

REVIEW ARTICLE

LYSOLECITHIN

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LYSOLECITHIN belongs to the group of naturally occurring lipids known as the glycerophosphatides. Its parent substance is lecithin, the most important member of this group, from which it is derived by the removal of one fatty acid radical. Although knowledge of the properties and behaviour of lysolecithin has accumulated very slowly its importance in mammalian lipid metabolism is now fully recognised.

Probably the first mention of the enzymatic hydrolysis of phosphatides appeared in a paper by Bókay (1877), who showed that lecithin reacted with pancreatic juice to yield a mixture of glycerophosphoric acid, fatty acids and choline. Little interest was shown, however, until Kyes (1903) and Kyes and Sachs (1903) working in Ehrlich's laboratory found that cobra venom reacted with lecithin to yield a product possessing haemolytic activity which they called "Cobralecithid". This material was thought to be a complex but Willstätter and Lüdecke (1904) and Lüdecke (1905) reported that the haemolytic agent was an enzymatic breakdown product of lecithin. This view was finally accepted when Delézenne and Ledebt (1911, 1912) and Delézenne and Fourneau (1914) treated an emulsion of egg yolk with cobra venom and obtained a crystalline product having the elementary composition of monopalmitoyl lecithin which they called "lysolecithin". In 1920, Fourneau confirmed that this material was lecithin from which one unsaturated fatty acid had been removed.

Lysolecithin was first shown to be present in the animal body by Belfanti (1924) who isolated it from the pancreas and salivary glands of horses and cattle. A year later he showed it to be a strongly toxic substance which dissolved erythrocytes and leucocytes; it injured the brain and its capillary endothelium and produced oedema and haemorrhage. Inactive samples isolated from other tissues were thought to be inhibited by cholesterol. Within eight years he, and also Nikuni (1932) quite independently, separated the specific enzyme lecithinase A from pancreas extract and showed that lysolecithin was the enzymatically catalysed hydrolytic product of lecithin. In 1931, Pighini and Delfini isolated lysolecithin from the brain of the guinea-pig and also from egg yolk; lysolecithin was also found in blood serum by Bergenhem and Fahreus (1936) and by Singer (1940).

A few workers claim to have isolated lysolecithin from plant material. Hirao (1931) found lysolecithin in wheat, rye, millet and barley as well as in rice, but whether lysolecithin was a constituent or a breakdown product during isolation should, in the writer's opinion, be investigated. Francioli (1934) isolated lysolecithin from the fungus *Lycoperdon giganteum* and Colmer (1948) found small amounts of lysolecithin in cultures of *Bacillus cereus*, *B. cereus* var. *mycoides*, *B. cereus* var. *anthracis* and cultures

labelled *Bacillus praussnitzii*. An example of the presence of lecithinase A in bacteria (a strain of *Serratia plymuthicum*) was reported by Hayaishi and Kornberg (1954) but they were unable to isolate lysolecithin owing to the excessive lysolecithinase activity of the enzyme fractions.

PREPARATION OF LYSOLECITHIN

Early Preparations

The first extensive investigation of the preparation, physical and chemical properties of lysolecithin was made by Levene and his colleagues. In 1923, Levene and Rolf prepared lysolecithin by the digestion of egg yolk in M/15 phosphate buffer with cobra venom as a source of lecithinase A. They found the optimum activity of the catalysing enzyme for this reaction to be at pH 7.0 ± 0.5 ; above pH 8 the activity of the enzyme was reduced to zero. The presence of various antiseptics was also found to impair the activity of the enzyme. The lysolecithin extracts obtained by Levene and co-workers possessed a nitrogen and phosphorus content higher than expected, whereupon further investigation resulted in the isolation of an ethanolamine-containing material which was named "lysocephalin"—this is probably the first report of the existence of lysocephalins.

A year later Levene and co-workers (1924) separated lysolecithin from lysocephalin by differential solubility in organic solvents of the metal complexes formed by treating the lysophosphatide with cadmium chloride. A similar separation of the choline-containing and ethanolamine-containing phosphatides at the lysophosphatide stage was also attempted by Fairbairn in 1948. He reported a complete separation of lysolecithin and lysocephalin through the use of the colloidal iron-magnesium sulphate method of Folch and Van Slyke (1939). Analytical figures for the lysolecithin obtained by Levene were in close agreement with Fairbairn's values. Levene found that the lysolecithin melted at 263° with blackening and decomposition; it was very hygroscopic and insoluble in acetone and also in ether. Hydrolysis of the material showed that the fatty acid fractions present in the lysolecithin obtained from egg yolk were mainly palmitic and stearic.

An alternative cadmium chloride complex formation method was examined by Contardi and Latzer (1928) who found that the cadmium chloride complex of the parent substance lecithin could be attacked by lecithinase A to yield the lysolecithin complex.

In 1933, King and Dolan prepared lysolecithin by a similar method to that of Levene, Rolf and Simms but using the venom of the fer de lance (*Bothrops atrox*) as a source of lecithinase A. The reaction mixture was digested for 18 hr. at 40° and pH 7 in 0.066M phosphate buffer; fractional precipitation of the lysophosphatide material showed that the first fraction was lysocephalin and later fractions contained lysolecithin; the yields compared favourably with those obtained by Levene and others and analysis of the lysolecithin gave a nitrogen content of 3.2 per cent and phosphorus 5.6 per cent.

Preparations of lysolecithins from plant sources have been reported from Japanese laboratories. In 1930, Iwata isolated lysolecithin from the

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alcoholic extracts of polished rice by formation of the cadmium chloride complex. His yield of 0.3 per cent lysolecithin in the polished rice is surprisingly high and although it is identical with the amount obtained by the action of venom on lecithin his value should be confirmed.

Much of the earlier work on the physical and chemical properties of lecithin and lysolecithin was carried out on material prepared by formation of the cadmium chloride-phosphatide complex. The presence of traces of any unremoved salt could, however, have adverse effects on its physical characterisation: properties such as surface tension, monolayer formation and solubilisation would be affected; chemical reactivity of the lysophosphatide could also be inhibited. More recently other techniques, such as chromatography and ion exchange, have been used in the purification stages of preparations. By these methods the possibility of the presence of any interfering ions is substantially reduced.

Pure Lysolecithin from Natural Sources

Hydrolytic products of lysolecithins isolated from natural sources have been found to possess mainly a C_{16} or C_{18} fatty acid. On this basis theoretical values for the nitrogen and phosphorus contents in the molecule of lysolecithin can be calculated and used as criteria for the standard of purity of the preparation. Fatty acids of slightly higher or slightly lower molecular weights and possessing some degree of unsaturation may also occur in lysolecithin. Deviation from theoretical values due to this will be small compared with the deviation in values resulting from the presence of impurities such as lecithin, the cephalins, glycerides and hydrolytic products, all of which are difficult to remove.

In 1954, Hanahan, Rodbell and Turner devised a method for preparing lysolecithin in good yield based on their discovery that snake venom, dissolved in a small quantity of water, reacts with lecithin in ethereal solution. Previously Desnuelle and Constantin (1953) had been able to show that pancreatic lipase possessed no catalytic activity under similar conditions. Hanahan and his colleagues prepared two homogeneous lysolecithins, monopalmitoleyl lecithin from (dipalmitoleyl)-L- α -lecithin and monopalmitoyl lecithin from (dipalmitoyl)-L- α -lecithin with snake venoms from *Naja naja* and *Crotalus adamanteus*.

The enzyme solutions were prepared in distilled water at pH 6.5–7.0. This narrow pH region was not critical, however, since there appeared to be very little loss of activity over the pH range 4.0–7.5 but the reaction rate decreased outside these limits. An increase or decrease in the enzyme concentration was found to produce a correspondingly proportional increase or decrease in the amount of fatty acid released from the lecithin substrate.

After 40 per cent completion of the reaction, lysolecithin and some unreacted lecithin substrate precipitated without affecting the course of the reaction. The unreacted lecithin which was carried down was ultimately found to be converted to lysolecithin which suggested that some of the snake venom catalysing the reaction must have been precipitated together with the lecithin. This observation, plus the fact that the

snake venom is insoluble in ether and complete mixing of the two phases is necessary to initiate enzymatic degradation, leads to the view that an enzyme-lecithin complex must have been formed in the substrate.

In an extension of this work Hanahan and his colleagues were able to show that the enzyme system attacked a saturated lecithin, that is, a lecithin molecule with no C-C double bonds present in the long chain fatty acids, at a much faster rate than an unsaturated lecithin (Fig. 1).

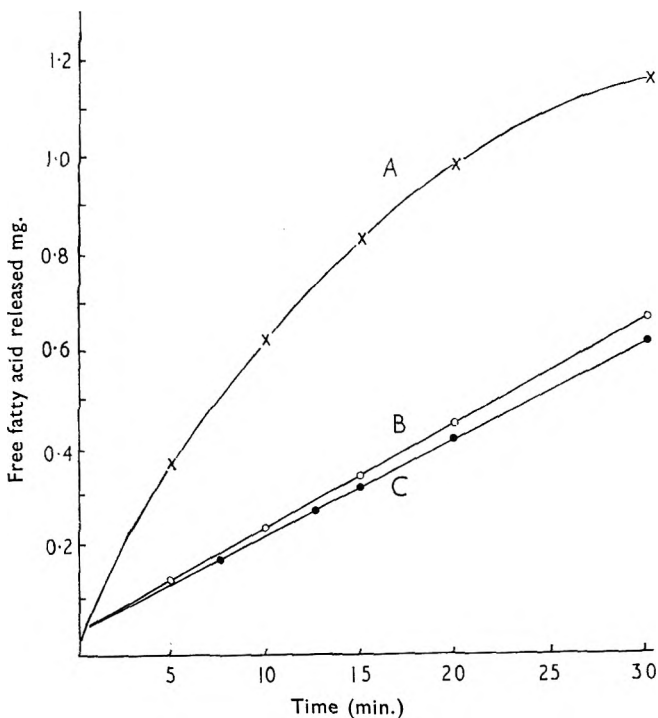


FIG. 1. Effect of *Crotalus adamantus* venom on unsaturated and saturated lecithin in a solvent of 95 per cent ether: 5 per cent ethanol. The control, containing an unsaturated lecithin and the venom, was dissolved in ether only. A, Saturated lecithin, ethanol added. B, unsaturated lecithin, ether only. C, unsaturated lecithin, ethanol added. (With acknowledgements to Hanahan, Rodbell and Turner, 1954.)

This led to the conclusion that enzymes were present in the venom which were capable of cleaving either a saturated or an unsaturated fatty acid, but not both.

Saunders (1957) added a further step to the method of Hanahan at the final purification stage of lysolecithin to ensure the complete removal of traces of venom. The amorphous material obtained by ether precipitation from chloroform was washed with acetone, dried and dissolved in a small volume of ethanol. This solution was slightly cloudy from traces of proteinous matter from the venom; these were removed by centrifugation followed by repeated crystallisation of lysolecithin from ethanol.

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His method also differed from Hanahan's in the choice of Russell's viper venom as a source of lecithinase A.

A quantitative estimation of lysolecithin has often been made by measuring its haemolysing action on red blood cells, but it is possible that lysing material other than lysolecithin is present which produces the same effect. A more satisfactory method has been suggested by Fairbairn (1945) whereby lysolecithin content is determined by solvent extraction of the unsaturated fatty acids which are then titrated against sodium hydroxide; phenolphthalein is used as an indicator.

Although lysophosphatides are normally insoluble in ether they are likely to remain in solution in this solvent in the presence of ether-soluble phospholipids which will have a co-solubilising effect; this anomalous behaviour could have adverse effects on methods of separation which depend on differential solubility or partition between solvents. Chromatographic methods for the complete separation of lysolecithin from its parent substance lecithin and also from cephalin material have therefore received much attention.

