBr_2 + H_2O \rightarrow R_2SO + 2HBr.$$

A similar type of reaction possibly occurs between iodine and cetomacrogol, the oxygen atoms of the ether groups of cetomacrogol providing a mechanism of solubilisation and a source of the hydrogen iodide. Osol and Pines (1952) suggested that a bond of the Lewis acid-base type was responsible for the solubility of iodine in glycols and glycerol. Thus the iodine functions as an electron pair acceptor (presumably by the formation of I^-I^-), whilst the oxygen atoms function as electron donors.

The molecular ratios of iodine to ethers given in Table V, show however that neither an ethylene oxide unit, nor a molecule of ether, holds one molecule of iodine, and therefore the association would appear to be more complex than postulated above. However, the molecular ratios of iodine to surface-active agent do show a noticeable increase as the

number of ethylene oxide units increases, providing evidence of an association between iodine and the ethylene oxide units. Further evidence of the formation of a complex between iodine and the surface-active agent is obtained from a consideration of the absorption spectra of such systems. The occurrence of complexes between iodine and solvent are characterised by the appearance of a new absorption maximum at a value lower than that obtained for non-complex forming solvents (540 mμ), e.g., 353 mμ for iodine in acetone (Benesi and Hilderbrand, 1950). Allawala and Riegelman (1953b) show the maximum for iodine/surface-active agents in solution as 370 mμ, while Ross and Olivier (1959) report a value of 360 mμ in aqueous and non-aqueous solutions. Elworthy (1960) found a value of 390 mμ and this same value was obtained for the ethers used in this work. A recent investigation of an iodine/cationic surface-active agent complex (Cohen, Economou and Libackyj, 1962), shows that it had an absorption maximum of 360 mμ in chloroform solution. With iodine/polyvinylpyrrolidone complexes, Oster and Immergut (1954) suggested, from observation of absorption spectra, that iodine induces polyvinylpyrrolidone to assume a helical form, the centre of which is occupied by iodine. This explanation provides a mechanism similar to that for starch and cyclodextran complexes, the form of which is described by Brown (1962), and could be also involved in the solubilisation of iodine by non-ionic surface-active agents.

The concept that the mechanism which caused the binding of a solubilisate to a non-ionic surface-active agent was also responsible for solubilisation has been postulated by Patel and Kostenbauder (1958) for *p*-hydroxybenzoates/polyoxyethylene glycol ethers. Blaug and Ashen (1961) noted that esters of *p*-hydroxybenzoic acids had a low solubility in polyethylene glycol but were soluble in polyethylene ethers, thus being analagous with the instance of iodine. They also found small amounts of binding between *p*-hydroxybenzoates and polyethylene glycol but greater binding with the ethers. They explained this difference on the suggestion of Higuchi and Lack (1954), that the presence of hydrophilic and hydrophobic moieties in the interacting molecules rendered the replacement of water molecules hydrogen bonded to the basic ether oxygen groups more favourable.

The foregoing work and explanations do not provide a complete elucidation of the means whereby cetomacrogol and similar compounds can bring iodine into homogeneous aqueous systems. Nevertheless, cetomacrogol can be employed to provide an aqueous iodine system and the properties of such a system will be described.

Acknowledgements. The authors thank Professor G. E. Trease for facilities in the Department of Pharmacy, and to Boots Pure Drug Company for supplying the analytical data in Table I.

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THE APPLICATION OF THE COULTER COUNTER TO PROBLEMS IN THE SIZE ANALYSIS OF INSOLUBLE DRUGS

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A method of programming an electronic computer has been developed which fits the results of size analyses obtained by means of a Coulter counter to a logarithmic normal curve from which a specific surface area figure is derived. Applications of the method to suspensions of phenothiazine are discussed.

The Coulter counter described by Kubitschek (1960) offers a new technique of counting solid particles in the sub-sieve range, which in common with automatic "flying spot" microscopes, such as those introduced by Dell (1954), Hawksley (1954) and Roberts and Young (1951), has two important advantages over classical methods of microscopy; that of greatly reducing the time taken to complete a size distribution, and the removal of operator bias. Recent developments in optical microscopy, such as the double-image micrometers of Timbrell described by Barnett and Timbrell (1962) and Cooke, Troughton and Simms described by Dyson (1960) have greatly reduced operator bias by increasing the precision with which the statistical diameters are measured. These methods still leave the operator with the job of locating and sizing the particles under a bench microscope, and the time involved is considerable.

The Coulter counter is well suited for sizing particles between 1 μ and 76 μ in diameter. This size range adequately covers most of the finely-divided drugs such as phenothiazine (*see* Kingsbury, 1958), and griseofulvin (*see* Atkinson, Bedford, Child and Tomich, 1962), where it has been proved that particle size has a biological effect.

Information fed to the "Mercury" Computer

The Coulter counter sizes relatively non-conducting particles according to changes in the resistivity of an electrolyte solution in which they are suspended. The electrolyte is made to flow through a small diameter (30 μ and upwards) cylindrical aperture in the wall of a glass vessel and the passage of a particle creates an impulse proportional to its size which is amplified in an electronic circuit.

To use the services of a "Mercury" electronic computer to obtain the data required, certain changes in the method of calculating the results were made. To make these intelligible, the functioning of the counter is briefly discussed.

When performing a size determination on a sample, the instrument, by means of two controls, is set to count all particles with diameters greater than a certain threshold value. The first, the threshold control, is a potentiometer with a continuous scale graduated from 0 to 100 whose readings is denoted by t', and the second, the current selector switch,

has ten settings numbered from 1 to 10, denoted by I. The aperture resistance, Ω, has to be determined for the supporting electrolyte used and this is done directly with a good DC voltmeter. From the I value and the aperture resistance, a "scaling factor", F, can be derived from tables supplied with the instrument. This, together with a calibration factor, k, translates the dial settings into particle diameters, d, according to the expression

$$d = k\sqrt[3]{t} \qquad \text{where } t = t'F$$

It should be noted that t has the dimensions of volume, which, for particles of equal density, is proportional to weight, and hence the t values are also used to transform the numbers of particles counted into weight fractions.

It would have been cumbersome to store the full table of F values in the computer, so a linear approximation was derived. Using this for a particular current setting I and aperture resistance Ω, the F value is given by

$$F = a_I + b_I \frac{(\Omega - 30{,}000)}{5{,}000}$$

The constants for each of the ten settings of I are given in Table I.

TABLE I

CONSTANTS FOR EACH OF 1 TO 10 SETTINGS OF I

Setting I	a_I	b_I
1	1·0	0
2	0·5014	0·00022
3	0·2521	0·00026
4	0·12744	0·00030
5	0·06515	0·000336
6	0·03401	0·000348
7	0·018481	0·0003649
8	0·010818	0·0003960
9	0·007178	0·0004551
10	0·005743	0·0005722

For Ω in the range 15 to 40 K ohms the values given by this approximation have a maximum error (when compared with the tabulated values) of about 0·5 per cent which occurs for setting 10: for I = 1 up to 9, the maximum error is 0·1 per cent. At present, this seems adequate but only a slight amount of work would be required to derive a quadratic approximation which fitted the tabulated values even more closely.

When the t values have been derived, a value has to be assigned to the calibration factor k, so that the particle diameters may be calculated. This factor can best be obtained by direct calibration with mono-disperse spheres of a diameter calculated by other means, and the value of k supplied by the manufacturers has been used for the experiments to be described. There is a further variable control on the instrument, the gain selector rheostat, which has six positions and gives pulse height increase in a $\sqrt{2}$ progression which affects the calibration factor (k) proportionately. This control, therefore, offers a means of varying the size range

covered by the machine without changing the diameter of the aperture or the conductivity of the supporting electrolyte.

In practice, when using the counter for routine control of a single product, a regular pattern of values of t′ and I, which is known by experience to give a reasonable change of particle diameter, is used, and once the particle diameters have been determined for each setting of the control knobs, these are used for each size analysis without the need for further calculation. However, in the computer programme, the calculation is done automatically on every occasion and any independent variation of t′ and I can be used with equal facility. If, as a result of making such variations, the particle diameters in descending order of magnitude are not known, this is of no importance, since the computer automatically sorts out the values into the correct order before proceeding with the calculation.

It is usual to calculate 12 to 16 values of d, each representing lower limits of size above which total particle counts are obtained experimentally. The counting is done automatically and the total number of particles larger than each particular value of d is recorded on the instrument. There are, however, corrections for coincidence, that is the counting of two or more particles as a single particle, and for background count, which have to be applied to the mean count $\bar{n}'$. The equation for calculating the coincidence factor, P, is given as

$$P = 2{\cdot}5\,(D/100)^3\,(500/V)$$

where D is the aperture diameter in microns and V is the volume of suspension in which the particles are counted, in microlitres. P is therefore an instrumental constant and its relationship to n″, the correction to be added to the number of particles to allow for coincidence, is

$$n'' = P(\bar{n}'/1{,}000)^2$$

The background counts, b, are determined experimentally at each dial setting, using the supporting electrolyte only, and the corrected expression for the total number of particles n, above any particular diameter, d, is

$$n = \bar{n}' + P(\bar{n}'/1{,}000)^2 - b$$

All the information required by the computer before it can perform the necessary calculation has now been briefly discussed. The data it requires are: The coincidence factor, P; the calibration factor, k; the aperture resistance, Ω, and, for each setting of the controls: the threshold control setting, t′; the current selector switch setting, I; the mean count, $\bar{n}'$; the background count, b.

Information Printed Out by the "Mercury" Computer

The actual number of particles, Δn, in each size range bounded by successive values of d, is obtained by subtracting adjacent n values. The mean volume factor t is the arithmetic mean of adjacent t values and hence $\Delta\bar{n}t$ gives the contribution to the total weight of each size fraction.

To calculate the weight percentage of particles in each size range, a figure representing the total weight of particles in the sample must be derived. This is a difficulty common to all counting methods of size analysis, because the contribution to the total weight of particles smaller in diameter than the smallest particle which has been counted, is unknown. It is usually possible by experience and inspection of the weights of sample in each size fraction to form a "reasonable" estimate of the total weight and in many cases, it may be assumed that the weight of particles below the smallest size counted is negligible. Such assumptions, however, need not be made if the distribution can be shown to obey a mathematical

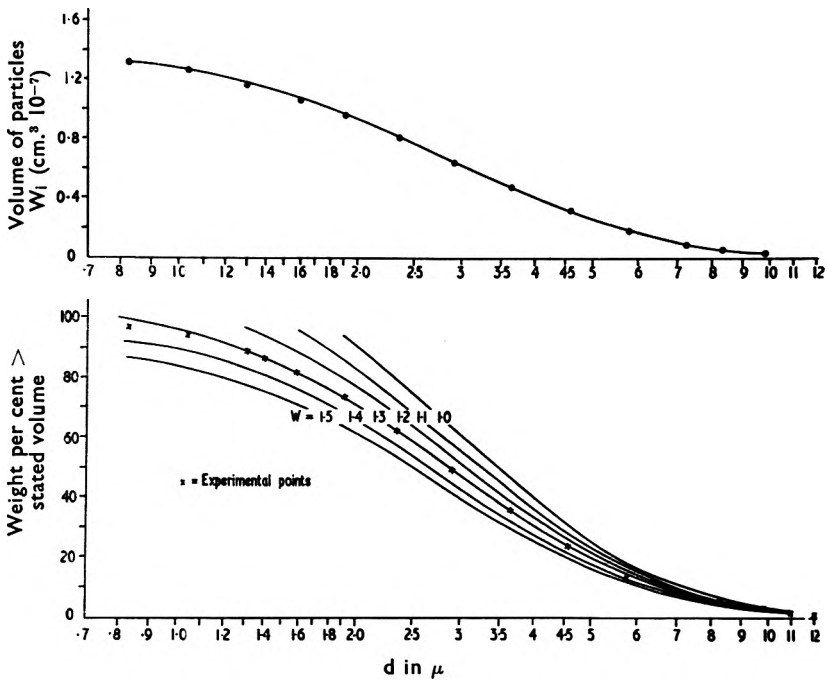

FIG. 1. (*a*) Upper figure, data obtained by summation of particle volumes given in the last column of Table III plotted against particle diameters in fourth column of Table II. (*b*) Experimental weight percentages from Table II, columns 4 and 5. Theoretical distributions using different values of W in the relationship $P = (w_1/W)$.

relationship. Several years' experience of determining particle size distribution by various methods has demonstrated that, for milled insoluble drugs, the distributions follow closely a logarithmic normal law (Thornton, 1959). This fact has now been used in programming the computer to obtain a complete size distribution. The equation for a logarithmic normal distribution may be written as:

$$\mathbf{P} = \int_1^{\infty} \left\{ \frac{1}{\sqrt{2\pi}\,\ln\alpha}\,\frac{1}{u}\exp\left[\frac{-(\ln u - \ln d_w)^2}{2(\ln\alpha)^2}\right]\right\} du$$

where **P** is the percentage by weight of spherical particles with diameters

greater than d, d_w is the weight mean diameter, i.e. the diameter of a hypothetical sphere such that 50 per cent by weight of the particles have larger diameters and 50 per cent have smaller diameters, and α is the standard deviation.