Trappe (1940) first applied adsorption chromatography to the separation of mixed lipids into their main constituent classes. By the use of a variety of adsorbents and solvents he showed that lipids were more strongly adsorbed as their polarity increased from hydrocarbons, through sterol esters, triglycerides, sterols and fatty acids to phospholipids. More recently Kornberg and Pricer (1952) and also Kennedy (1953) have employed paper chromatography in conjunction with radiographs or direct counting of eluted samples to establish the enzymatic synthesis of the acyl esters of phosphatidic acids and phospholipids. In 1954, Hanahan, Uziel and Huennekens reported the separation and identification of a series of pure, well-characterised lecithin and lysolecithin fragments from an homogeneous unsaturated (dipalmitoyl)-L- α -lecithin; the technique was also found suitable for resolving certain mixtures such as lecithin and lysolecithin, saturated and unsaturated lecithins or corresponding lysolecithins and other hydrolytic degradation products of lecithin. Whilst Bevan, Gregory, Malkin and Poole (1951) achieved a separation of phospholipids by both the paper and column chromatographic techniques, they were unable to report the isolation of a lysolecithin fraction. Difficulties were also encountered by Rhodes and Lea (1956, 1956a) who were unable to separate an unsaturated lecithin (Iodine Value = 80) and the same hydrogenated material (Iodine Value = 2); these and various individual synthetic lecithins all gave practically the same R_f values on silica-impregnated papers. Much valuable work has been done in this field by Lea and Rhodes (see Lea, 1956; Rhodes, 1956).

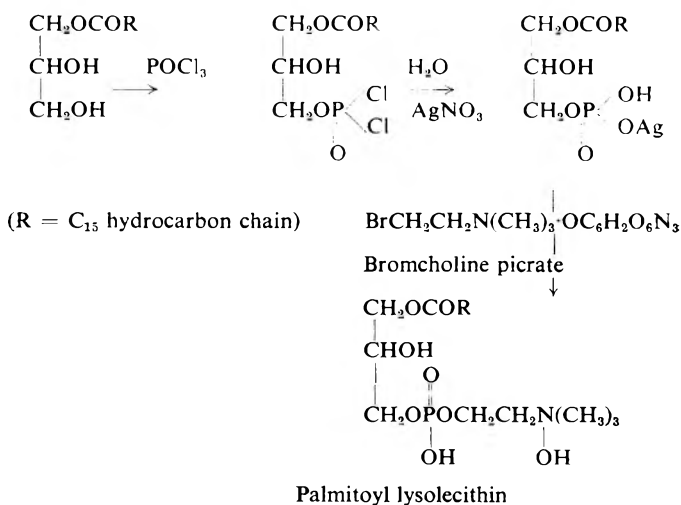
Using filter paper impregnated with silicic acid previously activated at 120°, Lea and Rhodes (1954) and Lea, Rhodes and Stoll (1955) separated the amino-nitrogen fraction (approximate R_f 0.9) of ether soluble phospholipids of egg yolk from the slower moving choline-containing fractions (approximate R_f 0.6 and 0.1) and found that the ether-insoluble residue retained by the column showed haemolytic activity. Lysolecithin was subsequently found to have the lowest R_f value (0.3) whilst other lipid

material, for example, cephalins, plasmalogens, and sphingomyelins varied between R_f values of 0.5 and 0.85.

In 1955, Lea and his colleagues also experimented with ion-exchange resins to obtain a separation of lecithin and cephalin components. Since phosphatidylcholine-containing material is approximately neutral and phosphatidylethanolamine-containing lipids are acidic, egg phospholipid was run on cross-linked sulphonated polystyrene or strongly basic resins with quaternary ammonium groups. Both these resins showed very little capacity (1–3 per cent of theoretical) for retaining fractions, probably on account of the comparatively large size of the phosphatide molecules; furthermore, it appeared that considerable hydrolytic decomposition of the phosphatides had taken place using the strong resins. Perrin and Saunders (1960) have since been successful in separating the two major phosphatide components in egg yolk by using the bicarbonate form of Dowex resins—the separation has also been equally successful with yeast, soya-bean, ground-nut and cotton seed phosphatides.

SYNTHESIS OF LYSOLECITHIN

Various attempts have been made to synthesise lysolecithin. In 1938, Kabashima claimed to have synthesised lysolecithin by the following reaction:

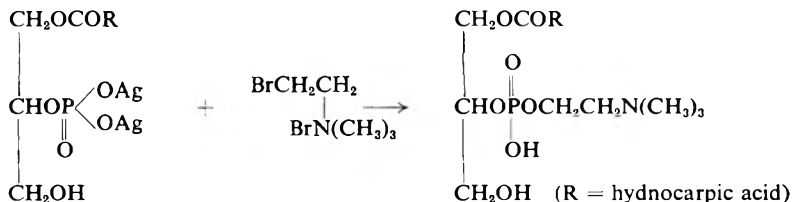


Kabashima's product decomposed at 262° and its haemolytic activity was found to be about one-fourth of that of lysolecithin prepared from egg lecithin.

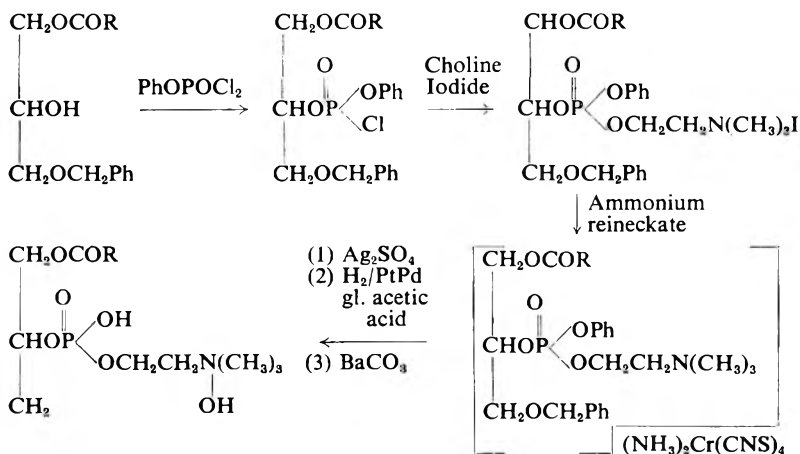
Arnold (1940) claimed a lysolecithin by the action of β -bromomethyl ammonium bromide on the silver salt of the monohydric acid ester of glycerophosphoric acid according to the equation at top of p. 327.

Baylis, Bevan and Malkin (1956) synthesised lysolecithins starting with 3-palmityl-1-benzylglycerol. This was dissolved in dry pyridine

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and added in drops with stirring to an ice-cold solution of phenyl phosphoryl dichloride in dry pyridine. Choline iodide was added and the resulting oil was converted into the reineckate; this was converted into the sulphate and hydrolysed. The equation for the reaction is shown below.



The position of the phosphate-choline group was not definitely established. The melting points were not sharp; they sintered between 50° and 60° and gave a meniscus between 230° and 240°.

Last year de Haas and van Deenan (1960) and Kogl, de Haas and van Deenan (1960) obtained high yields of lysolecithin by acylation of L- α -glycerophosphorylcholine. The primary interest of the workers at Utrecht was the synthesis of "mixed-acid" lecithins, that is, lecithins with different fatty acids esterified to the glycerol nucleus. In studies on hydrolytic reactions they found that lecithinase A catalysed the degradation of (α -stearoyl- β -oleoyl)-L- α -lecithin and (α -oleoyl- β -stearoyl)-L- α -lecithin, liberating stearic acid and oleic acid respectively; hydrolysis occurred exclusively in the α position. The methods appear to offer scope for the synthesis of a wide variety of lysolecithins and lecithins, especially the physiologically important highly unsaturated lecithins.

ACTIVITY OF THE CATALYSING ENZYME, LECITHINASE A

The conditions in which the enzyme lecithinase A catalyses the specific hydrolysis of lecithin to lysolecithin has been investigated extensively, a major contribution coming from Italian laboratories.

Enzyme extracts probably contain several active principles and sometimes one constituent can possess two or more active groups, for example,

the neurotoxin and phosphatidase actions of a single protein constituent of *Crotalus terrificus*. In work carried out before fractionation procedures of the enzyme extracts were used, unwanted enzymatic degradation products of lecithin complicated the isolation of a pure phosphatide material; subsequent isolation of the reaction products is necessary to obtain a pure homogeneous lysolecithin. In studying the hydrolytic reaction conditions it is essential to use a pure lecithin sample for attack by the enzyme lecithinase A.

The enzyme appears to be present in a very active form in the salivary secretions of various types of cobras and also in reptiles of the viper and crotalus families; it has also been found in the poison of bees and scorpions. According to Francioli (1934) its action appears to be inhibited *in vivo* probably by the presence of other enzymes or protecting materials. Francioli was not able to isolate lysolecithin from fresh animal organ, whilst the dried materials were found to contain lysolecithin indicating that interfering substances were denatured or destroyed in the drying process.

Flexner and Noguchi (1902) also found that lecithinase A was inhibited from catalysing lecithin by the presence of other enzymes and substances having a protective action; it was, however, comparatively resistant towards heat and Gronchi (1936) was able to confirm that heat treatment or drying processes broke down the spurious enzymes and bacterial interference leaving the more stable lecithinase A active. Ogawa (1936, 1937) found that inactivation by heat of lecithinase A itself depended upon the enzyme concentration; heating for 30 min. at 40° or 50° brought about a noticeable inactivation which increased very greatly when the enzyme was heated to 60° or 80°, and inactivation was complete at 100°. He found that the enzyme remained active for 18 hr. at 37° and was most stable at pH 4.6, but at a pH greater than 7.0 it became inactive. Belfanti, Contardi and Ercoli (1935) observed that an exact amount of homologous anti-serum inactivated lecithinase A obtained from snake poison.

Many workers have reported the activating effect of calcium ions on lecithinase A during the catalytic degradation of lecithin. Delézénne and Fourneau (1914) and also Kudicke and Sachs (1916) found that the activating effect of calcium ions was concentration dependent, but if the concentrations of these ions was high the enzyme became inactivated. A similar effect was observed with magnesium ions.

In 1955, Davidson, Long and Penny found that the presence of calcium ions was essential for consistent results in the hydrolysis of ovolecithin with moccasin venom. Examination of different natural and synthetic lecithins showed that the lecithinase A in moccasin venom was virtually specific for L- α -lecithins and had little or no activity on D- α -lecithins or β -lecithins.

Several other substances have been found to increase the activity of lecithinase A. Habermann (1957, 1958) observed that lecithinase A from bee toxin attacked lecithin more actively in the presence of surface-active substances such as Tween 20 or deoxycholic acid in addition to calcium ions. Serum albumin and lysolecithin itself activated strongly, whilst fluoride ions, citrate ions and the ions of heavy metals, including Hg and

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Zn, were found to inhibit as did also oleic acid. The optimum hydrogen ion concentration for bee venom lecithinase A activity on pure lecithin was found to be pH 8 and on egg yolk pH 8.3 when 1 g. of lecithinase A split 27 moles of lecithin in 30 minutes.

Sodium chloride has been found to inhibit the enzymatic activity to a slight extent (Belfanti and Arnaudi, 1932) whilst potassium cyanide, urethane and eserine have no effect (Davidson, Long and Penny, 1956; Habermann, 1957).

In 1935, Hughes used the venoms of cobra, daboia, black tiger, copperhead and black snake to examine the rate of hydrolysis of lecithin to lysolecithin as measured by the rapid fall in surface potential of a surface film and was able to show that the rate was dependent on the lecithin and venom concentrations and also on pH. At the same time, he observed that the addition of cholesterol to the surface film of lecithin decreased the rate of hydrolysis of the lecithin. His ingenious surface potential technique was very sensitive—as little as 0.025 μ g. of venom (*Denisonia superba*) gave a measurable effect.

SITES OF ACTION OF THE LECITHINASES

Lecithinase A attack on Lecithin

When lecithin is attacked by the specific enzyme called lecithinase A (after Contardi and Ercoli, 1933) one fatty acid ester linkage is cleaved with the formation of lysolecithin and a long-chain fatty acid. Accumulated evidence shows that the fatty acids present in the parent substance lecithin are variable in chain-length (usually C_{16} or greater) and degrees of unsaturation. Most of the unsaturated acids appear to be attached to the α -carbon atom of the glycerol nucleus whilst the saturated fatty acids occupy the β -position.