Fig. 1a shows results on the sample of phenothiazine suspension discussed later, and Fig. 1b shows the w_i values of Fig. 1a scaled down by different factors (1/W). The basis of the method is to choose a particular value of W which best fits a logarithmic normal curve so that $\mathbf{P} = w_i/W$ and this is obtained by the method of least squares according to the following argument. The curve is fixed when $\ln d_w$ and $\ln \alpha$ are known, and it is convenient to work in terms of two constants derived from these, viz.

$$x = -\ln d_w/\ln\alpha$$

and

$$c = 1/\ln\alpha$$

The value of **P** for a particular value of d is denoted by $\mathbf{P}(x + c \log d)$; it may be seen that

$$\mathbf{P}(x + c \log d) = \int_d^\infty \frac{c}{\sqrt{2\pi}} \cdot \frac{1}{u} \exp -[\tfrac{1}{2}(x + c \log u)^2]du$$

Suppose that there are n "reference diameters" $d_1, d_2, \ldots d_n$ with associated results, $w_1, w_2, \ldots . w_n$.

The sum of squares of deviations is then

$$\left\{\frac{w_1}{W} - \mathbf{P}(x + c \log d_1)\right\}^2 + \left\{\frac{w_2}{W} - \mathbf{P}(x + c \log d_2)\right\}^2 + \\ + \ldots \left\{\frac{w_n}{W} - \mathbf{P}(x + c \log d_n)\right\}^2$$

which is denoted by

$$S = \sum_{i=1}^{n} \left\{\frac{w_i}{W} - \mathbf{P}(x + c \log d_i)\right\}^2$$

The aim is to choose W, x and c so that S is minimised, i.e. so that the curve fits the experimental points in the "best" manner.

It is not possible to write down explicit expressions for the values of W, x and c which do this, so an iterative method must be used. Initial approximations for W, x and c are obtained and corrections to these are calculated by the Newton-Raphson method: suppose these are ΔW, Δx, and Δc. The usual procedure would be to take $W + \Delta W$, $x + \Delta x$, and $c + \Delta c$ as better approximations, calculate corrections to these, and repeat until the approximations were sufficiently close to the true answer. However, in this case, this procedure has been modified to speed up the calculation.

The modification is to calculate the values of S for the following set of values

$$(W - \Delta W, x - \Delta x, c - \Delta c)$$
$$(W \quad , x \quad , c \quad)$$
$$(W + \Delta W, x + \Delta x, c + \Delta c)$$

and continuing with

$$(W + 2\Delta W, x + 2\Delta x, c + 2\Delta c)$$

. . .

. . .

. . .

up to $(W + 20\Delta W, x + 20\Delta x, c + 20\Delta c)$ if necessary, in order to find three values which straddle the minimum. Having found three values, a quadratic curve is fitted to the three corresponding values of S and the point $(W + \lambda\Delta W, x - \lambda\Delta x, c + \lambda\Delta c)$ determined which minimises this quadratic function. This is then used as the next approximation to the solution.

The computer stops this process when the solution is "sufficiently" accurate, this being tested in the following way.

At each iteration $\frac{\Delta W}{W}$, $\frac{\Delta x}{x}$ and $\frac{\Delta c}{c}$ give the fractional corrections to the three "unknowns". The computer takes these three quantities with positive signs, adds them and stops the iteration procedure when the sum is less than 0·00001. This insures that the last correction made to each of the unknowns is less than 0·001 per cent and this will also represent the accuracy of the unknowns (or the maximum inaccuracy).

The relationship between the two parameters of a logarithmic normal weight distribution which serve to define it (namely the weight mean particle size, d_w, and the standard deviation, α) and the surface mean diameter, d_s, which is required to calculate the specific surface area, is obtained from the Hatch-Choate relationship (Herdan, 1960)

$$\frac{2 \ln d_s}{3 \ln d_w} = \frac{2 \ln M + 2 \ln^2 \alpha}{3 \ln M + 4{\cdot}5 \ln^2 \alpha}$$

M is the geometric mean diameter of a frequency distribution and α is, by definition, the ratio of the diameter above which 84·13 per cent of the distribution occurs, to the corresponding 50 per cent diameter. These percentages may be either by weight or by number.

From the above expression,

$$\log d_s = \log d_w - 1{\cdot}151 \log^2 \alpha$$

The specific surface area of spherical particles having the calculated distribution is then

$$S.A \times p = 6 \times 10^4 / d_s$$

where $S.A$ is the surface area in cm.^{2}/g., p is the absolute density of the drug in question, and d_s is the surface mean diameter in microns.

This calculation is easily fed to the computer, which finally prints out the following data for each setting of the controls: The threshold control setting, t′. The current selector switch setting, I. The true count, n. The particle diameter, d. The cumulative weight greater than each value of d, w_1/W.

The corrected cumulative weight derived from the log normal curve, and, for the entire distribution:

$$\text{The value of } S.A \times \text{p in cm.}^2/\text{cm.}^3$$

Experimental

The experiments made to test the performance of the Coulter counter and the usefulness of this computer programme were on samples of commercial suspension containing 50 per cent w/v of phenothiazine.

The particles were between 1 and 10 μ in diameter, hence an instrument tube with a 50 μ aperture, and a nominal sample volume of 0·05 ml., were used. The t′ and I values and the gain index, were selected to give sixteen calculated values of particle diameter between 0·82 and 12·3 μ and the dilution was such that about 35,000 particles greater than 0·82 μ were counted. The information printed out by the computer on a typical suspension is shown in Table II.

TABLE II

Size analysis of phenothiazine suspension

(Data obtained by means of the Coulter counter printed out by the "Mercury" computer)

Instrument controls		Total particles above corresponding value of d	Particle diameter in μ	Cumulative weight per cent greater than d	
t′	I	n	d	Experimental	From log normal curve
100	1	−0·5	12·30	0·00	0·96
70	1	2·0	10·92	1·53	1·57
50	1	3·5	9·76	2·18	2·43
30	1	13·0	8·23	4·91	4·46
20	1	21·0	7·19	6·35	6·89
20	2	82·5	5·72	13·00	13·21
20	3	263·2	4·55	22·80	22·65
20	4	727·7	3·62	35·49	34·91
20	5	1659·7	2·90	48·43	48·75
20	6	3601·7	2·34	62·35	62·29
20	7	6602·9	1·91	73·77	73·73
20	8	10606·2	1·60	82·34	81·99
20	9	14130·3	1·40	87·04	86·97
20	10	15171·3	1·31	88·05	89·11
10	10	25262·2	1·04	94·68	94·54
5	10	35091·3	0·83	97·91	97·56

(Specific Surface Area) × (Density) = 25,690 cm.²/cm³.

In obtaining a particle size distribution by any counting technique one fundamental assumption must be made. Generally speaking an estimate is made of the number or weight of particles too small to have been included, or these are neglected altogether and the number or weight counted is assumed to represent adequately the entire sample. The assumption made here is that the distribution is logarithmic normal.

It is, however, theoretically possible to account for the weight of phenothiazine used by the Coulter counter for each count, by direct weighing of the original sample and use of accurate methods of dilution of the suspensions. For these experiments, an electrolyte containing 0·9 per cent NaCl and 0·01 per cent Perminal BXN (an anionic wetting

agent) was used for making the dilutions which finally contained approximately $2{\cdot}5 \times 10^{-7}$ g. of phenothiazine in 0·05 ml. of suspension. The method of obtaining a total weight figure using the relevant data from Table II is shown in Table III, where the last column gives the total volume of solids in each size range assuming the particles are spherical. The total weight of phenothiazine is then calculated to be $1{\cdot}8 \times 10^{-7}$ g.

TABLE III

ESTIMATED WEIGHT OF PHENOTHIAZINE IN 0·05 ml. OF DILUTED SUSPENSION CONTAINING A CALCULATED QUANTITY OF $2{\cdot}5 \times 10^{-7}$ g., USING THE SIZE ANALYSIS DATA OF TABLE II

Number of particles between successive values of d N	Mean diameter in each size range (μ) $\bar{d}$	Volume of a sphere of diameter $\bar{d}$ ($cm.^3 \times 10^{12}$) V	Total volume in each size range ($cm.^3 \times 10^{12}$) N.V
2·5	11·6	817·5	2,044
1·5	10·3	572·2	858
9·5	9·0	382	3,630
8·0	7·71	240	1,920
61·5	6·46	140	8,610
180·7	5·13	72	13,010
464·5	4·08	37	17,190
932·0	3·26	18·1	16,870
1,942·0	2·62	8·5	16,500
3,001·2	2·12	4·9	14,700
4,004·3	1·75	3·0	12,012
3,524·1	1·50	2·0	7,048
1,041·0	1·36	1·45	1,509
10,090·9	1·17	1·0	10,090
9,829·1	0·93	0·5	4,915
—	<0·82	—	Say 4,094
			$\Sigma = 135{,}000 \times 10^{-12}$ cm.^{3}

$\therefore$ Weight of phenothiazine (density 1·36) = $1{\cdot}84 \times 10^{-7}$ g.

The fact that the weights of phenothiazine do not agree more closely can be ascribed to a number of factors. Perhaps the more important of these relate to the instrumental constants. The calibration factor, k, was that given by the makers since our initial experiments showed that a comparison of size analyses by Andreasen pipette, which was our standard method but was only applicable to the top size ranges, and the Coulter counter, were in excellent agreement. Also the volume of suspension used for each count is nominally 0·05 ml. but since this does not affect the performance or calibration of the instrument an absolute value has never been obtained. However, departure from this figure would materially affect calculations, e.g. if the volume were 0·04 ml. the weight of phenothiazine used in each count would be $2{\cdot}0 \times 10^{-7}$ g. Other points are that the particles are not spherical and hence there will be an effect caused by unknown shape factors, and that no solid can be absolutely insoluble in the suspending liquid. On this last point, however, no difference has been detected in size analyses between dilutions of phenothiazine in the electrolyte alone and in a similar solution previously saturated with phenothiazine. This is not the case with many so-called insoluble drugs, however, and the effect of solubility of the smallest particles must be carefully watched.

Whatever the reasons may be, the direct method of estimating the total weight of solid in the sample being counted would introduce serious

errors if it were used as the basis of the calculated size distribution. The effects of three different total weights, estimated by experience, and not by the method of direct weighing, used in compiling Table III are shown in the logarithmic probability graph (Fig. 2). This method of graphical representation of a size distribution as a straight line exaggerates departures from linearity at the "tails" of a size distribution but the effect is nevertheless real and will be more marked when the particles are smaller so that a greater proportion are beyond the limit of resolution of the instrument. If it is accepted that the distribution should be logarithmic normal, there is no need to estimate a figure for the total weight and the way this is done in programming the computer has been outlined. The calculated line derived from the computer figures is also shown in Fig. 2.