Many attempts have been made over a number of years to determine whether the α - or the β -ester linkage in lecithin is attacked by lecithinase A or whether the enzyme is preferentially active at a saturated or an unsaturated fatty acid ester linkage.

As early as 1904, Willstätter and Lüdecke found that the fatty acid liberated from lecithin was invariably unsaturated. This was substantiated by Delézenne and Ledebt (1911) who reported that the remaining fatty acid radical attached to the glycerol nucleus was saturated. The subject was pursued further by King (1934) who found that a catalytically hydrogenated natural lecithin was attacked as rapidly as the original material. More recently, however, Zeller (1952) found that the saturated fatty acid was split from L- α -dimyristoyl lecithin which had been incubated with snake venom at 37° and he postulated that lecithinase A attacked exclusively the α - or β -ester linkage irrespective of whether the fatty chain was saturated or unsaturated. Chargaff and Cohen (1939) showed that no fractionation of lecithin occurred in the course of formation of lysolecithin; this was demonstrated by stopping the hydrolysis of lecithin before completion when iodine value determinations gave the same figures for unsplit lecithin and for the original material.

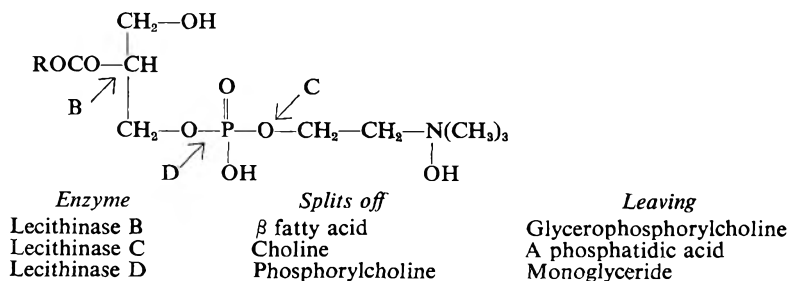
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Recently, Hanahan (1954) and Long and Penny (1954) have made experiments which show fairly conclusively that lecithinase A attacks the α -ester linkage only; their work is discussed in more detail under the heading "Chemical Structure of Lysolecithin".

Lecithinase B, C and D attack on Lysolecithin

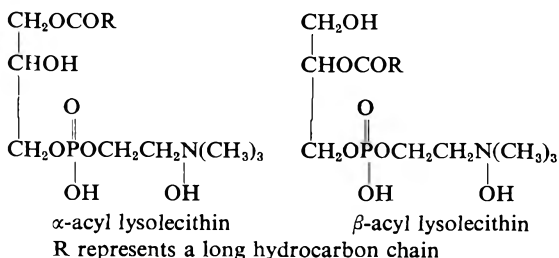
The three ester linkages of lysolecithin can be split by the specific enzymes lecithinase B, C and D. Lecithinase B attacks lysolecithin and cleaves the fatty acid ester linkage. Hanahan (1954) suggested that this takes place in two stages: (i) migration of the fatty acid residue from the β - to the α -position, (ii) removal of the fatty acid. The migration from the β - to the α -position is commonly experienced with mono- and diglycerides—the puzzling case is when the fatty acid remains in the β -position. Malkin (1956) suggested that the hydrogen of the adjacent phosphate hydroxyl group in the lysolecithin molecule may form a hydrogen bond with the ester-keto group thus reducing the tendency of the fatty acid residue in the β -position to migrate.

The modes of action of lecithinases C and D have been inconsistently reported. The modes of action according to Wittcoff (1951) (and also Hanahan (1957)) are given here; Lea (1957) gives the reversed nomenclature for lecithinases C and D.



CHEMICAL STRUCTURE OF LYSOLECITHIN

In lysolecithin one of the hydroxyl groups of a glycerol molecule is esterified to the long chain fatty acid and another to the phosphoryl choline group. Although the evidence is not conclusive, it is generally recognised that the phosphorylcholine group occupies the α' -position of the glycerol nucleus—lysolecithins from natural sources almost exclusively show this structure. This leaves two positions to which the fatty acid may be esterified:



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The main objective has therefore been to identify the position occupied by the fatty acid radical and to decide whether it is a saturated or an unsaturated moiety. One or two attempts have been made to synthesise a lysolecithin by acylation of glycerophosphorylcholine but more concentrated efforts have been directed towards a study of the breakdown products of lysolecithin. In 1937, Levene and Mehtretter attempted to determine the position of the fatty acid radical attached to the glycerol nucleus of the lysolecithin molecule by synthetic methods but they were unsuccessful. Hanahan and others (1954) attempted to resolve the problem by acylation of the alcohol group under a variety of conditions and solvents but no reaction could be effected. Some progress was made, however, in the same year when Hanahan reported that lecithin from beef, rabbit, dog, guinea-pig and rat livers had only unsaturated fatty acids on the α -position, and saturated fatty acids on the β -ester linkage of lecithin. He showed that lecithinase A of snake venom attacked the α -ester linkage of lecithins from liver and that the liberated fatty acid fractions contained all the unsaturation present in the intact lecithin. He suggested, however, that one could not generalise on selected positioning of the fatty acid ester linkages since completely saturated lecithins had also been isolated from animal and plant sources.

Zeller (1952) followed the hydrolysis of lecithin to lysolecithin by measuring the amount of haemoglobin released in the haemolysis of erythrocytes and reported for the first time that *Naia naia* and *Vipera espis* venoms could attack a pure saturated lecithin. He considered the position of the fatty acid rather than the nature of the fatty acid was the decisive factor in the cleavage of the ester linkage. Subsequently Hanahan, Rodbell and Turner (1954) showed that both (dipalmitoleyl)-L- α -lecithin and (dipalmitoyl)-L- α -lecithin were attacked by venom, that is to say, an unsaturated as well as a saturated fatty acid linkage was cleaved, the saturated lecithin being attacked at a faster rate than the unsaturated ester.

It can therefore be inferred that lecithinase A is specific for either the α - or β -linked fatty acid radical rather than for the unsaturated fatty acid radicals generally.

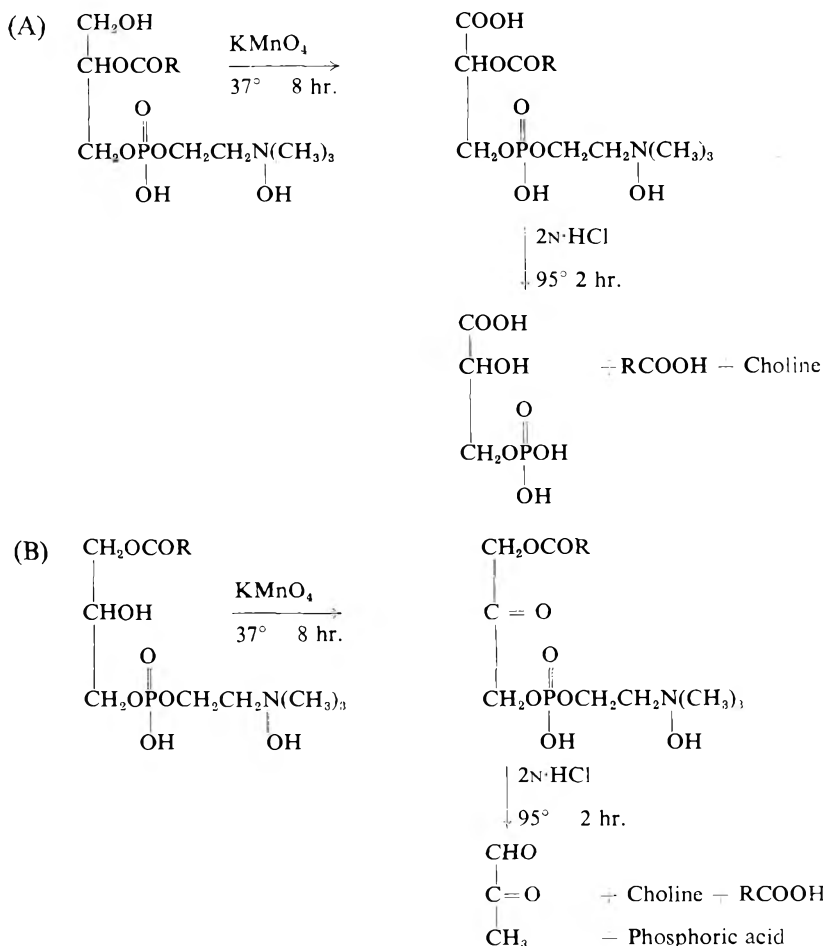
The problem of the position of attachment of the fatty acid radical to the glycerol nucleus of lysolecithin was finally solved by Hanahan (1954) and Long and Penny (1954). Hanahan found that monopalmitoyl lysolecithin was oxidised almost immediately by potassium permanganate to the corresponding acid; subsequent acid hydrolysis yielded phosphoglyceric acid, isolated as the barium salt, free fatty acid and choline. These results can be explained by the equations (A) shown on p. 332, if it is assumed that the fatty acid ester linkage is in the β -position of the glycerol nucleus.

Breakdown products from an α -acyl lysolecithin would have been methylglyoxal, phosphoric acid, fatty acid and choline according to the equations (B) overleaf.

Long and Penny (1954) approached the problem by examining the alcoholic group exposed when lecithin was treated with lecithinase A.

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One mole of the lysolecithin, after catalytic hydrogenation, reacted with four equivalents of acidified dichromate indicating the presence of a primary alcohol group.



β to α Acyl Migration

It is known that the fatty acid moiety in a β L-monoglyceride readily migrates to the α-position, in fact special precautions are necessary to obtain a pure β product. It is surprising, therefore, to find that β-(acyl) lysolecithin is stable—this is discussed elsewhere. Nevertheless, β- to α-migration of the fatty acid fraction in the lysolecithin molecule can be made with comparative ease.

In 1957, Uziel and Hanahan reported the migration of β-(acyl) lysolecithin to the α compound by a lysolecithin migratase enzyme. The yield of migratase enzyme from 20 g. pancreatin was sufficient to catalyse the conversion of 1 g. of β-(acyl) lysolecithin in a 60–70 per cent yield in 12 hr. at 25° whilst using a *Penicillium notatum* source of enzyme, 30 mg. of

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the extract was sufficient to catalyse to the same extent. The enzyme preparations were specific to saturated lysolecithins with no effect on the unsaturated compound or on saturated, partially saturated, or unsaturated lecithins.

The β - to α -acyl shift could also be catalysed by incubation of the substrate in 0.05N HCl for 12 hr. at 25°. Cyanide was a good inhibitor of the enzyme and non-enzyme-catalysed migrations, and it was also found to inhibit the lysolecithinase B action on a saturated lysolecithin.

Uziel and Hanahan's α -(acyl) lysolecithin precipitated in microcrystalline form from the aqueous solution of the enzyme-catalysed reaction mixture. Some of the physical constants of the α -compound showed a significant difference from corresponding values of the β -compound which is to be expected but it is puzzling to find that Uziel and Hanahan's material was insoluble in water since the functional groups in the α - and β -compounds in relation to water solubility are similar. The physical characteristics of their α -acyl material are compared with the β -acyl compound in Table I.

TABLE I

Property	α	β
M.p. [α] _D ^{25°}	236°-237° - 3.78°	257°-258° - 2.87°
Solubility in water at 25°	Insoluble	Soluble
Solubility in 0.1M NaCl (25°)	Insoluble	Soluble
Permanganate oxidation	Slow, 12-14 hr.	Rapid, 2-3 hr.
Products of oxidation followed by acid hydrolysis	Free fatty acid Choline Inorganic phosphate pyruv-aldehyde	Free fatty acid Choline Phosphoglyceric acid
Chromatogram, using water as solvent	No movement from origin	Single well defined spot, R_F 0.79

α -(acyl) Lysolecithin

Klenk and Debuch (1954, 1955) and Debuch (1956) have shown that natural plasmalogens contain a fatty acid and a fatty aldehyde residue. Gray (1958) and Gray and Macfarlane (1958) hydrolysed choline-phosphatide-containing plasmalogen fractions of ox heart muscle by incubation with 90 per cent (v/v) acetic acid for 18 hr. when all the plasmalogen was split into free aldehyde and lysolecithin. Permanganate oxidation and subsequent acid hydrolysis of the hydrogenated lysolecithin product yielded phosphorylcholine and methyl glyoxal—the lysolecithin must therefore have had the α -acyl structure. This lysolecithin material gave, on analysis, 2.20 double bonds per fatty acid by iodine uptake; it was also water soluble and showed marked surface activity (Robinson and Saunders, 1958a).