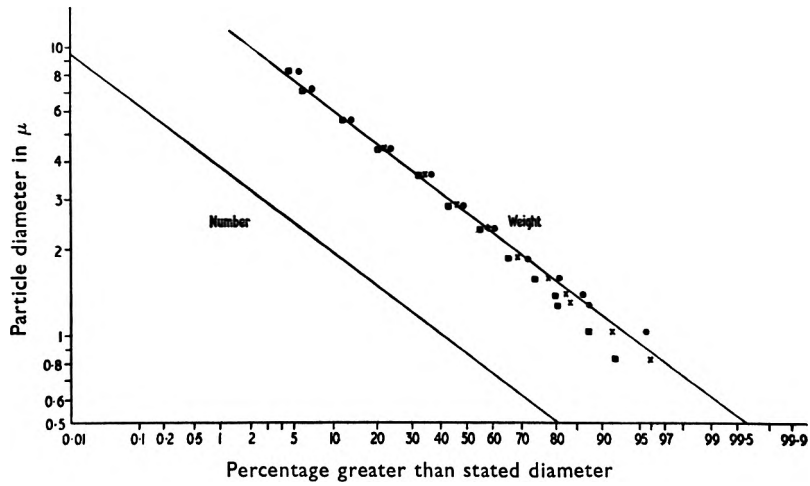

FIG. 2. The experimental points are plotted, assuming three different totals for the volume of *all* particles, including those smaller than the limit of resolution of the Coulter Counter. The weight line is that corresponding to the distribution printed out by the Mercury computer. The number line is derived theoretically from the weight line (see text). ■ $\epsilon = 1{\cdot}40 \times 10^{-7}$ cm.^{3} × $\epsilon = 1{\cdot}35 \times 10^{-7}$ cm.^{3} ● $\epsilon = 1{\cdot}30 \times 10^{-7}$ cm.^{3}

The absolute lower limit of size with the apparatus described is about 0·8 μ. From Table III it can be seen that the number of particles counted in each size range, N, is still very large at this diameter, suggesting that the sample contains many particles too small to be counted. As a theoretical check of this, the logarithmic normal weight curve can be transformed into a frequency, or number, curve which will then give the number average diameter or that diameter below which the number of particles should begin to decrease. The relationship between weight and number of particles is

$$\log d(w) = \log d(n) + 6{\cdot}907 \log^2\alpha$$

where d(w) and d(n) are diameters of particles such that the percentage greater than d, by weight and number, has the same numerical value.

Thus, from Fig. 2, 50 per cent by weight of the particles are greater than 2·85 μ (d $= 2{\cdot}85\mu$) and $\alpha = 1{\cdot}875$. The corresponding value of d(n) from the above equation is 0·87 μ which means that 50 per cent by number are greater than 0·87 μ. This is also the geometric mean diameter, M. From Fig. 2, also, it can be seen that 97 per cent by weight of the particles have diameters greater than 0·87 μ. Since the minimum diameter counted by the instrument is 0·82 μ (see Table II), only about half the theoretical total number of particles in the suspension have been counted, but because these are the larger particles, they represent 97 per cent of the total weight. The data, is, therefore, not sufficiently complete to enable a full distribution by number to be determined experimentally but, by conversion into a weight distribution, which is common practice, assuming the weight of a particle is proportional to the cube of its diameter, the data covers 97 per cent of the distribution.

The calculated surface area figure is 25,690 cm.^{2}/cm.^{3} or 18,900cm.^{2}/g., taking the density of phenothiazine to be 1·36 g./cm.^{3}.

Discussion

The experimental results, which are typical of many that have been obtained, show that the Coulter counter is a useful apparatus for the determination of particle size distribution within a range of diameters between 1 and 15μ. This range can be extended upwards by substituting a larger aperture but extension below 0·8 μ is not a feasible proposition with the present instrument. By using an electronic computer to calculate the results, fitting them, if applicable, to a logarithmic normal curve by the method described, and calculating the surface area of the particles, a considerable saving in time is effected and operator error is largely eliminated.

It is not universally accepted that particles in a powder or suspension produced by a grinding technique should show a logarithmic normal distribution of size. Our experience is, however, that when there are no gross artificial limitations placed on the spread of particle sizes, such as a sieve in the mill discharge or the collection of "fines" in a separate container, the results fit a logarithmic normal curve within the limits of experimental error. Programmed in the way described, the Mercury computer cannot print out the theoretical distribution if the experimental results are not sufficiently close to a logarithmic normal distribution, and if this occurs, there is strong evidence that the assumption of logarithmic normality cannot be made. This has never happened with the many routine samples of phenothiazine suspension sized by the Coulter counter, but when it does, the information obtained is still of value since the cumulative weight percentages are printed out and this alone is a time-saving operation.

The usefulness of the logarithmic normal distribution, apart from the elimination of the effect of particles too small to be counted, is that it allows an exact value of the specific surface area of the solid particles to be calculated, on the assumption that they are spherical and that their surface

is smooth, i.e. there are no cracks or fissures. Air permeability methods of measuring the surface area of powders make similar assumptions regarding the powder surface so that a direct comparison of surface areas of solids in suspension and in the dry state is possible (Table IV).

TABLE IV

COMPARISON OF SPECIFIC SURFACE AREA OF PHENOTHIAZINE
(Data obtained from Coulter counter and Rigden air permeability apparatus)

Surface area in cm.²/g.	
Coulter counter	Rigden apparatus
11,500	12,400
16,000	17,100
19,200	24,000
23,400	24,300
23,500	27,200
29,200	31,100
32,600	38,800
33,500	43,500

This is of value because the direct measurement of surface area of a dry powder is a simple means of characterising it, although it is not so specific as a complete particle size distribution because different distributions can have the same surface area. Such a method cannot be applied to a suspension of particles in a liquid since the powder must be in the dry state, and the filtering and drying of a suspension often leads to the formation of aggregated particles whose size bears little resemblance to that of the original particles, particularly if surface-active agents are present in the suspension. Difficulties also exist in the reverse process of sizing a dry powder by counting the particles after they have been suspended in an aqueous medium, particularly when the powder is hydrophobic, since true dispersion is hard to achieve without subjecting the suspension to a vigorous shearing process which has a grinding effect on the particles.

If then, it is desired to compare the particle size of a drug when formulated into an aqueous suspension with that of the same drug after it has been finely milled in a dry-grinding machine, the use of the Coulter counter to produce a specific surface area figure for the particles in the suspension and a direct air permeability method to give a similar figure on the dry powder has much to commend it. The need for such measurements occurs when the biological effect of a suspension formulation is to be compared with a tablet formulation of the same drug.

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THE EFFECT OF SOME ANTI-ACETYLCHOLINE DRUGS ON THE RESPONSES OF THE ISOLATED RABBIT INTESTINE TO SYMPATHETIC NERVE STIMULATION AND TO THE ACTION OF ADRENERGIC NEURONE BLOCKING AGENTS

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The effects of atropine and some quaternary derivatives on the inhibitory responses of the Finkleman preparation to stimulation of the peri-arterial sympathetic nerves and to added adrenaline were examined. None of the compounds tested appeared to affect the inhibitory responses with the exception of *N*-diphenylmethylatropine, which much enhanced the inhibitory effect of the electrical stimulation. In addition, this compound antagonised *in vitro*, but not *in vivo*, the activity of several adrenergic neurone blocking compounds, but not guanethidine.

ACCORDING to some recent work (Burn and Rand, 1959; Burn, 1961, 1962), a cholinergic mechanism is believed to play a role in the liberation of the sympathetic mediator at postganglionic sympathetic nerve endings. We thought it of interest to use the isolated rabbit intestine as described by Finkleman (1930), to find out whether the responses of this preparation would be influenced by agents known to interfere with the mechanisms of cholinergic mediation.

In the experiments to be described we investigated the influence of anti-acetylcholine drugs on the inhibition of intestinal motility, either by electrical stimulation of the sympathetic postganglionic fibres or by adrenaline. In addition, the possible alterations induced by these drugs on the adrenergic neurone blocking effects of bretylium, guanethidine, xylocholine, hemicholinium and dimethylphenylpiperazinium (DMPP) were sought. According to Wilson (1962) and Bentley (1962), DMPP seems to possess adrenergic neurone blocking properties, besides the known nicotinic ones.

EXPERIMENTAL

Methods

Most of our experiments were made on the isolated intestine segments of the rabbit, prepared with the extrinsic sympathetic supply intact as described by Finkleman (1930). Rabbits of either sex, weighing 2–3 kg., provided isolated intestinal loops of 10–12 cm. which were suspended in 100 ml. baths (Zamboni, 1940), filled with Tyrode solution at a constant temperature of 32° and connected to an isotonic lever (ratio 1 : 4; load 2 g.). The neurovascular hilum was placed on platinum electrodes immersed in the perfusion fluid and connected to an electronic stimulator. Rectangular pulses of 1 msec. duration were applied at a frequency of 20–25/sec. for 30 sec.

A few experiments were also made in the heparinised rat, by recording the arterial pressure through a mercury manometer connected to the cannulated carotid artery and estimating the antagonistic effect exerted by

bretylium against the hypertensive responses seen after intravenous administration of eserine (150–200 μg./kg.). Rats were anaesthetised with urethane given intraperitoneally (1·5 g./kg.), since the typical pressor response to eserine is partially suppressed by barbiturate anaesthesia (Della Bella and Gandini, 1962).

The following drugs were used: atropine sulphate, atropine *N*-methylbromide, scopolamine *N*-butylbromide, *N*-diphenylmethylatropine bromide, adrenaline chloride, bretylium tosylate, guanethidine sulphate, dimethylphenylpiperazinium iodide, xylocholine bromide, hemicholinium bromide, eserine sulphate, cocaine hydrochloride. The concentrations used are expressed as salts.

RESULTS

Influence of Atropine and its Derivatives on the Responses of the Finkleman Preparation to Adrenaline and Electrical Stimulation

Atropine concentrations from 0·1 to 10–20 μg./ml. were used. We observed somewhat unexpectedly in about ten preparations that whereas the first application of atropine at, say, 0·1–0·25 μg./ml. induced the typical

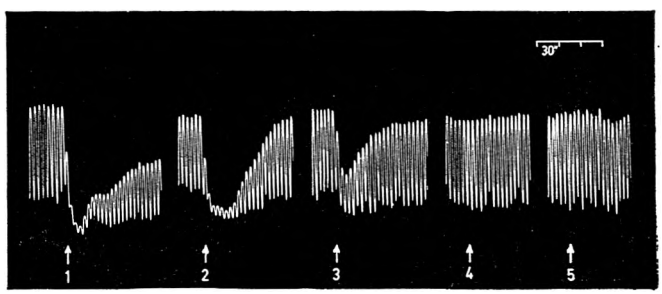

FIG. 1. Isolated rabbit intestine. At 1, 2, 3 and 4, atropine, 0·2, 0·5, 2 and 10 μg./ml. respectively, for 5–10 min. After each dose a prolonged washout. Interval between each dose, 10 min. The desensitisation to atropine motor inhibitory effects is progressive and complete and is followed by unresponsiveness to the diphenyl derivative (10 μg./ml.) (at 5).

marked reduction in spontaneous activity, a second application, after washing and recovery, at a concentration of 3–5 times gave a much smaller inhibition. In the course of 4 or 5 administrations made at intervals, such as to allow the reversion of the effects of each, concentrations of 20–30 μg./ml. may be attained without any apparent effect on the motor activity of the preparations (Fig. 1).

Once unresponsiveness to atropine had developed, the isolated rabbit intestine reacted differently to acetylcholine compared to nicotine. Whereas at the beginning of three experiments the intestine reacted to acetylcholine 0·005 μg./ml. or to nicotine 0·0025 μg./ml. with a strong contraction and a marked increase in tone, after repeated atropine treatment at increasing concentrations and in the presence of atropine at 20 μg./ml., the intestine reacted to nicotine only at 0·5 μg./ml. or more, and did not react to acetylcholine even at 5–10 μg./ml.

A similar rapid loss of responsiveness to the inhibition of spontaneous contractions induced by the quaternary nitrogen derivatives of atropine and scopolamine was observed. The phenomenon appears to be a cross-desensitisation: in fact the intestine no longer sensitive to atropine is

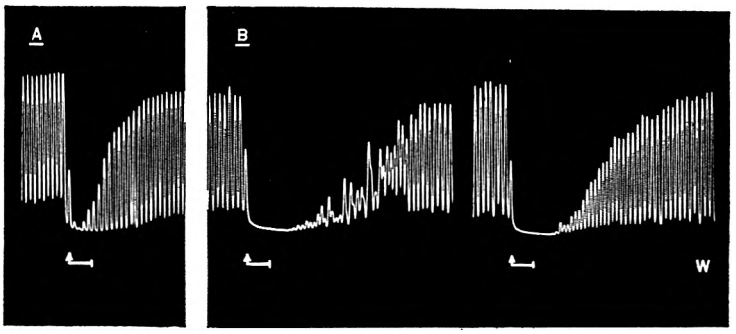

FIG. 2. Isolated rabbit intestine prepared according to Finkleman. In A: response to 30 sec. electrical stimulation of sympathetic postganglionic fibres (at arrow), before *N*-diphenylmethylatropine. In B: after desensitisation to *N*-diphenylmethylatropine: responses to sympathetic stimulation (at arrows) in the presence of *N*-diphenylmethylatropine (15 μg./ml.). Interval between each stimulation period, 15 min. At W: prolonged washing.

unresponsive to quaternary compounds (see Fig. 1); similarly the responses to atropine disappear after the desensitisation to quaternary derivatives.