CHEMICAL PROPERTIES

Esterification of the "Free" Alcoholic Groups

In a study of the site of action of lecithinase A on lecithin, Hanahan (1954) reported that the usual derivatives of a "free" alcoholic group

present in the β isomer were difficult to prepare; no reaction took place with reagents such as stearoyl chloride, palmitoyl chloride, *p*-nitrobenzoyl chloride, phthalic anhydride or acetic anhydride; the preparation of the α naphthyl or 3-nitronaphthylurethane was unsuccessful.

Attempts have been made to obtain labelled lecithins by acylation of β -lysolecithins of known constitution. Levine and Mehlretter (1937) reported the preparation of lecithin from lysolecithin by reaction with fatty acid anhydrides and sodium acetate under anhydrous conditions; later Hanahan (1957) tried to repeat this preparation but was unsuccessful.

Other similar procedures investigated by Hanahan were (a) fatty acid anhydrides in the presence of trifluoroacetic acid, (b) free fatty acid in the presence of trifluoroacetic acid anhydride and (c) replacement of the hydroxyl group by halogen using selected reagents and reacting with the silver salt of the long chain fatty acid.

Enzymatic Hydrolysis of Lysolecithin by Lecithinase B

The cleavage of the fatty acid radical from lysolecithin by a specific enzyme was probably first reported by the Italian workers Contardi and Ercoli (1933). They isolated the hydrolytic enzyme which they called "lecithinase B" from old rice bran (lecithinase B in new bran was contaminated with the C enzyme) and the mycelia of *Aspergillus oryzae*. On incubating lysolecithin for 9 days with extracts of rice bran, part of the phosphatide phosphorus became water-soluble without the liberation of phosphorus or choline and this material was interpreted as glycerophosphorylcholine; in the process the lysolecithin completely lost its haemolysing power.

Other sources of the enzyme have since been reported. Schmidt, Hershman and Thannhauser (1945) isolated pure glycerophosphorylcholine from incubated beef pancreas whilst Fairbairn (1948) stated that a much richer concentration of the enzyme was present in *Penicillium notatum*. The high concentration of lecithinase B compared with lecithinases A and C present in *Penicillium notatum* (Fairbairn, 1948; Shapiro, 1953) and *Serratia plymuthicum* (Hayaishi and Kornberg, 1954) led Hanahan (1957) to suggest that this enzyme may be present with the specific function of removing lysolecithin rapidly from the cellular environment. Lecithinase B has also been isolated from bacteria (Hayaishi and Kornberg, 1954) and in a highly purified form from ox pancreas (Shapiro, 1953); the ox pancreas sample had an activity 40 times that of the original extract.

Early experiments by King and Dolan (1933) suggested that the enzyme may catalyse the hydrolysis of lecithin as well as lysolecithin. They investigated the enzymatic hydrolysis of egg lecithin and lysolecithin by enzyme-containing extracts of rabbit intestinal mucosa and young rachitic rat bones and found that the former enzyme extract attacked twice the quantity of lysolecithin as lecithin and at a slightly higher pH optimum (7.8), whilst the extract of young rachitic rat bones hydrolysed almost three times the quantity of lysolecithin compared with that of lecithin to which it was relatively inert: the actual volume hydrolysed by the intestinal

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extract was noticeably greater than the bone extract. Several workers have since attempted to catalyse the hydrolysis of lecithin with lecithinase B and have been unsuccessful. Shapiro (1953) showed that his lecithinase B preparation from ox pancreas was inactive towards lecithin and triolein but it possessed a maximum activity with lysolecithin at pH 6 and a substrate concentration varying from 3 to 8 μ moles/ml. The enzyme was also found to be stabilised by glycerol but unaffected by calcium, magnesium and potassium ions as well as cysteine. Fairbairn (1948) also found that the enzyme was specific for lysophosphatide and repeated attempts

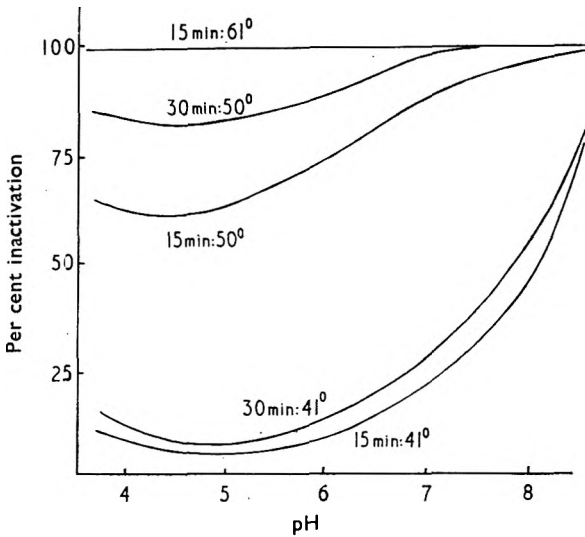


FIG. 2. Stability of lecithinase B (0.6 ml. of extract of *Penicillium notatum* per ml.) to heat and hydrogen ion concentration. Inactivation rapidly increases above pH 7 at temperatures of 41° or higher. (With acknowledgements to Fairbairn, 1948.)

showed that it was inactive with lecithin and cephalin; other reactions showed that the enzyme was readily inactivated by heat in slightly alkaline conditions (Fig. 2) and also by heavy metal ions. Cyanide was a potent inhibitor. The optimum activity for this enzymatic hydrolysis reaction was in the range pH 3.8–4.4 which is in close agreement with Contardi and Ercoli (1933), who gave 3.5 for the corresponding enzyme from *Asperigillus oryzae*, but considerably lower than Shapiro's figure (6.0). The colloidal iron-magnesium sulphate method of Folch and Van Slyke (1939) for the separation of lysophosphatides and fatty acids from glycerophosphorylcholine was used by Fairbairn (1948) to analyse for lysophosphatide in a quantitative determination of the hydrolysis of lysophosphatide to glycerophosphorylcholine and fatty acids. He found that, at 30° and within a reaction time of 15 min., a first order reaction took place, the appropriate constant k being proportional to the enzyme concentration over a wide range (Fig. 3).

More recently Hanahan and Uziel (1956) produced further evidence to show that, for enzymatic hydrolysis by lecithinase B, the substrate must be a monoacyl-substituted glycerophosphorylcholine and the only base present must be choline. They found that lecithinase B from the mycelia of *Penicillium notatum* was more active toward an unsaturated lysolecithin than toward a saturated compound (Fig. 4). The two reactions were markedly different, the former proceeded smoothly and could be carried to completion whilst the latter proceeded until gelation at 50 per cent completion stopped the reaction; this phenomenon was attributed to the simultaneous reaction of an isomerase.

Lysolecithin can also be hydrolysed by treatment with acid; complete hydrolysis occurs on autoclaving with 3 per cent sulphuric acid at 105°

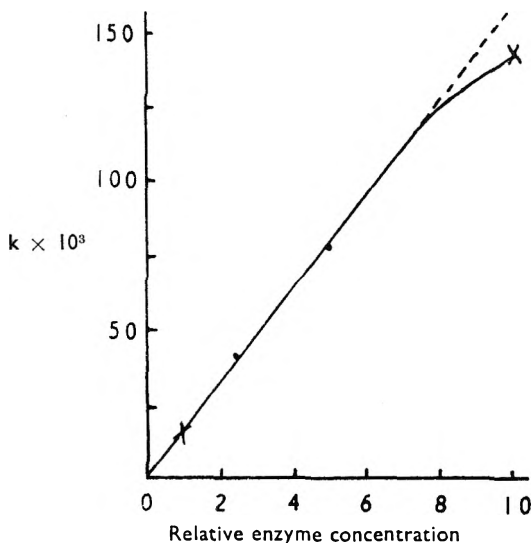


FIG. 3. Hydrolysis of lysophosphatide by lecithinase B obtained from *Penicillium notatum*. Plot of the appropriate constant k against relative concentration of enzyme. The first and last points marked X denote 7 and 53 per cent hydrolysis respectively in a reaction time of 5 min. (With acknowledgements to Fairbairn, 1948.)

for 8 hr. (Levene, Rolf and Simms, 1924). The reaction, whether enzyme or acid catalysed can be followed by titration of the free fatty acid with or without prior isolation or, alternatively, by colorimetric assay (Hanahan and Uziel, 1956) of the unreacted lysolecithin.

Precipitation Reaction

Lysolecithin is precipitated by KI_3 in N HCl at 0°, a property which has been used as a basis of a colorimetric assay; under the same conditions phosphorylcholine and glycerophosphorylcholine are not precipitated (Hayaishi and Kornberg, 1954; Hanahan and Uziel, 1956).

Antioxidative Effects

Inactivation of the enzyme systems of oxidative phosphorylation and thromboplastin by lysophosphatide intermediates has been suggested by

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Habermann (1954). He observed that oxidative phosphorylation in homogenised liver was inhibited by lecithinase A present in bee toxin in dilutions ranging from 3.3×10^{-6} to 6.7×10^{-7} ; in the absence of this enzyme the toxin inhibited only in doses required to cause inflammation (1.3×10^{-4} dilution): the toxins of *Naja nigricollis*, *Naia naia* and *Vipera ammodytes* had a similar effect. Habermann suggests that lipid material plays an essential role between respiration and phosphorylation and the presence of lecithinase A interferes with this mechanism either directly or

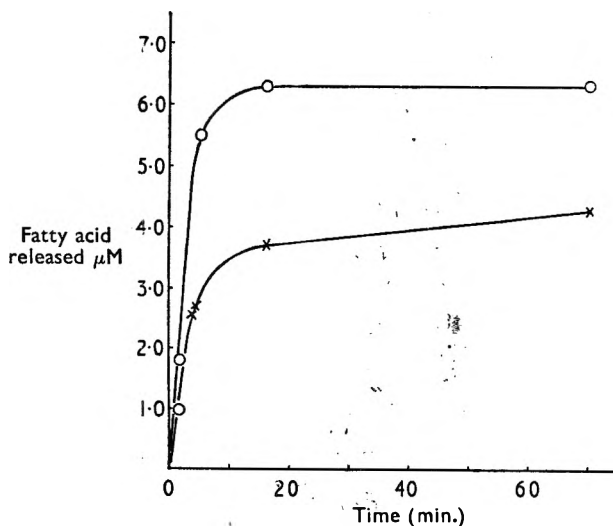


FIG. 4. The rate of hydrolysis of a saturated (×) (β -palmitoyl-L- α -glycerol phosphorylcholine) and unsaturated (○) (β -palmitoleyl-L- α -glycerol phosphorylcholine) lysolecithin using an enzyme extract of the mycelia of *Penicillium notatum*. (With acknowledgements to Uziel and Hanahan, 1956.)

by forming intermediary lysophosphatides; the latter proposition appeared to be the more likely since he was able to show the adverse effect of additions of lysolecithin to the reaction mixture.

PHYSIOLOGICAL ACTIVITY

Haemolysis

Probably the first evidence of the mechanism of snake venom poisoning came from Kyes (1903) and Kyes and Sachs (1903) who compared their "Cobralecithid" with the parent substance lecithin and observed that it had the property of haemolysing red blood cells with the subsequent liberation of haemoglobin.

Flexner and Noguchi (1902) had already shown that blood serum was necessary in the haemolysis of red blood corpuscles by Cobra toxin, the red cells washed free of toxin were incapable of lysis. A year later Kyes and Sachs found that the haemolytic effect of cobra toxin was inactivated by the presence of lecithin without the serum being present.