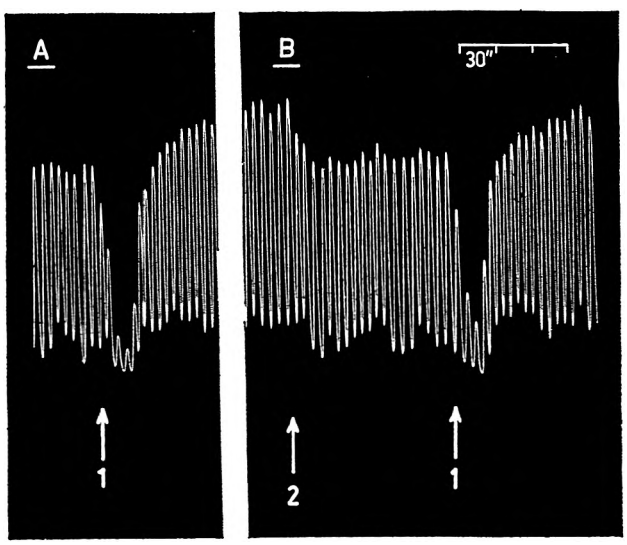

FIG. 3. Isolated rabbit intestine. In A: inhibitory response following adrenaline (0·01 μg./ml.) (at 1). The drug is removed after 30 sec. contact, by thorough washing. In B: after progressive desensitisation, the presence of *N*-diphenylmethylatropine (15 μg./ml.) (at 2) does not practically modify the inhibitory response to adrenaline (0·01 μg./ml.) (at 1).

The influence of the same anti-acetylcholine agents on the motility inhibition due to sympathetic electrical stimulation or adrenaline administration, was also investigated. The compounds under test, at varying concentrations, did not modify the intestine responses to chemical or electrical stimulation. *N*-Diphenylmethylatropine was the single exception and it enhanced significantly the responses to electrical stimulation, in both intensity and duration (Fig. 2), while being completely ineffective on the adrenaline-induced responses (Fig. 3). This enhancement, which is illustrated in Fig. 2, subsides, after the drug is removed by washing, in the course of 3–5 responses. Once the responses have returned to normal, a further treatment with *N*-diphenylmethylatropine appears to be ineffective.

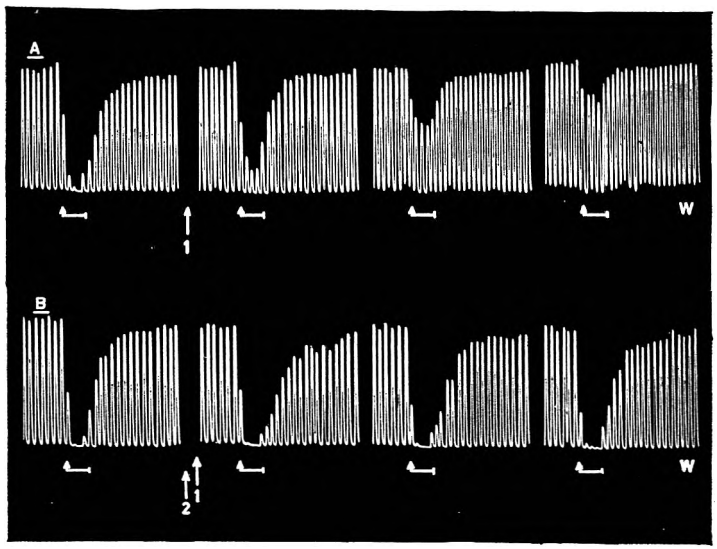

FIG. 4. Isolated rabbit intestine prepared according to Finkleman. In A and in B: responses of two preparations from the same animal to 30 sec. electrical stimulation of sympathetic postganglionic fibres (at arrows). Interval between each stimulation period, 15 min. In A: bretylium 10 μg./ml. (at 1) is left in contact with the drug until washing (at W). In B: same treatment as in A. At 2: progressive desensitisation to *N*-diphenylmethylatropine is developed and the preparation is left in contact with this drug at 15 μg./ml. Under such conditions, the effect of bretylium (10 μg./ml.) (at 1) fails to develop.

Interaction of Atropine and Adrenergic Neurone Blocking Agents in the Finkleman Preparation

The finding that the *N*-diphenyl derivative affected the responses to the sympathetic electrical stimulation made it the more interesting to investigate whether it or the other compounds under study altered the suppressive effect exerted by the adrenergic neurone blocking agents on the inhibition of spontaneous contractions induced by sympathetic stimulation. To elucidate this point two groups of experiments were made.

In the first, the Finkleman preparation was exposed to increasing concentrations of atropine or quaternary compounds, care being taken that the motor activity was no longer affected; then the responses to the appropriate stimulation were standardised and, in the presence of the anti-acetylcholine drugs, the treatment with the adrenergic neurone blocking agent was applied at such a concentration as to reduce the response by 80 per cent or more within 30–45 min. The agents used and their concentrations, μg./ml., were as follows: bretylium, 10–15; guanethidine, 5–7·5; xylocholine, 20; DMPP, 2·5–5; hemicholinium, 20–40.

The results obtained in about thirty preparations showed that among the derivatives under test, only *N*-diphenylmethylatropine antagonised the adrenergic neurone blocking effect; with atropine, in some experiments, an earlier onset of such an effect was observed, whereas *N*-methylatropine and the *N*-butylscopolamine derivative were completely ineffective. Fig. 4 illustrates the antagonistic activity of *N*-diphenylmethylatropine towards bretylium. In particular, it may be seen that the *N*-diphenyl derivative in the presence of bretylium, unlike the response previously demonstrated

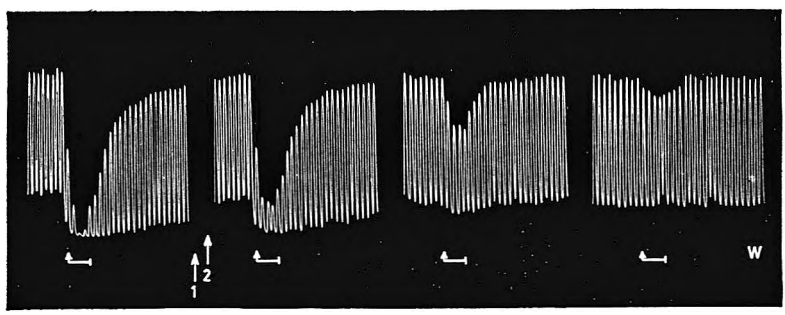

FIG. 5. Isolated rabbit intestine prepared according to Finkleman. Responses to 30 sec. electrical stimulation of sympathetic postganglionic fibres (at arrows). After desensitisation to *N*-diphenylmethylatropine, the preparation is treated with the same drug at 15 μg./ml. (at 1) and subsequently with 5 μg./ml. guanethidine (at 2). At W: prolonged washing. The guanethidine effect is not affected by the presence of *N*-diphenylmethylatropine.

in Fig. 2, does not enhance the postganglionic electrical stimulation effects. Furthermore we observed that the antagonism by *N*-diphenylmethylatropine of a nerve blockage does not persist on washing. In addition, guanethidine was the only adrenergic neurone blocking agent that was not antagonised (Fig. 5).

With the second group of experiments we wanted to investigate whether the adrenergic neurone-blocking effect would be more easily reversed on addition of the anti-acetylcholine drugs. Again, it was observed that *N*-diphenylmethylatropine was the only compound able to enhance the reversion of the sympathetic block, with the exception of the sympathetic paralysis caused by guanethidine.

Influence of N-*Diphenylmethylatropine on the Suppression by Adrenergic Neurone Blocking Agents of the Eserine Hypertensive Responses in the Rat*

Some experiments were performed in the intact rat to determine if *N*-diphenylmethylatropine, as demonstrated for cocaine (Della Bella and Gandini, 1962; Lešić and Varagić, 1961) (Fig. 6), could modify the inhibition by adrenergic neurone blocking agents of the eserine hypertensive responses (Varagić and Vojvodić, 1962). Atropine was not tested, since it is known to possess a direct antagonistic activity (Dirnhuber and Cullumbine, 1955), while, in our hands, *N*-diphenylmethylatropine, in doses up to 4–6 mg./kg. intravenously, does not significantly modify the hypertensive responses.

On the other hand, as previously described, the responses to eserine are much reduced after bretylium or guanethidine administration: this effect occurs in a few minutes and generally increases in the subsequent responses.

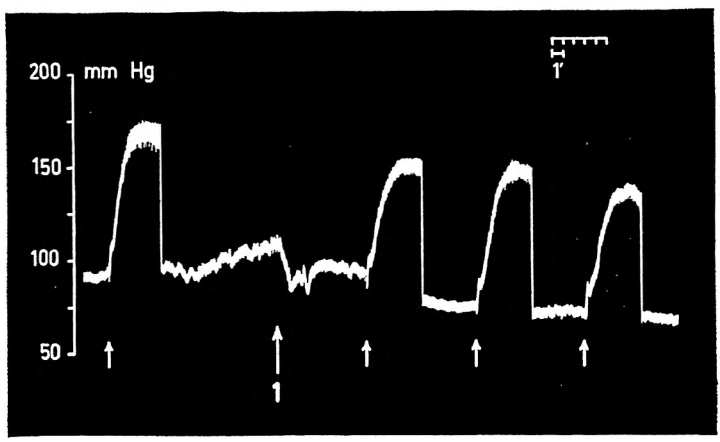

FIG. 6. Rat, 270 g. Recording of the carotid blood pressure. Pretreatment with 5 mg./kg. of cocaine given subcutaneously. At the arrows: eserine (150 μg./kg.) is injected into the jugular vein. At 1: bretylium (4 mg./kg.) i.v. Cocaine pretreatment counteracts the reduction of hypertensive responses by bretylium.

In contrast to the *in vitro* observations, no antagonism towards the adrenergic neurone blocking effect of bretylium was exhibited by *N*-diphenylmethylatropine (Fig. 7).

DISCUSSION

Our results clearly indicate that the effect of adrenergic neurone blocking agents can be effectively antagonised by a compound known to possess anti-acetylcholine properties.

Among the compounds tested, *N*-diphenylmethylatropine, a drug possessing atropine-like as well as ganglion-blocking activity on the peripheral, vagal and sympathetic synapses but devoid of direct adrenaline potentiating activity, when applied to the Finkleman preparation, appeared to be able to potentiate the effects of the electrical stimulation of the sympathetic postganglionic fibres, and also to prevent the sympathetic paralysis caused by adrenergic neurone blocking agents.

The lack of antagonism towards the suppression by bretylium of the eserine hypertensive responses *in vivo* might be ascribed either to the different animal species, or to a different body distribution of the two drugs, preventing *N*-diphenylmethylatropine from reaching effective concentrations at the sympathetic postganglionic neurone, where bretylium, on the contrary, is known to be selectively concentrated (Boura, Copp, Duncombe, Green and McCoubrey, 1960).

It is interesting to note that *N*-diphenylmethylatropine *in vitro* was able to antagonise only those compounds possessing a quaternary nitrogen, namely xylocholine, bretylium, hemicholinium, DMPP, whereas no antagonism towards guanethidine was observed. It has been already suggested that guanethidine may differ from the other agents in mechanism and site of action (Cass and Spriggs, 1961), and our experiments support this.