Manwaring (1910) and Delézenne and Ledebt (1911) subsequently elucidated the mechanism of the toxin by finding a lecithinase present in the

toxins which split the unsaturated fatty acid group from lecithin, resulting in a residue of haemolysing lysolecithin. Later observations of Belfanti (1925, 1928), Guerrini (1925) and Houssay (1930) suggested that the formation of lysolecithin may play a dominant role in the symptomatology of snake venom poisoning. This was supported by symptoms observed when lysolecithin itself was injected into animals and which in some respects resembled those caused by snake venom. Emulsions of heart, liver, kidney and spleen material were then treated with snake venom but there was no evidence of haemolytic activity in these isolated systems probably because of solvent conditions and inhibitors.

From experiments on incubated mammalian blood, Bergenhem and Fahreus (1936) reported that lysolecithin was highly adsorbed on red blood cells and was probably responsible for a retardation of the sedimentation rate, reduction in aggregation and a change in shape of the erythrocytes from the biconcave to the spherical form; the process could be inhibited by quinine in small concentrations whereas sodium arsaniolate was inactive. They actually demonstrated that the haemolysing substance could be isolated in larger quantities from incubated serum than from native serum (since confirmed by Singer, 1941, and Collier and Wilbur, 1944) and suggested that, *in vivo*, lysolecithin could be formed by enzymatic action in the relatively stagnant blood of the spleen whilst the rapid movement of blood in the peripheral circulation would inhibit the formation of lysolecithin. This was again confirmed by Singer (1941) and Collier and Wilbur (1944) who also obtained an increased production of lysolecithin in stagnant blood from the splenic vein and varicose veins; at about the same time Singer (1945) found that splenectomy caused definite alterations in lysolecithin metabolism.

The lysolecithin content of blood has been quantitatively investigated and expressed as the antihaemolytic value (AHV) of the blood (Collier and Wilbur, 1944). Experimentally it is the amount of added lysolecithin, in mg., required to produce 50 per cent haemolysis of 1 ml. of blood under standard conditions. Changes in the concentration of lysins, or of antihaemolytic substances present in the blood, will be reflected in a measure of the AHV; it can otherwise be regarded as an approximate measure of the resistance to lysis by the various lytic substances in the blood.

Collier and Wilbur determined the antihaemolytic value (AHV) of whole blood, erythrocytes and serum by titration and found that the AHV of whole blood or serum decreased markedly on incubation (Fig. 6*a*) p. 342 (see also Bergenhem and Fahreus, 1936), a rise in temperature also accelerated the change with no evidence of a temperature optimum (Fig. 6*b*); the optimum pH was about 7.5 (Fig. 6*c*).

The change in AHV of incubated serum was not markedly affected by agitation, reduction in oxygen tension, or lipase inhibitors. This may have been due to a decrease in free cholesterol content on incubation, or the result of various changes, for example, those following denaturation of serum or plasma proteins, lipids and lipoprotein complexes. These factors controlling the change do not appear to be identical with those

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that affect the sedimentation velocity of Bergenhem and Fahreus. Furthermore, high temperatures accelerated rather than inhibited the change in AHV which suggests a process primarily non-enzymatic. The effect of pH was different from that found by Bergenhem and Fahreus and closer to the effect observed by Hughes (1935) with snake venom on lecithin films.

Gorter and Hermans (1943) found that the haemolytic activity of lysolecithin (containing some lysocephalin) was rapid for the first few minutes and then ceased; the lysolecithin appeared to be adsorbed and was not available for lysing neighbouring cells, suggesting that a certain amount of lysophosphatide haemolysed a fixed number of erythrocytes.

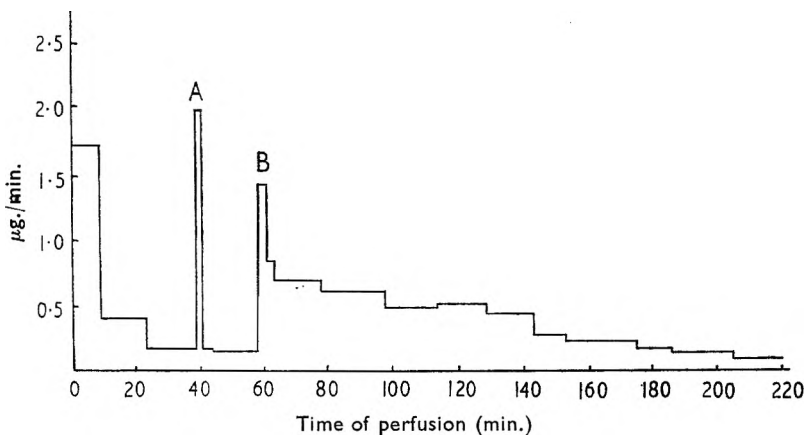


FIG. 5. Release of adrenaline from the perfused left adrenal of a cat weighing 3.3 kg. A, denotes injection of 1 $\mu\text{g.}$ of acetylcholine chloride; at B, injection of 2 $\mu\text{g.}$ of lysolecithin. (With acknowledgements to Feldberg, 1940.)

They estimated that the amount of lysolecithin necessary to haemolyse one cell was equivalent to a unimolecular layer, but some allowances had to be made in this estimate for a possible change in shape of the cell. On this basis the number of erythrocytes haemolysed by 1 mg. of lysolecithin was 0.4×10^9 for rabbit cells, 5.5×10^9 for human red blood cells and $15 \pm 2 \times 10^9$ for sheep cells. Other estimates of the haemolysing activity of lysolecithin have also been made. King and Dolan (1933) estimated that one part of lysolecithin would haemolyse one thousand parts of red cells; Ogawa (1936) found that a 0.002 per cent solution of lysolecithin was capable of haemolysing completely within 30 sec. an equal volume of 2 per cent sheep cell suspension; the lysolecithin retained its activity after 21 hr. provided the pH was less than 7.4, but at pH 8.1 or 9.6 it was strongly inactivated.

Several other workers have reported the haemolytic activity of lysolecithin. Kabashima (1938) found that a dilution of 3×10^{-5} showed lytic action whilst Magistris (1929, 1932) found that lysolecithin prepared from different sources possessed haemolytic activity in dilutions varying from 1 part in 26,400 (egg yolk source) to 1 part in 6,100 (horse brain),

but the lower estimates may have been due to inhibition of the lysing action resulting from the presence of protein and carbohydrate impurities.

A comparison between the haemolytic properties of lysolecithin and saponin described by Wilbur and Collier (1943) showed that the rate of haemolysis was closely related to cell volume. Saponin haemolysis progressively slowed as the cell volume increased whilst lysolecithin haemolysis was accelerated as the initial volume was increased; they concluded that the effects of the two lysins upon the cell membrane was different.

Collier (1952) and also Nygaard, Dianzani and Bahr (1954) observed that the quantity of lysolecithin required to lyse a given number of cells varied with the concentration of the cell suspension. Collier also found that the lysing action was temperature dependent; the rate of haemolysis decreased with lowering of temperature but the extent of haemolysis increased, indicating that more lysin was adsorbed on the cells at lower temperatures. These factors suggested that an equilibrium existed between the lysophosphatide in solution and that adsorbed on the cell; furthermore, the extent of lysing was probably related to the surface area of the cell. Gorter and Hermans (1943) had previously estimated that a monolayer of lysophosphatide on the cell surface would cause complete haemolysis but whilst Collier agreed with this he went further by suggesting that it was secondary to the proposition that lysophosphatide reacted mainly to form a complex with free cholesterol which "neutralised" the lysolecithin within the cell membrane.

The ability of cholesterol to inhibit the haemolysing activity of lysolecithin was probably first recognised long ago by Minz (1908) who thought that the inhibiting effect arose from the molecular fixation of cholesterol by lysolecithin. Shortly afterwards this was confirmed by Delézenne and Fourneau (1914) who found that, although no compound formation actually took place, a complex was formed between cholesterol and lysolecithin and this had no haemolytic activity.

Collier and Chen (1950) observed that the AHV of rabbit plasma was increased by any treatment that caused an increase in plasma-free cholesterol—they actually demonstrated that oral administration of cholesterol to normal or splenectomised rabbits induced hypercholesterolemia and the increase in the AHV was parallel to the increase in free cholesterol in equi-molecular proportions; the normal level of free cholesterol in the rabbit contributed very little to the AHV.

In 1952, Collier observed that a marked contraction in the volume of erythrocytes undergoing lysis took place in the presence of calcium ions, the cations themselves normally having no effect on the cells. This is probably related to the observations of Leathes (1925) who found that calcium salts inhibited myelin formation and also to those on film formation by Alexander, Teorell and Åborg (1939), who suggested that binding of the phosphate groups of cephalin by calcium ions was responsible for the stability of cephalin monolayers at a benzene-water interface. Collier (1952) observed that the presence of lysophosphatide slightly increased the hypotonic fragility of red blood cells but sublytic concentrations of

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lysolecithin, sufficient to cause disc-sphere transformation, decreased fragility to hypotonicity; thiourea penetration was also found to increase substantially in the presence of lysolecithin.

Singer (1940, 1941, 1945) has described the extraction of lysolecithin from serum and the quantitative measurement of its haemolytic power in a study of the fragility of various types of red blood cells. He found no parallelism in the resistance of erythrocytes to lysolecithin and to hypotonic salt solutions, suggesting that the corresponding mechanisms of haemolysis via spherocytosis by these haemolytic agents were different. The comparative haemolytic effect of lysolecithin on human and dog erythrocytes is shown in Table II.

TABLE II
COMPARATIVE HAEMOLYTIC EFFECT ON HUMAN AND DOG ERYTHROCYTES
(Singer, 1940)

Amount of haemolysis per cent	Lysolecithin dilution								
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Human erythrocytes ..	+	+	+	+	-	-	-	-	-
	100	100	100	98	42	0			
Dog erythrocytes	+	+	+	+	+	+	+	-	-
	100	100	100	100	100	100	100	58	0

Haemolysis above 90 per cent is regarded as being complete.

The results show that dog erythrocytes are approximately 8 times less resistant to lysolecithin than human erythrocytes. Singer also found that spherocytes of congenital haemolytic jaundice were less resistant towards lysolecithin than normal erythrocytes; if a change in shape is a precursor to haemolysis this may be accounted for by a simple defect in intrinsic shape (see Ponder, 1948).

Dameshek and Schwartz (1939) considered spherocytosis to be a morphological expression of an alteration in the structure of erythrocytes due to different types of haemolytic substances. Evidence from observations on spherocytes of congenital haemolytic jaundice and various acquired kinds showed a different susceptibility towards lysolecithin which could have been explained by varying alterations in the cell structure caused by different chemical substances. Singer reached the conclusion that the spherocytes, although morphologically similar may be quite different physiologically.

Habermann and Molbert (1954) investigated the morphological differentiation of haemolysis produced by bee venom, lysolecithin, snake venom and digitonin by the optical and the electron microscopes. They found that the bee venom caused a contraction and net-like pattern at the cell wall of the shadow of human and frog erythrocytes and stromatolysis also occurred; the effect of snake venom was similar to bee venom except that very little contraction of the membrane was observed and digitonin did not cause stromatolysis. (The lecithinase A activity found in bee venom has been extensively studied by Habermann and Neumann, 1954). Lysolecithin dissolved the shadows and caused contractions, stromatolysis and instability of the nucleus of the frog erythrocytes.

Some excellent electron microscope studies obtained by Nygaard, Dianzani and Bahr (1954) showed the effect of lecithinase A and lysophosphatides on the morphology of isolated mitochondria from rat liver. Lecithinase A caused a characteristic disruption and dislocation of the membrane whilst lysolecithin caused disintegration of the mitochondria and inactivation of the succinoxidase enzyme systems; lecithinase C and trypsin did not have these effects.