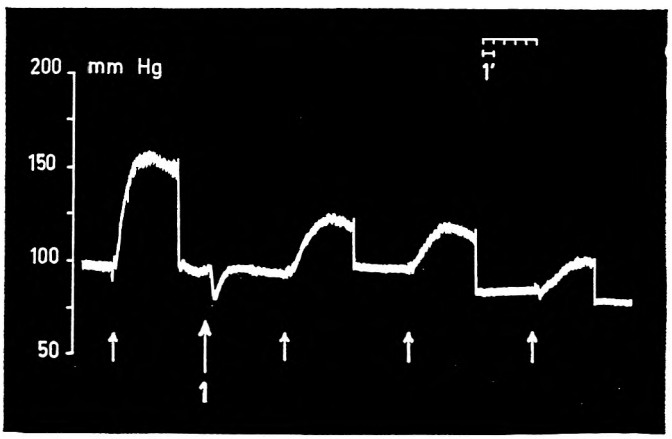

FIG. 7. Rat, 310 g. Recording of the carotid blood pressure. Pretreatment with 6 mg./kg. of *N*-diphenylmethylatropine, i.v. in three divided doses. At arrows: eserine (180 μg./kg.) is injected into the jugular vein. At 1: bretylium (4 mg./kg.) i.v. A considerable reduction of the hypertensive responses occurs, in spite of the *N*-diphenylmethylatropine pretreatment.

Perhaps the most interesting point emerging from our experiments is that it is possible to antagonise the adrenergic neurone blocking effect with a compound whose structure and properties are clearly different from those possessed by the antagonists considered so far and represented, as it is known, by sympathomimetic amines, or by drugs interfering with their metabolic fate such as the monoamine oxidase inhibitors (Day, 1962; Bain, 1960; Boura and Green, 1959; Wilson and Long, 1960).

On the basis of current structure-activity considerations and of available data, *N*-diphenylmethylatropine is a drug prevailingly anti-acetylcholine in its action, and this may represent an indirect argument in favour of the hypothesis of a cholinergic mechanism being involved in the liberation of the sympathetic mediator at the sympathetic postganglionic neurone endings.

By analogy to that suggested by Day (1962) for sympathomimetic amines, a possible explanation of the antagonism towards the adrenergic nerve blocking agents shown by *N*-diphenylmethylatropine might be found in the work of Stephenson (1956) on the mechanism at play in drug antagonism: i.e. it might be that both the agents and *N*-diphenylmethylatropine have an affinity for the sympathetic postganglionic nerve endings and that only their efficacy is different.

Finally we would like to call the attention to the rapid decrease in sensitivity of the rabbit ileum to the direct motor inhibitory effects of atropine. The fact that such desensitisation appeared also towards the quaternary nitrogen derivatives of atropine and scopolamine, despite their different pharmacological properties, thus behaving like a cross-desensitisation, raises the problem of a common denominator in the phenomenon, the meaning of which is worth investigating.

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THE HYDROLYSIS OF PROPYL BENZOATE IN AQUEOUS SOLUTIONS OF CETOMACROGOL

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The alkaline hydrolysis of n-propyl benzoate in aqueous cetomacrogol solutions has been investigated. The rate of hydrolysis of solubilised ester in the presence of 100 per cent excess of sodium hydroxide is a first order process in which the rate decreases with increase in cetomacrogol concentration. This indicates that incorporation of ester within the micelles protects it against hydrolysis. The rate of reaction depends on the degree of saturation of the dispersion and not on the actual concentrations of ester or cetomacrogol except in so far as these control the degree of saturation. The degree of saturation is expressed as a "Saturation Ratio" which is the ratio of the ester concentration to its solubility.

The alkaline hydrolysis of certain esters in aqueous solutions of cetrimide, a cationic surface-active agent, has been studied previously (Mitchell, 1962). It was found that the initial hydrolysis rate of emulsions increases with cetrimide concentration while that of solutions decreases. In the later stages of the reaction, the rate of hydrolysis of both emulsions and solutions decreases with increasing cetrimide concentration. This paper reports studies on the alkaline hydrolysis of n-propyl benzoate in solutions of cetomacrogol, a non-ionic surface-active agent.

Increasing concentrations of both cetrimide and cetomacrogol reduce the hydrolysis rate of solutions indicating that solubilisation within the micellar "phase" of the dispersion protects the ester against hydrolytic attack.

The rate of hydrolysis in cetomacrogol solutions is shown to depend on the degree of saturation of the dispersion where this is expressed as a Saturation Ratio R, in which

$$R = \frac{c}{c_s} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where c is the concentration of ester, and c_s its solubility in the solution of surface-active agent.

Experimental

Materials

Cetomacrogol 1000 B.P.C. m.p. 40°, $n^{60°}$ $1{\cdot}499_0$, hydroxyl number (B.P.C. 1959 method) 51·6. Assuming a molecular weight of 1,300, concentrated stock solutions were prepared, stored in the dark and diluted as required. n-Propyl benzoate fractionally distilled under reduced pressure, b.p. 231°, $n^{22°}$ $1{\cdot}499_1$.

Determination of Solubility

The solubility of propyl benzoate at 35° in varying concentrations of cetomacrogol solution was determined using the method previously described (Mitchell, 1962).

Determination of Hydrolysis

The rate of hydrolysis was measured at 35° in the presence of sodium hydroxide sufficient to provide 100 per cent in excess of that needed for complete hydrolysis. The technique was similar to that described earlier except that samples withdrawn at definite intervals were added to excess hydrochloric acid and the remaining acid back-titrated with sodium hydroxide using bromothymol blue as indicator. The reaction between sodium hydroxide and propyl benzoate was stoichiometric.

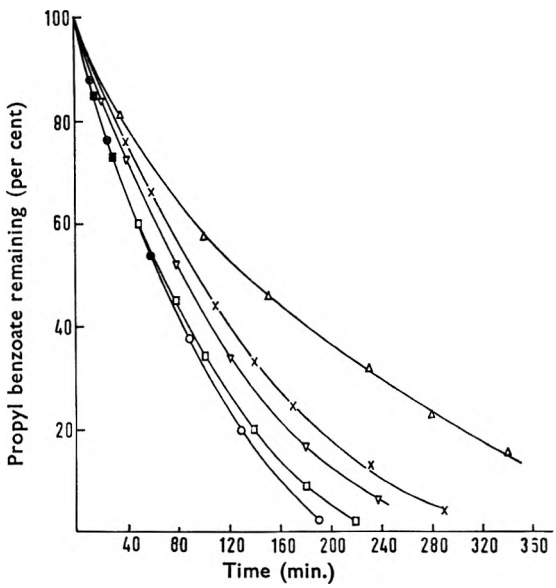

FIG. 1. Influence of cetomacrogol concentration on the alkaline hydrolysis of n-propyl benzoate (0·05 moles/litre) at 35°. Cetomacrogol concentration (moles/litre). ○ 0·01. □ 0·02. ▽ 0·06. × 0·08. △ 0·128. ● emulsion; ○ solution.

RESULTS

The solubility curve of propyl benzoate in cetomacrogol solutions at 35° is linear; the solubilities of propyl benzoate being respectively 0·195, 0·0514, 0·102, 0·136, 0·169 and 0·237 moles/litre for cetomacrogol solutions of 0·01, 0·03, 0·06, 0·08, 0·10 and 0·14 moles/litre. The influence of cetomacrogol concentration on the hydrolysis of propyl benzoate is shown in Fig. 1. The initial rate of reaction of suspensions and emulsions of ester was the same while that of solutions decreased with increase in cetomacrogol concentration. In the later stages the rate decreased

progressively with increase in cetomacrogol concentration. Figs. 2 and 3 show the dependence of the reaction on the Saturation Ratio. From Figs. 2 and 3 it is evident that the hydrolysis of solutions of propyl benzoate in cetomacrogol in the presence of 100 per cent excess of sodium hydroxide is a first order reaction, whereas the hydrolysis of emulsions cannot be represented by a first order rate plot. The initial rates of reaction, first order rate constants k, and half lives $t_{\frac{1}{2}}$, for various Saturation Ratios are given in Table I.

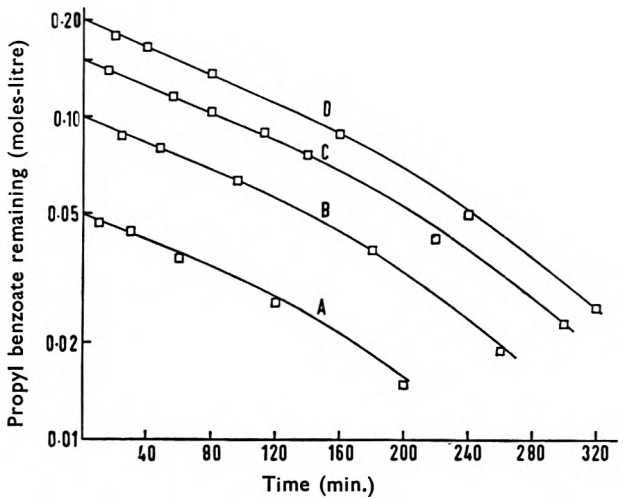

FIG. 2. Alkaline hydrolysis of n-propyl benzoate in cetomacrogol solutions at 35° plotted as a first order reaction. Saturation Ratio = 2·0. Cetamacrogol (moles/litre). A 0·0140; B 0·0285; C 0·0440; D 0·0585.

DISCUSSION

The rate of oxidation of some aliphatic aldehydes in aqueous solutions of cetomacrogol has been shown to depend on the degree of saturation of the dispersion where this is expressed as the Saturation Ratio, R (Carless and Mitchell, 1962). For a saturated solution $R = 1$, while for an emulsion $R > 1$ and for a solution $R < 1$. For any given value of R the rate of oxidation was found to be independent of both aldehyde and cetomacrogol concentrations except in so far as these control the Saturation Ratio. The results presented in Figs. 2 and 3 and Table I show that the hydrolysis of n-propyl benzoate in cetomacrogol also depends on the Saturation Ratio. At each value of R for solutions the first order rate constants k, and the half lives $t_{\frac{1}{2}}$, are in close agreement. Moreover the initial rate of hydrolysis divided by the ester concentration is a constant K, Table I.

$$\text{i.e. initial rate} = Kc \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

Where from

$$\text{Equation 1, } c = Rc_s \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

TABLE I

DEPENDENCE OF ALKALINE HYDROLYSIS OF PROPYL BENZOATE IN CETOMACROGOL SOLUTIONS ON THE SATURATION RATIO

Saturation Ratio R	Propyl benzoate moles/litre c_s	Propyl benzoate moles/litre c_s	Cetomacrogol moles/litre	Initial rate moles/litre/min × 10^4	K $\min^{-1} \times 10^3 / \left(\frac{\text{Initial rate}}{c}\right)$	k $\min^{-1} \times 10^3$ Ist order rate constant	$t_{\frac{1}{2}}$ min.	$t_{\frac{3}{4}}$ min.
0·25	0·01250	0·050	0·0285	0·78	6·3	5·6	126	280
	0·01875	0·075	0·0440	1·20	6·4	5·1	130	282
	0·0250	0·10	0·0585	1·56	6·3	5·1	131	275
	0·050	0·20	0·1280	3·16	6·3	5·4	130	273
0·5	0·0125	0·025	0·0140	1·02	8·2	8·4	83	160
	0·0250	0·050	0·0285	2·05	8·2	8·4	83	168
	0·0375	0·075	0·0440	2·85	7·6	8·5	82	158
	0·050	0·10	0·0585	3·91	7·8	8·8	78	159
	0·10	0·20	0·1280	8·30	8·3	8·7	81	164
1·0	0·025	0·025	0·0140	2·55	10·2	9·8	71	140
	0·050	0·050	0·0285	4·80	9·6	9·7	72	138
	0·075	0·075	0·0440	7·06	9·4	10·3	69	129
	0·10	0·10	0·0585	10·0	10·0	10·4	67	129
2·0	0·05	0·025	0·0140	4·51	9·0		65	114
	0·10	0·050	0·0285	8·02	8·0		69	118
	0·15	0·075	0·0440	12·1	8·1		71	121
	0·20	0·100	0·0585	18·0	9·0		69	120

$R = c/c_s$

where c = ester concentration

c_s = solubility of ester in cetomacrogol

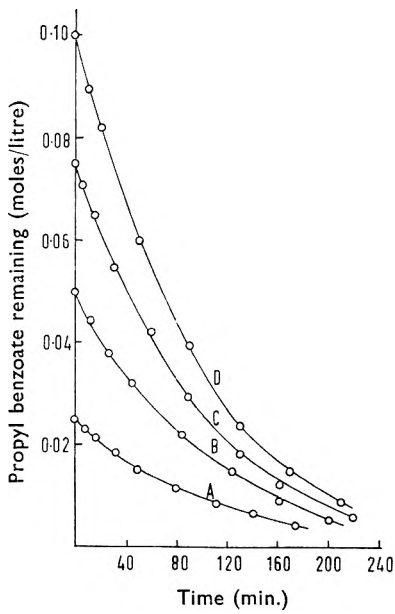

FIG. 3. Alkaline hydrolysis of n-propyl benzoate in cetomacrogol solutions at 35° plotted as a first order reaction. Saturation Ratio = 2·0. Cetomacrogol (moles/litre). A 0·0140; B 0·0285; C 0·0440; D 0·0585.

For emulsions e.g. R = 2·0, it is not possible to calculate a first order rate constant, but values for $t\frac{1}{2}$, $t\frac{3}{4}$ and K are in good agreement.

From a consideration of the effect of cetomacrogol on the hydrolysis of n-propyl benzoate it is possible to consider the part played by emulsion droplets and micelles in the reaction. By keeping the amount of ester constant and increasing the amount of cetomacrogol, dispersions are produced ranging from emulsions at low concentrations of cetomacrogol to solutions at higher concentrations. The transfer of ester from the emulsified to the solubilised state with increase in cetomacrogol concentration leads to an enormous enlargement of the interfacial area. The initial rate constant of emulsions is independent of cetomacrogol concentration however, and is the same as that for saturated solutions of ester in cetomacrogol. This indicates that the reaction is not controlled by the interfacial area of dispersed ester.

In the solubilised state the rate decreases progressively with increase in cetomacrogol concentration. Solubilisation of water-insoluble compounds in aqueous solutions of surface-active agents is associated with the presence of micelles. The dissolved material is distributed between the true aqueous "phase" and the colloidal micellar "phase" of the dispersion.

Hydrolysis will occur most rapidly in the aqueous "phase" where the ester molecules are accessible to hydroxyl ion attack. Increasing the concentration of cetomacrogol and thereby the number of micelles, increases the distribution of ester in favour of the micellar pseudo-phase and the rate of hydrolysis decreases.

Acknowledgment. I wish to thank Mrs. Lucy Wan Sai Cheong for the determination of the physical constants of cetomacrogol.

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PHARMACOPOEIAS AND FORMULARIES

THE BRITISH PHARMACOPOEIA 1963*

REVIEWED BY LLOYD C. MILLER, PH.D.
Director of Revision of the United States Pharmacopeia

THE "B.P. 1963" has appeared, on schedule, five years after publication of the revision it supersedes. Within its familiar red covers is satisfying and solid evidence of a truly large-scale voluntary effort and exceptionally good administrative direction. It is the ninth revision of the first British Pharmacopoeia, which appeared in 1864, and thus becomes the centenary edition. Greater significance attaches to holding now to a five-year schedule when attention is drawn to the fact that only five revisions had appeared by 1948.

The Secretary's office aids reviewers, among others called upon early to look closely at each new revision, with a discussion in the Introduction of "the principal changes made" in the new edition. In this way, those nearest the revision have a means of ranking the importance of the changes brought about. The topic given the greatest attention in the Introduction is the full achievement of the change-over from the Imperial system to the metric system of weights and volumes. The many complex problems met in dealing with this conversion are considered in detail. A reference is made to the careful advance preparations in the form of the announcement in B.P. 1958. They received other publicity as well. The older forms of penicillin are the subject of a further systematic change in potency declaration represented in the virtual abandonment of activity units in favour of metric units in stating dosage.

The introduction notes also an important inclusion, in many monographs of a brief statement under the heading "Action and Use". Experience elsewhere suggests that this will prove to be a most welcome aid to the users of the new compendium. However, "The statements are in no sense intended as a complete guide to the use of the substance (see General Notices, page 9). The information is not usually repeated in the monographs on preparations of the substance and in some instances it has been found more convenient to include the information under the statement on 'Dose'."

Of the many changes that strengthen the standards in the individual monographs, those that affect capsules as a group provide, for the first time, limits on disintegration and uniformity of weight of the contents. Permission to add colours to capsules is granted in a few cases, albeit reluctantly, inasmuch as the Commission goes on record as being of the opinion that "the practice of colouring preparations may have serious disadvantages and should be exercised with restraint".

A significant change in the approach to standards is represented by the case of Phenobarbitone Injection whereby standards are set up for the

* Pp. xxviii + 1210. Published for the General Medical Council by The Pharmaceutical Press, 17, Bloomsbury Square, London, W.C.1. 100s. (Inland postage 2s. 9d.).

material from which the Injection is prepared, i.e., the sterile contents of a sealed container, instead of merely for the solution prepared therefrom which ordinarily is in existence only long enough to be injected into a waiting patient. Another significant revision in respect to preserving sterile solutions is that throughout the general chapter on Injections the term "bactericides" has replaced "bacteriostatics". This substitution will have far reaching implications for the bacteriologist called upon to advise in the preparation of pharmaceuticals.

The growing need for the use of authentic specimens as reference standards is cited and notice taken of the support the Commission has given the programme administered by the World Health Organisation in providing Authentic Chemical Substances from its Centre in Stockholm.

A discussion of what has come to be known among those most concerned as "the thyroid problem" includes notice to the effect that the "study of this complex problem is continuing, and the introduction in this Edition of an assay based on the determination of the total iodine in thyroid combination with a limit test for inorganic iodide should be regarded as a measure subject to review when the results of further work... become available".

While continued recognition is accorded the practice of packaging parenteral solutions in multiple-dose containers, emphasis is placed on the risk associated with such containers arising from their "improper use".

A good measure of the value of a modern pharmacopoeia to its users is the attention given to the general methods by which compliance with the established standards may be determined. A convenient guide to this is the space devoted to the appendices and on this score the B.P. 1963 rates well, for not only is more space devoted to this important section but there is ample evidence that full use has been made of the recent advances in the technology of pharmaceutical analysis. An Appendix dealing with infrared absorption spectra is new, as is an entire section dealing with chromatographic analysis, nonaqueous titration, and the use of the oxygen flask combustion technique in the preparation of samples for analysis. Among the other innovations is the introduction of directions for sterilising powdered substances, a pharmaceutical procedure of increasing importance in view of the relatively large number of injectable drugs that are too unstable to be distributed in the form of sterile solutions.

The Tests for Sterility are modified in an important way for the antibiotics other than penicillin, the only antibiotic that can be inactivated by procedures that do not affect bacteria that may be present. Thus the other antibiotics are put into solution and passed through a sterile membrane filter disc as a means of "concentrating" any viable micro-organisms to improve the chances of detecting their presence. The disc is then divided into two portions, which are tested for anaerobic and aerobic organisms, respectively.

Giving emphasis to the section on methods, however, should not be allowed to detract from the significance of the additions to and deletions from the list of articles recognised, the monographs for which make up

some 70 per cent of the space of the book. Despite the deletion of a long list of titles for articles that are no longer regarded as deserving of pharmacopœial recognition, the monographs section now occupies 160 pages more than in the preceding edition, mainly because of the many new monographs provided. It is scarcely possible to pick out any individual article or group of articles as having greater significance than others. Pharmacologists will certainly note, however, the return to grace of Diamorphine Hydrochloride, best known as heroin; it was last official in B.P. 1948. This admission is especially noteworthy because of the statutory ban on the distribution of heroin in many parts of the world on account of its pronounced liability to cause addiction.

The British Pharmacopoeia 1963 is the third edition brought out under the direction of the present Secretary of the British Pharmacopoeia Commission since he assumed office in 1951. As an addition to a notable series, it reflects great credit on the Secretary personally and the entire Secretariat; it gives evidence as well of able and unstinting help from the Commission and the many committees appointed to assist it during the revision period.

THE BRITISH PHARMACEUTICAL CODEX, 1963*

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FEW books can exist for a dual purpose and effectively fill both needs; generally either one or both of the aims suffers in such an ambitious undertaking. The British Pharmaceutical Codex is a rare exception to this generalisation, inasmuch as it has a long history of commendable service both as a compilation of highly authoritative and useful therapeutic (actions and uses) information as well as a valuable compendium of recognised and accepted standards and specifications for many pharmaceuticals and dosage forms not included in the corresponding British Pharmacopoeia. The latest revision of the Codex continues in this fine tradition and the reader is pleased to note that further improvements have been made which serve to extend and increase the value and usefulness of the book.

The present volume is the eighth edition in a series dating from 1907, and is intended to become effective, in the United Kingdom on January 1, 1964. Its publication follows by only three and one-half years the appearance of the previous edition, the B.P.C. 1959. The explanation for this accelerated publication programme rests in a mutual desire on the part of the British Pharmacopoeia Commission and the Council of the Pharmaceutical Society of Great Britain to provide a parallel publication schedule for the two books, and so enable them to come into effect on the

* Pp. xxxvi + 1433. The Pharmaceutical Press, London. 105s. (Inland postage 2s. 9d.).

same dates. This arrangement has certain obviously desirable features, particularly for those agents for which standards are newly included in one book and are simultaneously deleted from the other.

The general format and arrangement of the new Codex is essentially that of the previous edition, which proved to be popular and convenient to use. In number of pages, the present volume exceeds its predecessor by over 140, and this may be attributed principally to the appearance of a substantial increase in new monographs.

The section devoted to monographs on drugs is listed as Part I and dramatically emphasises the two-fold purpose of the Codex. As in the past, the monographs generally include two main sections: one headed "Standard" and the second "Actions and uses". In addition, there is generally other information relative to synonyms, storage, dose and preparations. The "Actions and uses" information is authoritative and results from the careful consideration and evaluation of each article by a panel of acknowledged medical experts. The "Standard" is a set of pharmaceutical tests and assay specifications, and is supplied for each of the monographs which is not carried in the current British Pharmacopoeia; hence the desirability of concurrent publication of the two books. A significant innovation in the Codex monograph standards is the introduction of infra-red identification procedures in a number of the monographs. This has also necessitated the establishment of a programme whereby authentic specimens of those drugs are made available on order for use as comparison standards in the new infra-red procedures.

Close examination of the individual monographs reveals a number of other changes which are more of an editorial nature, but which should be noted for the benefit of veteran users of the Codex who are familiar with the previous style. Formerly, tolerance statements were provided in a separate paragraph titled "Content" at the beginning of the Standard section; in addition, the method for quantitative determination was previously given in another paragraph titled "Assay". In the present edition, however, the two paragraphs have been merged into a single one titled "Content", which appears at the end of the section. Another editorial change has been to convert the order of the elements in the molecular formulae to correspond with that of *Chemical Abstracts*. Now, after carbon and hydrogen, all the elements are given in alphabetical order; formerly, oxygen was listed immediately after hydrogen, and then the remaining elements were given alphabetically.

Of the 818 drug monographs, over 200 are new admissions to the Codex. In addition to providing this large number of new monographs, the Codex Committee has seen to the complete revision of the remaining monographs which appeared in the 1959 volume.

As in the past, Parts II to VI include monographs on immunological products, human blood preparations, surgical sutures, surgical dressings, and formulary preparations, respectively. It seems somewhat inappropriate to classify each of these categories as a separate "Part", with the same apparent emphasis as that given to Part I. Altogether Parts II to

VI total only about one-half the number of pages of Part I alone. In the opinion of this reviewer, the Codex Revision Committee might well consider consolidation in the next edition of Parts II to V into a single Part with suitable sub-sections. This would appear logical from the standpoint of the relatively brief nature of each of these Parts, and also because of the close natural relationship of the topics covered. Of these, Part V, dealing with surgical dressings, has been the most extensively revised and brought up to date.

Several new and very useful formulae have been added to Part VI. Because the policy has been continued of adding new formulae only if the recipe ingredients are freely available to pharmacists, the number of new formulae is consequently limited. A notable change in the formulary section has been the major conversion of weights and measures to the metric system. While a very few preparations had been so converted in the previous edition, this change has been largely carried to near completion in the present volume.

A number of new appendices have been added bringing the present total to 22. Furthermore, a couple of the 17 appendices in the previous edition have been discontinued. Among those added are a set of methods for the examination of aerosol propellents, a system for the classification of powders, a table of standard wire gauges, and additional analytical information to aid the examination of various pharmaceutical products.