Conflicting accounts have been given on the lysing action of lysocephalin. Dunn (1934) found that lysocephalin prepared by the method of Levene and Rolf (1923) produced complete haemolysis of washed beef

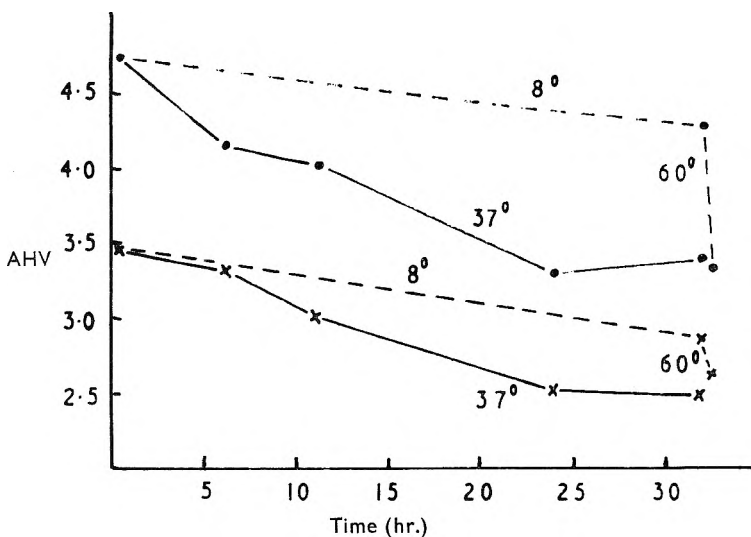


FIG. 6a. Change in the antihæmolytic value on incubation of human serum from two individuals. (The number of mg. of added lysolecithin which will bring about 50 per cent haemolysis of 1 ml. of blood or its equivalent, in 60 sec. at room temperature is called the antihæmolytic value AHV.) Broken lines are control samples held at 8° with final heating at 60° for 30 min. Note the close approach to the test samples on heating to 60°. (With acknowledgements to Collier and Wilbur, 1944.)

erythrocytes in one hour at a dilution of 1:20,000, whilst Magistris (1929) was unable to find any hæmolytic activity with lysocephalin. The problem should be re-investigated using pure specimens of lysocephalins obtained by the now available improved techniques of purification.

OTHER PHYSIOLOGICAL AND PHARMACOLOGICAL ACTIONS OF LYSOLECITHIN

The action of lysolecithin on the adrenal medulla has been investigated by Feldberg (1940). He set out to determine whether adrenaline was released and brought into circulation by the lytic action on medullary cells when lysolecithin was either injected arterially into the adrenals or formed from their lipids following an arterial injection of venom. If adrenaline release was the direct outcome of the lytic action of lysolecithin

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it could be demonstrated on a cell suspension from the adrenals *in vitro*. On the other hand, the adrenaline release may have followed the intermediate liberation of histamine acting as a secretory stimulus; since histamine causes adrenaline release in cats but not in rabbits, the effect of lysolecithin, if acting in this way could be examined. Feldberg found that injection of lysolecithin into the central stump of the coelic artery after evisceration caused a long lasting output of adrenaline from the adrenals of cats. If an isolated cat adrenal had been perfused with Locke solution, the adrenal release often lasted for over two hours. This was regarded as a response of the medullary cells to injury, contrasting with the rapid effect produced by acetylcholine or by nerve stimulation;

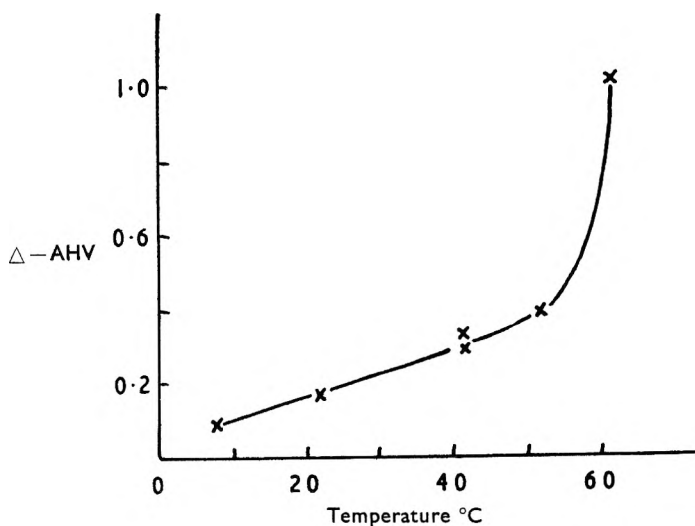


FIG. 6b. Effect of temperature on the change in AHV of rabbit serum. Δ — represents a decrease in AHV on incubation. (With acknowledgements to Collier and Wilbur, 1944.)

the difference in response to acetylcholine and lysolecithin is shown in Fig. 5, p. 339. He also found that adrenaline was released from a suspension of cellular material from the cat adrenal *in vivo* but little or no secretory action on the adrenal medulla of rabbit appeared to take place.

In 1938, Feldberg and Kellaway reported that snake venoms released histamine from the perfused lungs of guinea-pigs and cats and the amount of histamine released increased with the quantity of snake venom injected until the lung became almost depleted of its histamine. Subsequently Kellaway (1939) suggested that the part played by lysolecithin in snake venom haemolysis was parallel to its action in liberating histamine.

The pharmacological actions of alcoholic extracts of envenomed monkey's liver and lecithin treated with cobra venom were also investigated by Feldberg and Kellaway (1938). They found that the strongly haemolytic venom-free extracts caused the appearance of histamine, protein and other substances which brought about a slow delayed contraction of the

guinea-pig's jejunum and characteristic after-changes in its reactivity to histamine and to acetylcholine. On the other hand the purified lysolecithin preparation lacked the stimulant action on the guinea-pig's jejunum. When injected intravenously into guinea-pigs, lysolecithin caused symptoms resembling acute anaphylactic shock with the addition of haemorrhagic oedema of the lungs. Injected into the interior chamber of the rabbit's eye, lysolecithin brought about opacity of the cornea and irregular alterations of its curvature. Feldberg, Holden and Kellaway (1938) concluded from further experiments that the symptoms produced by venom could not be attributed to lysolecithin alone and suggested that a second substance must be present.

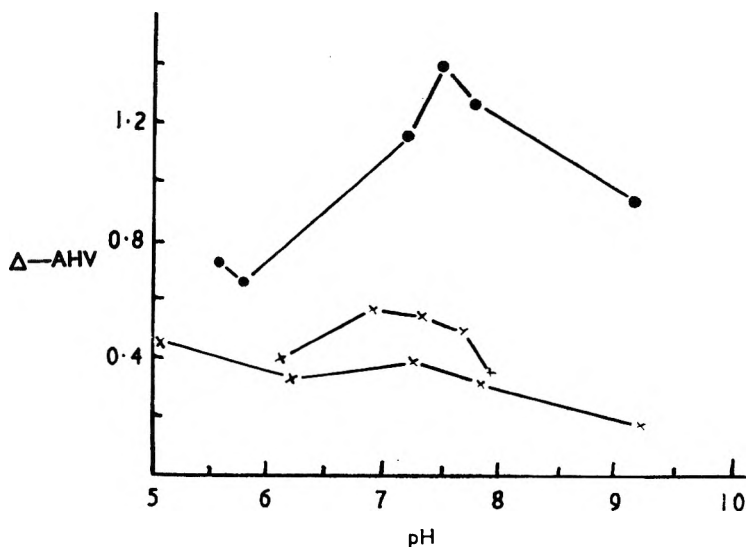


FIG. 6c. Effect of pH on change in antihaemolytic value of incubated serum. Δ —represents the decrease in AHV on incubation. \circ , human serum \times , rabbit serum. (With acknowledgements to Collier and Wilbur, 1944.)

Kellaway and Trethewie (1940) have shown that lysolecithin releases adenylic compounds from perfused hearts. Gautrelet, Corteggiani and Carayon-Gentil (1941) have examined the release of acetylcholine from a suspension of cellular material of guinea-pig's brain by lysolecithin. By treating the brain with lecithinase A free from proteinase activity, acetylcholine was released (together with lysolecithin and a fatty acid) indicating that some of the brain phosphatides and acetylcholine are present as a complex.

The amount of lysolecithin present in various types and conditions of blood has been investigated. Singer (1940, 1941, 1945) developed a method of extraction and quantitative determination of lysolecithin in blood and carried out experiments to determine some of the conditions that affect the lysolecithin content in blood. He found that the quantity of lysolecithin can be increased from two to eight times by incubating

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stagnant blood for several hours, but movement during the incubation period prevented any further increase in lysolecithin production, probably due to degradation of the lysing substance. This led to the possibility that circulating blood may contain less lysing material than stagnant blood.

If this was so Singer suggested that the relationship could be expressed by a factor called the lysolecithin quotient (LLQ) to relate the circulation of the blood to lysin production. The factor was defined as the ratio:

$$\text{LLQ} = \frac{\text{lysolecithin content of unincubated serum}}{\text{lysolecithin content of incubated serum}}$$

He found that normal peripheral blood consistently had a lysolecithin quotient of less than unity, usually one-half to one-eighth) and could be differentiated from relatively stagnant blood. For example, the reservoir blood in the spleen, temporarily isolated from circulation, rather like incubated stationary blood, had an LLQ of 1. LLQ's were obtained from several sources: dog splenic venous blood, 1; dog splenic artery, 1; varicose vein blood, 1, and hepatic venous blood, 1. In a study of haemolytic anaemia Singer found no difference in the blood of patients with congenital haemolytic anaemia from that of normal patients.

Rocha e Silva and Beraldo (1948) studied the inhibitory effects of lysolecithin upon the action of histamine, acetylcholine, and potassium chloride on the guinea-pig gut. They found that the effects depended on the time of contact of lysolecithin with the muscle; the inhibitory effect was not specific and appeared to be slightly greater towards acetylcholine than histamine. The rate of recovery of the gut from inhibition was independent of the number of additions of the spasmogenic agent—even large doses of histamine did not alter this rate of recovery much. If the recovery to lysolecithin was allowed to proceed at two different temperatures, 37° and 27°, the time of recovery changed much; 50 per cent recovery took place in 506 sec. at the higher temperature whilst it took 930 sec. at the lower temperature, suggesting that a chemical process is involved in recovery. Similar results were obtained with synthetic antihistamine drugs, indicating that recovery from inhibition follows the same law. These facts constituted a strong indication that recovery depends solely upon the intimate potentialities of the muscle and not simply from the dissociation of a complex formed between lysolecithin (or synthetic antagonist) and cell receptors.

Increasing interest is being shown in the important effects of lysolecithin and snake venoms on the nervous system (see McArdle, Thompson and Webster, 1960).

Many of the neurotoxic venoms contain an active lecithinase A component which, when freed from other active enzymes by heat treatment and introduced into the spinal cord of rats, has caused paralysis (Sanders, Akin and Soret, 1954; Quastel, 1958).

In 1959, Petrushka, Quastel and Scholefield showed that slices of rat-brain cortex exposed to cobra venom lecithinase A consumed oxygen for approximately one hour and then became inhibited to any further oxygen uptake. Although several workers have shown that lysolecithin

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and lecithinase A also depress the rate of oxygen uptake and of oxidative phosphorylation in isolated liver mitochondria, Petrushka found that slices of liver and kidney were not affected in this way.