While it is not listed as an appendix, and in fact is not even part of the bound book, note should be taken here of the pamphlet titled "Names of Substances". This 16-page booklet is enclosed with the 1963 Codex, and provides a handy cross-reference of non-proprietary and trade names for all the drugs appearing as monographs in the new Codex.

In summary, it is the reviewer's considered conclusion that the 1963 B.P.C. surpasses all of its predecessors both in the quantity of material covered and in the quality of treatment. Undoubtedly, the practitioner of medicine and the practitioner of pharmacy in the United Kingdom will continue to rely upon the Codex for the most concise and reliable information on today's leading pharmaceutical products. In doing so, both the physician and the pharmacist will be able to practice their individual professions just that much more securely.

LETTERS TO THE EDITOR

The Urinary Excretion of ^{14}C-Labelled Butylated Hydroxytoluene by the Rat

SIR,—The urinary excretion of tritiated butylated hydroxytoluene (BHT) in the rat was recently described by Golder, Ryan and Wright (1962). At dose levels of 100 μg. per rat the total amount of radioactivity recovered in the urine was 34·5 per cent after four days. It was, however, pointed out that this result might be in error because of the unknown amount of oxidation and hence loss of tritium from the molecule by metabolic reactions. Dacre (1961) has shown that the major metabolites of BHT in rabbits is 3,5-di-t-butyl-4-hydroxybenzoic acid, arising from oxidation of the methyl group. In the excretion studies by Golder, Ryan and Wright (1962), it was shown that nearly half the total tritium in the BHT was attached to the methyl group. It was, accordingly, highly desirable to confirm the previous excretion results and this has now been done using ^{14}C-labelled material.

TABLE I

URINARY EXCRETION OF BHT.^{14}C* BY THE RAT

Day	No. of experiments	Mean per cent of radio activity excreted
1	6	7
2	6	10
3	6	10
4	6	6
	Total excretion ..	33

* Dose (i.p.) 100 μg. ($5{\cdot}45 \times 10^5$ d.p.m.).

The results are set out in Table I. We have found using ^{14}C-labelled BHT that the recovery of radioactivity is very close to that found previously with tritiated material. This result is interesting since, coupled with the tritium work, it leads to the conclusion that at low dose levels, oxidation of the methyl group does not appear to be a major metabolic pathway in rats. This may be of some importance in the toxicity of this compound. We are at present attempting to identify the substances excreted at low dose level.

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Action of Guanethidine on the 5-Hydroxytryptamine Content of the Brain

SIR,—As guanethidine decreases the level of noradrenaline in the brain of the rat (Pfeifer, Vizi and Sàtory, 1962; Kroneberg and Schümann, 1962), it seemed interesting to us to investigate the effect of guanethidine on the 5-hydroxytryptamine (5-HT) content of the brain. In addition we compared α-methyldopa (an inhibitor of 5-hydroxytryptophan decarboxylase) and reserpine with guanethidine.

White rats of both sexes and of different ages were used. Brain 5-HT content was estimated by the procedure described by Bertaccini (1960). Guanethidine, DL-α-methyldopa and reserpine were administered once daily for 7 or 12 days. Animals were killed 16–24 hr. after the last drug administration.

TABLE I

EFFECT OF GUANETHIDINE, DL-α-METHYLDOPA AND RESERPINE ON 5-HT CONTENT, AS THE FREE BASE, OF THE BRAIN IN WHITE RATS OF BOTH SEXES AND OF DIFFERENT WEIGHTS FOLLOWING REPEATED SUBCUTANEOUS ADMINISTRATIONS

Compound	Sex	Weight of rats g.	No. of rats	Dose mg./kg. × days	Brain 5-HT content		P
					mean μg./g. tissue	per cent decrease	
NaCl 0·9 per cent							
Controls	male	200–300	24	—	0·39 ± 0·06	—	—
Guanethidine	male	200–300	16	10 × 7	0·24 ± 0·07	−38·5	<0·01
α-Methyldopa	male	200–300	8	200 × 7	0·19 ± 0·09	−51·3	<0·01
Reserpine	male	200–300	8	1 × 7	0·048 ± 0·034	−88	<0·01
NaCl 0·9 per cent							
Controls	female	280–320	6	—	0·57	—	—
Guanethidine	female	280–320	6	10 × 12	0·28	−50·9	—
NaCl 0·9 per cent ..							
Controls	male	140–160	20	—	0·250	—	—
Guanethidine	male	140–160	8	10 × 7	0·245	−0·2	—

It can be seen from Table I that rats weighing more than 200 g. have a significantly higher 5-HT level in the brain than rats weighing only 140–160 g.

Guanethidine, following repeated administrations at dosage which reduces blood pressure in the rat with renal hypertension (Bein, 1960), decreases cerebral 5-HT content in rats of both sexes weighing more than 200 g. Guanethidine seems to be ineffective in rats weighing 140–160 g. Except for a slight palpebral ptosis no overt behavioural changes were present in the animals treated by guanethidine. α-Methyldopa and reserpine seem to be more effective in reducing the brain 5-HT level than guanethidine.

We cannot explain why guanethidine decreases the brain 5-HT content of rats weighing 200–300 g. but not of those weighing 140–160 g. It has been shown (Green and Furano, 1962) that neoplastic mast cells contain two pools of amines: the endogenous amines are held in a pool separate from exogenous amines. Endogenous amines turn over at rates different from the exogenous amines (Day and Green, 1962). It may be, in our experiments there is a similar situation and possibly only a part of the amines present can be influenced by guanethidine.

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Urinary Phenols in Patients Treated with α-Methyldopa

SIR,—There is still some doubt about the mode of action of the hypotensive drug α-methyldopa.

It was originally introduced because Sourkes (1954) had shown that in high concentration it blocked the conversion of dopa to dopamine by inhibiting dopa decarboxylase and thus inhibiting catecholamine formation.

Other workers (Gillespie and others, 1962; Pletscher, 1963) have suggested that α-methyldopa's hypotensive action is due to its reserpine-like property of depleting the body stores of catecholamines.

Recently, Day and Rand (1963) suggested that it inhibited the conversion of dopa to dopamine by competing for dopa decarboxylase, being itself converted to α-methyldopamine. This, in turn, was converted to α-methylnoradrenaline which displaced noradrenaline from the storage sites at sympathetic nerve endings. Here it was held as a "false neurotransmitter" and was released in response to sympathetic nerve stimulation. Having a pressor activity much less than that of noradrenaline, the blood pressure fell.

It seemed likely that a study of the urinary excretion of the metadrenalines and the phenolic acids 4-hydroxy-3-methoxymandelic acid (HMMA) and homovanillic acid (HVA) after the administration of α-methyldopa might throw some light on this problem.

Hypertensive patients were fasted overnight. A control sample of urine was collected over a period of 2 hr. The patient was then given an oral dose of DL-α-methyldopa (usually 250 mg.) and no food was allowed for a further 4 hr.

Urine samples were collected over successive 2 hr. periods for 8 to 12 hr. The patient remained in bed throughout the test.

Phenolic acids were extracted and separated by two dimensional chromatography as described by Robinson and others (1959). The metanephrines were isolated by the method of Robinson and Smith (1962), separated chromatographically, and estimated spectrophotometrically after their oxidation to vanillin.

Two hours after the administration of the α-methyldopa the HVA excretion had fallen to about 50 per cent of that in the control period. Two hr. later, it could scarcely be detected on the chromatogram and 8 hr. after giving the α-methyldopa, HVA was undetectable on the chromatogram.

During the same period there was a slight rise in the excretion of HMMA and a significant rise in the excretion of the metadrenalines.

About 10 to 12 hr. after the dose of α-methyldopa, a compound appeared in the urine which was tentatively identified as α-methylnormetanephrine.

Our results are in accord with Day and Rand's hypothesis. The early reduction in HVA excretion reflects the dopa decarboxylase inhibition at this stage. The increased excretion of both metadrenaline and normetadrenaline

suggests that both adrenaline and noradrenaline may be displaced by their α-methyl homologues. The fact that α-methylnormetanephrine does not appear in the urine for 10 to 12 hr. is consistent with the α-methylnoradrenaline's being held at nerve endings until the noradrenaline is displaced.

A further point of interest was that there was a striking increase in the excretion of vanillic acid during these experiments. Eight hr. after the α-methyldopa was given, the vanillic acid excretion was about ten times greater than in the control period. The acid could not have arisen from exogenous sources since the patients were fasted for the early part of the test and the food taken later was known to be free from sources of vanillic acid.

This observation suggests that vanillic acid can arise from α-methylnoradrenaline by an analogous series of reactions to that in which it is known to arise from noradrenaline (Smith and Bennett, 1958; Rosen, and Goodall, 1962).

The results of these studies will be published *in extenso* at a later date.

This work was supported by a grant from the Research Sub-Committee of the Birmingham Regional Hospital Board, to whom we wish to express our thanks.

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September 12, 1963

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Effects of Reserpine in Rats Pretreated with α-Methyldopa

SIR,—Day and Rand (1963a) have described an arousing effect of α-methyldopa in rats sedated by reserpine. It was assumed that, when the brain stores of dopamine and noradrenaline had been emptied by reserpine, there was a repletion by the α-methylated catecholamines formed from α-methyldopa. Carlsson and Lindqvist (1962) have shown that α-methyldopa is decarboxylated *in vivo* and that α-methyldopamine is transformed into α-methylnoradrenaline in the brain. It was suggested by Day and Rand (1963b) that the α-methylated catecholamines may serve as "false transmitters", probably with a less potent activity than the natural amines.

In the present study we have further demonstrated a central action of the α-methylated catecholamines by pretreating rats with α-methyldopa before a depletion of the catecholamine stores is produced by reserpine.

The rats, weighing 300 g. were given α-methyldopa (Aldomet, Merck, Sharp and Dohme Ltd.) 150 mg./kg. intraperitoneally at 9 a.m. on the first and second days, followed by 300 mg./kg. at 4 p.m. on the second day. On the third day these pretreated rats, together with untreated control rats, were given reserpine, 2 mg./kg. i.p. Before the reserpine injection all animals appeared normal.

The controls were deeply sedated after reserpine, with decrease in muscular tone, eyelid ptosis and diarrhoea. Pretreatment with α-methyldopa before reserpine administration, however, resulted in the occurrence of exophthalmos (for 4–5 hr.), an increase in muscular tone with a state resembling catatonia (for 3–4 hr.) and an absence of diarrhoea (for 8–24 hr.). After 24 hr. all rats were mildly sedated and had diarrhoea. At this time the animals pretreated with α-methyldopa could no longer be distinguished from the controls given reserpine only.

The administration of a potent catechol-*O*-methyltransferase inhibitor α-propyldopacetamide (H22/54 Hässle Ltd.) 300 mg./kg. immediately before and 4 hr. after the administration of reserpine, did not influence the appearance or the duration of the symptoms. In a second series of experiments rats were pretreated with α-methyl-*m*-tyrosine in place of an equal dose of α-methyldopa. The results obtained from rats administered α-methyl-*m*-tyrosine plus reserpine, however, did not differ from those of the reserpine treated controls.

The syndrome noticed after reserpine administration in rats pretreated with α-methyldopa has been tentatively ascribed to a liberation of α-methylated catecholamines in the brain. Since α-methyldopamine is known to disappear from the brain within 16–20 hr. after the administration of α-methyldopa, the signs noted are probably caused by α-methylnoradrenaline. This substance is not attacked by monoamine oxidase, a fact which may account for the prolonged action observed in this experiment. When reserpine depletes the brain stores of naturally occurring catecholamines there are only rapidly transient signs of sympathetic activity. The absence of corresponding signs and symptoms after pretreatment with α-methyl-*m*-tyrosine may indicate that its corresponding decarboxylation product (metaraminol) is devoid of central action.

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Banana and Restraint Ulcers in Albino Rats

SIR,—Banana has been reported to markedly decrease histamine induced gastric acidity (Sanyal, Das, Sinha and Sinha, 1961), and to have prophylactic value in phenylbutazone-induced gastric ulcers in guinea-pigs (Sanyal, Gupta and Chowdhury, 1963). Encouraged by these results it was thought worthwhile to screen the efficacy of banana in another test preparation which simulates human peptic ulcer in its pathogenesis. Restraint ulcer technique (Brodie and Hanson, 1960) was selected, because the lesions produced by this method are consistently in the glandular portion of the stomach and are the result of a physiologically induced stress.