TABLE III
SOME PHYSIOLOGICAL ACTIONS OF SNAKE VENOM POISONING AND LYSOLECITHIN RELEASE

Substrate	Effect
Intravenous injection in rabbit (Iwata, 1934)	A syndrome occurs; fall in blood pressure. Lethal dose 1 mg./kg.
White rats (Iwata, 1934)	Infiltration and parenchymatous degeneration of liver and kidney; relationship of effect with vitamin B content of diet.
Cats and dogs (Feldberg and Kellaway, 1937)	Rapid fall in systemic blood pressure. In cats a lethal dose causes fluid loss from circulation peripheral vasodilatation, haemorrhagic oedema of lungs, heart failure
Guinea-pig (Arthus, 1912; 1913; Delézenne and Fourneau, 1914)	Symptoms resembling anaphylactic shock, haemorrhagic oedema of lungs
Subcutaneous injection	Oedema, necrosis
Heart of cat (Delézenne and Fourneau, 1914)	Changes in coronary circulation; reduction in force of beat; rapid heart failure; beating ceases in diastole
Isolated striated muscle (Houssay 1930; Houssay, Negrete and Mazzocco, 1922)	Contraction, fibrillation; increase in lactic acid content; loss of K ⁺ and phosphates; inexcitability
Perfused dog liver (Feldberg and Kellaway, 1937, 1938)	Output of protein, histamine, pigments from fluid from hepatic veins
Liver mitochondrial system of rat and guinea-pig (Rodbell and Hanahan, 1955)	Stimulation of oxygen consumption. Above 1 μ mole complete inhibition of oxygen uptake. Changes in morphology
Aqueous homogenates of brain, liver, kidney, muscle of rat (Webster, 1957; Habermann, 1954a)	Marked clearing action; maximum at 0.007-0.008M lysolecithin
Uterus, jejunum of guinea-pig (Feldberg and Kellaway, 1938)	Contraction of jejunum
Enzyme systems (Habermann, 1954, 1954a; Nygaard, Dianzani and Bahr, 1954)	Effect on oxidative phosphorylation, succinoxidase system, tissue thromboplastin, probably by a structural disintegration
Adrenal medulla cat (Feldberg, 1940)	Output of adrenaline
Adrenal medulla of rabbit (Feldberg, 1940)	No effects
Plasma of rabbit (Feldberg and Kellaway, 1938)	Retards clotting
Eye of rabbit (Feldberg and Kellaway, 1938)	Opacity of cornea, irregular alterations in its curvature
Rat brain cortex (Petrushka, Quastel and Scholefield, 1959)	Inhibition of oxygen uptake after 1 hr.
Spinal cord of rat (Sanders, Akin and Soret, 1954; Quastel, 1958)	Paralysis
Whole-cell preparations of rat brain and liver (McArdle, Thompson and Webster, 1960)	Release of glutamine-oxaloacetic transaminase from both tissues. Some cholinesterase released from brain

The importance of these findings led McArdle, Thompson and Webster to investigate the action of lysolecithin and snake venom lecithinase A on whole-cell preparations of rat-brain and liver in order to discover whether there were any differences in the lytic action on their tissue preparations as distinct from mitochondrial membranes. They found

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that both lysolecithin and lecithinase A caused a striking release of glutamic-oxaloacetic transaminase from each of these tissues, a somewhat smaller but nonetheless significant release of cholinesterase also took place from the rat-brain slices. In agreement with Quastel (1958) they observed that respiration of the rat-brain slices was more sensitive to inhibition than liver slices. Oxygen consumption was more rapidly inhibited by the venom lecithinase A.

Whilst McArdle, Thompson and Webster were unable to suggest a mechanism for the extensive release of glutamic-oxaloacetic transaminase on existing evidence, they suggested that measurement of the release of intracellular enzymes such as glutamic-oxaloacetic transaminase from brain, muscle and liver cells could be used for the detection and semi-quantitative estimation of the presence of substances capable of exerting a lytic action on these tissues. This important field of work is still in progress and results are therefore awaited with interest.

A summary of some of the physiological actions of snake venom poisoning and lysolecithin release is given in Table III.

PHYSICAL PROPERTIES

General

The physical constants and properties of lysolecithin (the β -acyl compound) reported by the earlier workers Levene, Rolf and Simms (1924) are in close agreement with values obtained more recently; in the light of the present day advances in techniques of isolation and purification their work is most commendable. They found that lysolecithin can be crystallised from hot pyridine into aggregates of fine needles and when thoroughly dry formed a pure white solid. Subsequent investigations have shown that it is readily soluble in chloroform, glacial acetic acid, pyridine and methanol and ethanol but insoluble in ether, light petroleum and acetone; the solubility of lysolecithin in several solvents has been reported by Robinson and Saunders (1958). Lysolecithin is very hygroscopic and dissolves in warm water eventually forming a viscous fluid which is optically quite clear. Levene, Rolf and Simms found that lysolecithin softened slightly on heating to 100° and decomposed at 263° (Hanahan, 1954, found that his material melted at 195 – 196°). They observed its optical activity in several solvents; chloroform $[\alpha]_D^{20} = -2.6^\circ$ (Hanahan gave -2.2°), pyridine $[\alpha]_D^{25} = +1.2^\circ$ and glacial acetic acid $[\alpha]_D^{25} = +0.8^\circ$. Robinson and Saunders (1958) found its optical activity in ethanol to be $[\alpha]_D^{20} = +2.6^\circ$. Levene and others determined the dissociation constants for lysolecithin and gave $K_1 = 0.18$ and $K_2 = 1.26 \times 10^{-12}$ with an isoelectric range between pH 2.75 and 9.90 and gave an intermediate value of pH 6.3 as the isoelectric point; this value agrees fairly closely with the accurate determination of the corresponding value for lecithin (6.7) by Chain and Kemp in 1934.

Surface Films

Hughes (1935) claims to have formed liquid-expanded type unimolecular films of lysolecithin at an air-water interface. These films possessed a surface area/mol. of 108\AA^2 (lecithin 116\AA^2) which could be compressed

to 65.5\AA^2 (lecithin 73\AA^2), the surface area per fatty acid chain indicating that lysolecithin formed a much more distended film than lecithin. The electric moment per molecule (vertical component) decreased from 4.3 to 4.1×10^{-19} e.s.u. (lecithin 8.8 to 7.1×10^{-19} e.s.u.) and was approximately half that for lecithin, possibly due to the removal of the fatty acid chain and the ester linkage; surface potential measurements also showed that lysolecithin ionises at a lower pH than lecithin, indicating that lysolecithin acts as a stronger acid. The lysolecithin film was oxidised very slowly by permanganate indicating that the fatty acid chain was saturated. Lysolecithin is completely soluble in water and it is surprising that Hughes was able to obtain significant surface potential and surface area measurements of the films. It is possible that the lysolecithin used by Hughes was contaminated by a considerable amount of lecithin.

The surface areas of soluble films of molecules of α - and β -lysolecithin calculated from surface tension measurements are discussed under the heading "Surface Activity".

Surface Activity

Robinson and Saunders (1958-60) have studied the surface-active properties of lysolecithin in some detail. The two distinctly different hydrophilic (phosphorylcholine) and lipophilic (long chain fatty acid) regions in the molecule would be expected to endow lysolecithin with strong surface-active properties. The high surface activity of lysolecithin in water was shown to be comparable with typical soaps. Lysolecithin has an isoelectric point close to pure water and in this solvent it behaves rather like a non-ionic surface-active substance and the lowering of the surface tension of water by the phospholipid is almost unaffected by the presence of electrolytes; this stability shown by lysolecithin sols has been confirmed by experiments on coagulation of the sols by electrolytes. Several methods have been used to obtain a critical micelle concentration of lysolecithin in water but all that can definitely be said is that it is very low (less than 10^{-2} per cent w/v) (Robinson and Saunders, 1958, 1958a, b).

A comparison between the surface activities of α - and β -acyl-lysolecithins has been reported by Robinson and Saunders (1958a). The distance between the hydrophilic and lipophilic regions in the α -acyl-lysolecithin is probably greater than in the β -acyl compound and hence the amphipathic character was expected to be more pronounced in the former. On the other hand, unsaturated linkages in the fatty acid radical of the α -compound (2.2 double bonds per molecule) gave the molecule an increased affinity for water which was likely to lessen its surface active properties. The surface activities of the two compounds were similar, both lowering the surface tension of water to 44 dyne/cm. or less at the very low concentration of 10^{-2} per cent w/v whilst their surface areas at zero surface pressure (calculated from the approximate adsorption isotherm) were 101\AA^2 and 90\AA^2 per molecule of the α - and β -compounds respectively (Fig. 7).

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A light scattering study of the size of the micelles by Robinson and Saunders (1959b) gave a micellar weight of nearly 100,000. For a monomer molecular weight of 515, the number of molecules per micelle was approximately 190; this value was somewhat lower than 273 monomers per micelle obtained from diffusion studies by Thomas and Saunders (1958). This discrepancy was possibly in part due to the difficulty in obtaining an accurate value for the density of lysolecithin (owing to

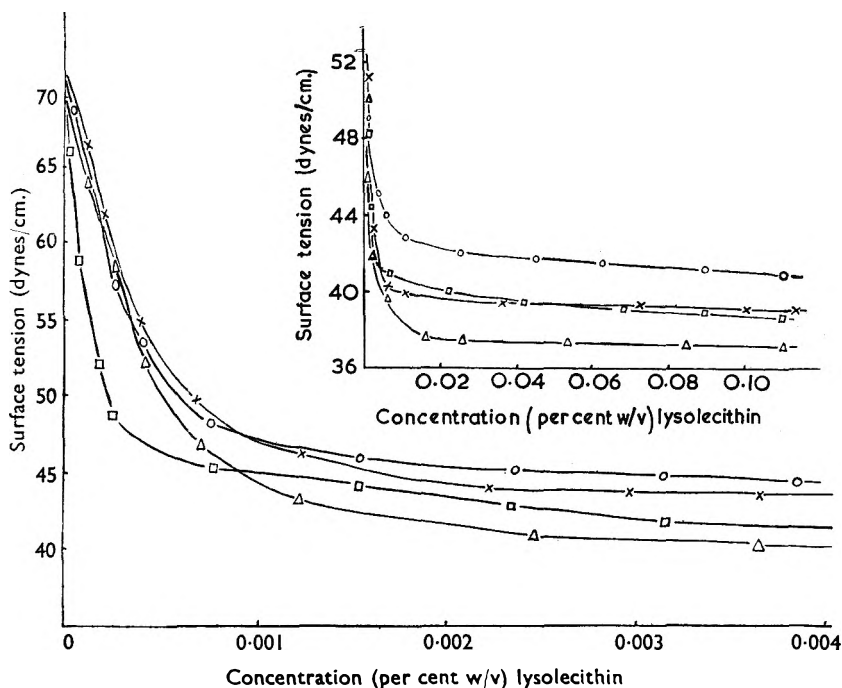


FIG. 7. Effect of α - and β -(acyl)lysolecithins on the surface tension of water \times , α -Lysolecithin at 25°; \circ , β -lysolecithin at 25°; \triangle , α -lysolecithin at 40°; \square , β -lysolecithin at 40°.

its hygroscopic character) which is used in the diffusion calculations. In diffusion, the rate is measured and the possibility of entraining water in the process must be considered. Assuming the discrepancy to be due to entrainment of water, the difference in the experimental values indicate that each lysolecithin micelle entrains approximately 2,370 molecules of water.