Male albino rats (140 to 170 g.) were divided into two groups. One group of 10 rats acted as control. In the second group of 15 rats the usual diet was

replaced by powdered, air-dried unripe banana pulp 48 hr. before the fasting stage of 18 hr. during which free access to water was allowed. Following the fasting period the rats were put individually into a tightly fitting metal corset after immobilisation of their limbs by adhesive tape. The tail was also immobilised by fixing it to the outer wall of the corset. The animals were killed after 24 hr. of restraint and the stomach removed and cut along the greater curvature to open the gastric mucosa which was washed carefully to remove superficial blood clots. Specimens were examined both macro- and micro-scopically. The presence of at least one area of erosion of the gastric mucosa was taken as the criterion for positive appearance of ulcer. Experiments on the control group were interspersed between those of the treated group. Evaluation of the pathology was always by the "blind" method. The results are in Table I.

TABLE I

THE EFFECT OF BANANA POWDER ON THE INCIDENCE OF RESTRAINT ULCERS IN ALBINO RATS

Group	No. of rats	Animals with ulcers		Animals with haemorrhage	
		No.	per cent	No.	per cent
Control ..	10	8	80	8	80
Banana fed ..	18	2 ($P < 0.01$)	13·3	7*	46·7 $P > 0.05$

* 5 rats showed only few punctiform haemorrhagic spots but no erosion.

Superficial mucosal ulcers in the glandular portion of the stomach were found in 80 per cent of the control rats. Ulcerated stomach was invariably accompanied with frank intragastric haemorrhage. Microscopically there was erosion of the mucosa, dilatation and congestion of the blood vessels and sometimes oedema of the mucosal and submucosal layers. In the banana-fed rats small ulcers were present in only 13·3 per cent of rats ($P < 0.01$). These rats had frank intragastric haemorrhage also. In all other rats either the stomach showed microscopically normal appearance or slight vascular dilatation.

The mechanism of restraint ulcer is not clear, but parasympathetic overactivity and nervous dysfunction related to corticovisceral activities during restraint have been suggested as the possible factors (Hanson and Brodie, 1960). The present work therefore, shows that banana powder, besides its prophylactic value against chemical (phenylbutazone) ulcers as reported earlier (Sanyal, Gupta and Chowdhury, 1963) can also afford significant protection against ulcers produced by a stress situation.

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The Use of β-Thiopropionic Acid for Stabilising the Fluorescence of Adrenolutine and Noradrenolutine

SIR,—The determination of small amounts of adrenaline and noradrenaline by the fluorometric trihydroxyindole method using two sets of filters for 405 and 436 mμ (e.g. Euler and Lishajko, 1959) is made difficult by the increasing fluorescence of the final solution with time—particularly at 436 mμ. Firstly, ascorbic acid breaks down in strong alkali with the formation of a pink coloured compound. The rate of breakdown is influenced by the strength of ferricyanide used for oxidation, the temperature and the presence of substances from biological fluids which have come through the alumina separation procedure. Fluorescence may also depend on the presence or absence of adrenolutine or noradrenolutine; blank solutions often increase in fluorescence more rapidly than those containing these lutines. The increase in blank fluorescence is particularly troublesome at low concentrations of the amines where the blank or control sample may often fluoresce more than the standard after a few min. Another factor which may contribute to the rising fluorescence is the presence of small quantities of plasma proteins (or related compounds) or other unknown substances which come through the alumina separation procedure. Precipitation of plasma proteins (Vendsalu, 1960) may reduce this effect but may also introduce variable losses because preliminary experiments with Sephadex G50 (Medium) have demonstrated the affinity of the plasma proteins for adrenaline and noradrenaline.

Euler and Lisajko (1961) have recommended the use of ethylenediamine for stabilising the fluorescence of the final alkali-ascorbic mixture in the analysis of adrenaline and noradrenaline in urine. At high concentrations ($> 0{\cdot}2$ μg./ml. of alumina eluate), ethylenediamine improves the stability of fluorescence particularly at 436 mμ. At concentrations of the amines of 5 ng. and below there are still difficulties, particularly when assaying plasma.

With ethylenediamine the fluorescence increases unsteadily to a plateau and may then decrease after a variable time. The rate of increase to this plateau depends critically upon the amount of ethylenediamine added, its purity and upon other unidentified factors which are related to the efficacy of washing the alumina in the separation procedure. The ratio of adrenolutine to noradrenolutine may also vary continuously. Increasing the concentrations of ethylenediamine above the authors' recommended figure (0·2 ml./10 ml. alkali-ascorbic mixture) produces a slight improvement in stability—though at the expense of reduced readings (particularly of adrenolutine) and also in an overall increase in background fluorescence.

To overcome the difficulties of stabilising the fluorescence many substances alone and in combination with ascorbic acid have been tried. β-Thiopropionic acid (TPA) was found to be easily the best and it effectively reduced the increase in fluorescence of both the alkali-ascorbic and plasma-alkali mixtures.

The alkali-ascorbic-TPA mixture is prepared as follows: TPA (0·2 ml.) is placed in a sample tube (3 ml.); and approximately 15 sec. before use ascorbic acid (0·1 ml. 5 per cent) followed by sodium hydroxide (1·9 ml., 5N) are added to this sample tube from a Seligson automatic pipette. This mixture is then added to a graduated flask (5 ml.) containing the pH-adjusted alumina eluate, previously oxidised by ferricyanide (0·25 per cent) for 2½ min. This method of preparation is simple and reliable—the 5N sodium hydroxide is stored in a plastic container (with CO_2 absorbent air intake) attached to the pipette, and the 5 per cent ascorbic acid is kept in a plastic bottle for up to a week in a refrigerator. The ferricyanide is conveniently stored and dispensed (0·1 ml.) from

a plastic ultra-micro-pipette. The glass containers were rinsed with normal hydrochloric acid, tap water and double distilled water between use, and a particular glass cuvette was used for a batch of analysis.

Results using β-thiopropionic acid show an easily obtained reproducibility of ± 100 pg. for adrenaline and noradrenaline in the hands of relatively unskilled laboratory personnel.

The improvement in the stability of the fluorescence is most useful at 436 mμ though the parallel improvement at 405 mμ makes this latter wavelength most suitable for estimations of small amounts of total adrenaline + noradrenaline. It is probably that a suitable secondary filter could be found which would equalise the fluorescence of adrenolutine and noradrenolutine (with added TPA) at 405 mμ. Without the addition of TPA the fluorescence of the two lutines was exactly equal at 405 mμ.

The ratio of adrenaline to noradrenaline at 436 mμ, using TPA was 2·6 (± 0·1) : 1 using 2A and 47B as primary filters* and 2A-15 as a secondary filter*). The ratio of adrenaline to noradrenaline at 405 mμ was 0·75 (± 0·05) : 1 (using a 405* and an Ilford Bright 623 as a primary and secondary filter respectively). The fluorometer used was a Turner model III.

The use of this thioacid opens up possibilities of reliably and routinely assaying adrenaline and noradrenaline in picrogram quantities.

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*G. K. Turner, Associatés, Palo Alto, California.

BOOK REVIEWS

POISONING. By Jay M. Arena. Pp. xvii + 440 (including Index). Charles C. Thomas, Springfield, Ill., U.S.A., 1963. $16.75.

The author of this book, Dr. Jay M. Arena, is clearly a man with a mission. He tells us that "when . . . a house officer in . . . Duke University Medical Center, it was my unfortunate lot to see and care for many children with acute caustic alkali poisoning". This experience evidently roused in him a horror of preventable (accidental) poisoning in young children which has been the motive for much of his work. This has lain in the field of paediatrics and poisoning plays an extraordinarily large part in child care in North America. As a result of the pressures so created, the first Poison Control Centre was organised in Chicago in 1953 and for 10 years has been collecting and analysing data concerning acute poisoning, as well as issuing information. It is on the data acquired by a service of this kind, installed in the Paediatric Department of Duke University, that this book is based. Now that Britain is taking its first officially sponsored step in the same direction with the installation of a Poison Information Centre at the Department of Forensic Medicine in Guy's Hospital, London, the content of this book is of exceptional interest.

It is divided into 11 sections, of which the most interesting is entitled "Public Safety Education" and comprises a survey of the methods of propaganda found useful to reduce the incidence of poisoning among children. Unfortunately it appears to be thought desirable to use a loose and journalistic style of presentation in this book which, in the reviewer's opinion, detracts from the weight of the views offered. This, in conjunction with the horrific dust cover, liberally decorated with skeletons and crossbones, gives an impression of undue levity. The two column format, paucity of illustration, and the inclusion of 120 pp. of close-set tables (lists of poisonous substances, etc. presumably derived from the files of the Information Centre) in the text are not attractive and the information is not likely to be very helpful in England largely because it concerns American products. A more serious criticism, perhaps, is the advocacy of gastric lavage in almost all cases when it has been repeatedly shown to be of severely limited use; the use of "universal antidote" which has been almost universally abandoned, and a number of medicaments and therapeutic procedures which have never been shown to be of real value. The author, though vastly experienced, is evidently not very critical in his choice of materials.

This book is not likely to appeal to many general practitioners in Britain, but it should prove to be a useful and interesting addition to the libraries of departments of Paediatrics and Toxicology, and a mine of information and sage advice to the discriminating reader. The index occupies no less than 80 pp. which makes easy the task of reference. J. D. P. GRAHAM.

A BIBLIOGRAPHY OF THE TABLETTING OF MEDICINAL SUBSTANCES. Compiled by A. J. Evans and David Train. Pp. 160 (including Index). The Pharmaceutical Press, London, 1963. 25s.

This book is a result of work carried out in the Department of Pharmaceutics and the Library of the School of Pharmacy, University of London. Everyone interested in the theories and the techniques involved in making medicinal tablets will be grateful to the compilers, the publishers and all those concerned with the project, for making this excellent bibliography generally accessible. Until this publication was issued no up-to-date and comprehensive collection of references on the subject had been available.

Presumably because of the limited number of copies so specialised a subject would require, a photolithographic method of reproduction from typescript has been adopted which has resulted in a serviceable if not very attractive format. Proof reading and typing would appear to have been very carefully done and errors are few; some apparent misprints are due to the method of reproduction used.

Within the limits set by the compilers a very comprehensive list of references has been provided and annotations have been added where the titles of the papers, or other works, do not adequately indicate their contents. The difficulties of devising a subject index have been satisfactorily overcome by a detailed classification of the material under five main headings (viz.: 1, General; 2, Tablets; 3, Tabletting Practice; 4, Materials; 5, Fundamentals) and 61 subsections. In any future editions when more references are included a more detailed subject index with cross references would be helpful. As it is, any specific reference, the authorship of which is not known, may often be found in a matter of seconds, the search rarely requires more than a very few minutes. A complete author index is, of course, included.

The subject matter of the bibliography covers a number of rapidly expanding fields of investigation and practice. It is very much to be hoped that authors will persist in their expressed intention to continue the collection of references and, by the publication of supplements to this volume, keep the bibliography up to date and perhaps also widen its scope.

If, as a result of a wider circulation the publishers were able to use a more costly method of printing another edition, an invaluable bibliography might have the presentation it deserves.

D. STEPHENSON.

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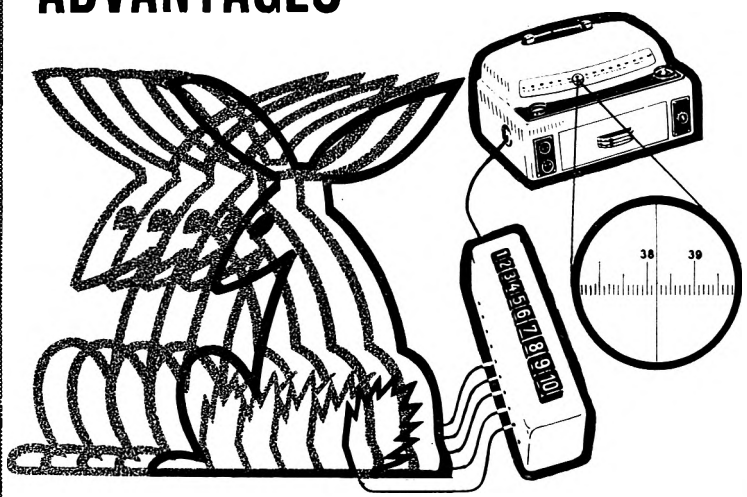

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