Solubilising Effects

The solubilising effects of lysolecithin have been studied under various conditions. Earlier investigations by Rousseau and Pascal (1938) showed that lysolecithin had a solvent effect on certain portions of the streptococcus cell whilst Hasegawa and Nakamoto (1939) observed that it dissolved live but not dead pneumococci. More recently it has been emphasised by Habermann and Neumann (1957) that if lysolecithin (or

the enzyme lecithinase A producing it) is present in the body, it may be very important physiologically in relation to permeability or solubilising processes of all kinds. In a study of the change in permeability of red cells brought about by animal poisons, Habermann (1955) found that melittin was more effective than lysolecithin and digitonin in reducing the erythrocyte membrane permeability to potassium ions and to haemoglobin. He also found that the permeability of the interstitium was increased by hyaluronidase whereas lysolecithin, phospholipase A and melittin did not contribute to this effect. The ability of lysolecithin to solubilise homogenates of whole fresh rat brain was investigated by Webster (1957); nearly all the brain tissue was rendered soluble by 0.007–0.008M lysolecithin with three hours (Fig. 8). He observed that 90 per cent of the clearing action of the whole rat brain was effected by a limiting amount of lysolecithin within a given time. This is in close agreement

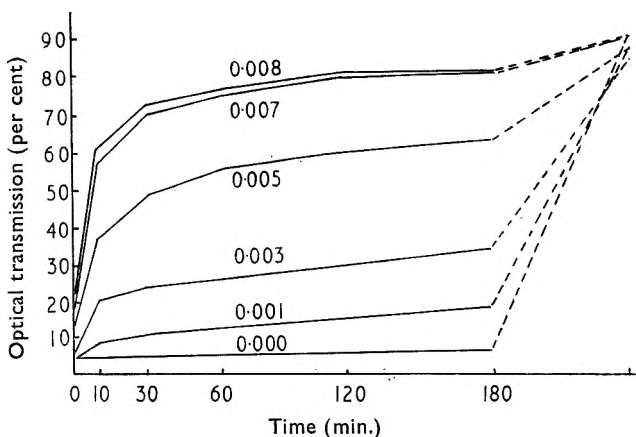


FIG. 8. Effect of different concentrations of lysolecithin on the rate and degree of clearing of fresh rat brain homogenates in 0.025 M sodium carbonate, pH 8.3. (With acknowledgements to Webster, 1957.)

with the time reported for the haemolysis of a fixed number of erythrocytes by a given amount of lysolecithin (Gorter and Hermans 1943). The haemolysing action is known to be dependent on the cholesterol content of the cells and Webster's work should lead to a further consideration of the relation between the haemolysing activity of lysolecithin and its solubilising power on cell contents. Webster points out that the clearing action may be used in the study of substrates and enzyme inhibitors normally inaccessible owing to the presence of organised lipid-rich myelin sheaths. In this respect he suggests that abnormal production of lysophosphatide material may disrupt and dislocate myelin formation, a factor contributory to the pathogenesis of demyelinating disease.

The property of lysolecithin to solubilise in heterogeneous systems, however, might be very different from its action on pure components in these systems when possible effects of other lipid material assisting the

action are absent. Robinson and Saunders (1959a) studied the solubilisation of cholesterol, triolein and monostearin, each representative of the different extents of chemical reactivity of lipids likely to be found in biological systems. The solubilising action in water of lysolecithin on these lipids was large, the amounts solubilised increasing in the order triolein, cholesterol and monostearin. The lysolecithin-triolein and lysolecithin-cholesterol sols were fairly unstable, but lysolecithin-monostearin sols having a mol ratio of 2:1 formed gels which were stable for a month. Lysolecithin can be regarded as a derivative of a monoglyceride (probably closest to β -monopalmitin) the only essential difference between the two molecules being a phosphate-choline group conferring complete water solubility on lysolecithin. The rigidity of the lysolecithin-monostearin sols was therefore attributed to structural similarities bringing about maximum van der Waal's forces and dipole interactions; such stability effects could contribute to the structural role of phosphatides in the cell membrane.

Viscosity of Sols

In some preliminary experiments on mixed lysolecithin-lecithin sols Saunders (1957) found that, under certain conditions, the viscosity of these sols was very high. This observation led to further work in this field in an attempt to support his suggestion that this viscous phosphatide system could be the structural foundation of a cell membrane.

Robinson and Saunders (1959) reported that the optically clear sols formed by lysolecithin possessed a viscosity similar to that of water, increasing approximately linearly with increasing concentration. The viscosity, however, varied with the viscometer used, indicating that the sols exhibited non-Newtonian flow. They observed that the viscosity of the sols increased in alkaline conditions when distinct ageing effects became apparent and these were found to be irreversible on neutralisation of the sols.

A study of the behaviour of mixed lysolecithin-lecithin sols has shown some interesting results.

Thomas and Saunders (1958a) found that introduction of lysolecithin into a lecithin sol caused a big increase in viscosity and at its maximum the sols became very thick liquids. The viscosity then decreased to a constant relative viscosity of 3.0 after 24 hr.; considerable interaction had clearly taken place. Their results indicated that a marked change in shape of the lysolecithin-lecithin complex took place between a weight fraction of lecithin of 0.4 and 0.6 total phosphatide from spherical to probably disc-shape. Robinson and Saunders (1959b) found that light scattering results gave a symmetrical micellar shape for lysolecithin itself but the aggregates formed by the parent substance lecithin were disc-shaped (Robinson 1960). This is in fair agreement with the viscosity results if one supports the view that, in the mixed phosphatide sols where the aggregates have a spherical shape, sufficient lysolecithin is present to solubilise the lecithin within the micelle: increasing the lecithin weight fraction to 0.6 gives this component a dominating influence over shape

and distorts the mixed aggregate in favour of the disc-like model shown by lecithin micelles.

The interaction of lysolecithin with lecithin and other lipid material to form highly viscous sols substantiates the theory for cell membrane formation proposed by Saunders (1957). In this proposition he states that since lysolecithin is an enzymatic breakdown product of lecithin these substances would be expected to be widely distributed, with lecithinase, in living systems. If sufficient lysolecithin is present in the internal fluid of a cell it can stabilise the lecithin against coagulation by the presence of monovalent metal salts, but can still permit precipitation of a phosphatide membrane when this fluid meets a solution containing divalent metal ions such as in plasma. The lysolecithin content would not be lytic at the weight fraction necessary to give precipitation and so the membrane would be stable. Other components present in fluids will also play a part but this phosphatide balance in the cell contents is the important factor in the primary formation of the cell membrane which is subsequently strengthened by adsorption of proteins and modified by other lipids.

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RESEARCH PAPERS

16 α ,17 α -ISOPROPYLIDENEDIOXY-6 α -METHYLPREGNA-1,4-DIENE-3,20-DIONE (B.D.H. 3144). A NEW ANTI-INFLAMMATORY AGENT

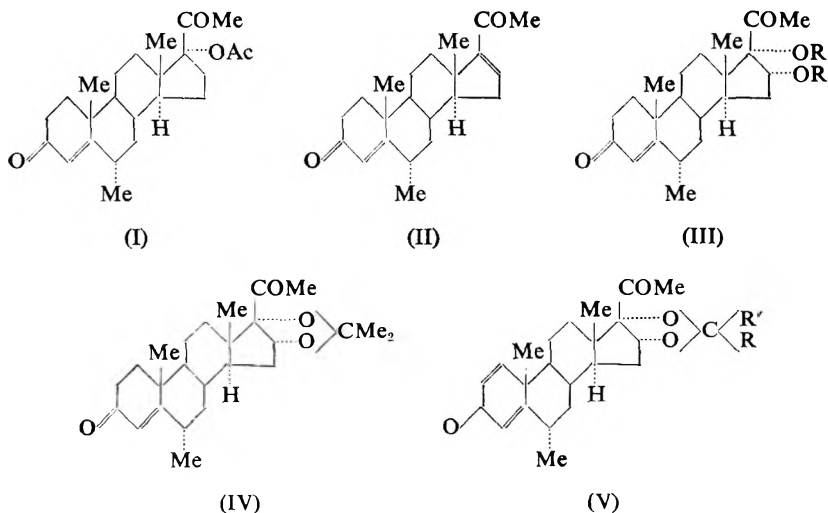
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The preparation and anti-inflammatory properties of the title compound and of some related structures are reported. B.D.H. 3144 (16 α ,17 α -Isopropylidenedioxy-6 α -methylpregna-1,4-diene-3,20-dione) is found to be a potent anti-inflammatory agent which approaches prednisolone in activity.

AFTER the preparation by Barton, Ellis and Petrow (1959) of 17 α -acetoxy-6 α -methylpregn-4-ene-3,20-dione (I), a highly potent progestational agent, we turned to the partial synthesis of the related 16 α ,17 α -diacetoxy-6 α -methylpregn-4-ene-3,20-dione (III; R = Ac). To this end 16 α ,17 α -dihydroxy-6 α -methylpregn-4-ene-3,20-dione (III; R = H) was prepared as described below. Its conversion into the diacetate (III; R = Ac), however, offered initial difficulty. In the meantime the parent diol (III; R = H) had been converted into the acetonide (IV) in excellent yield.



Biological study of the last compound confirmed its anticipated progestational potency, but also revealed a quite unexpected and substantial anti-inflammatory activity. In view of this significant result, (IV) was converted into its Δ^1 derivative (V; R = R' = Me; B.D.H. 3144), which forms the main subject of the present communication.

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Cooley, Ellis, Hartley and Petrow (1955) had shown previously that oxidation of 3 β -acetoxypregna-5,16-dien-20-one with potassium permanganate under controlled experimental conditions leads to the formation of the corresponding 16 α ,17 α -dihydroxy derivative in moderate yield. Attempts to apply this reaction to the readily available 6-methyl derivative of 3 β -hydroxypregna-5,16-dien-20-one, (Burn, Ellis, Petrow, Stuart-Webb and Williamson, 1957), however, proved uniformly unsuccessful owing to concomitant attack upon the C(5)-C(6) ditertiary unsaturated linkage. This difficulty was circumvented by using 6 α -methylpregna-4,16-diene-3,20-dione (Burn and others, 1957) (II) as starting material when the required 16 α ,17 α -dihydroxy-6 α -methylpregn-4-ene-3,20-dione (III; R = H)

TABLE I

THE ANTI-INFLAMMATORY ACTIVITY OF B.D.H. 3144, CORTISONE, HYDROCORTISONE, PREDNISONE, AND PREDNISOLONE

Compound	Total dose mg./rat	No. of implants	Route	Granuloma	
				Mean wt. mg.	Per cent reduction
B.D.H. 3144	0.625	17	Subcut.	125	17
	1.25	20	..	118	22
	2.5	20	..	105	31
	5.0	20	..	103	32
Hydrocortisone	5.0	17	..	139	8
Controls	—	20	—	154	—
Cortisone acetate	10	20	Subcut.	98	38
Hydrocortisone	7.5	20	..	106	32
Prednisolone acetate	5.0	19	..	52	67
Controls	—	20	—	157	—
B.D.H. 3144	2.5	20	Oral.	105	36
	10.0	20	..	84	49
Hydrocortisone	2.5	20	..	116	29
Prednisone	2.5	20	..	75	54
Prednisolone acetate	2.5	19	..	66	60
Controls	—	20	—	163	—

was obtained, albeit in only 25 per cent yield. The last compound condensed readily and nearly quantitatively with acetone in the presence of perchloric acid to give 16 α ,17 α -isopropylidenedioxy-6 α -methylpregn-4-ene-3,20-dione (IV). Conversion into its Δ^1 derivative (V; R = R' = Me) was achieved using 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (Burn, Kirk and Petrow, 1960), such oxidants as selenium dioxide having proved unsatisfactory.

The 2-methyl derivative of (V; R = R' = Me) was prepared by treating 16 α ,17 α -isopropylidenedioxy-6 α -methylpregn-4-ene-3,20-dione (IV) with diethyl oxalate in the presence of sodium hydride to give the sodio-salt of the 2-ethoxalyl derivative. Methylation with methyl iodide, followed by alkaline hydrolysis furnished 16 α ,17 α -isopropylidenedioxy-2 α ,6 α -dimethylpregn-4-ene-3,20-dione, which was converted into the required Δ^1 derivative by reaction with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone. The remaining congeners of (IV) and (V) included in the present biological study were prepared by an alternative route, which will be reported elsewhere.

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Anti-inflammatory Assay

The anti-inflammatory activity was estimated by a modification of the methods described by Meier, Schuler and Desaulles (1950), Cresseri and Meli (1953) and Christian and Williamson (1958). An agar pellet containing 5 per cent turpentine and measuring approximately 0.5 cm. × 1.0 cm. and weighing 220.2 ± 2.5 mg. was implanted subcutaneously under the dorsal skin on either side of the vertebral spine in young albino rats weighing between 70 and 90 g. The implantation was made under ether anaesthesia through a small medial incision. Immediately after the implantation the animals were given the steroid in an aqueous suspending medium either subcutaneously or orally twice daily for 5 days. Twenty-four hr. after the last injection the animals were killed, the granuloma capsule freed from the implant and weighed wet on a torsion balance.

TABLE II
ANTI-INFLAMMATORY ACTIVITY OF STEROIDS ADMINISTERED SUBCUTANEOUSLY

Steroid	Activity
1. 6 α -Methylpregn-4-ene-3,20-dione	none
2. 17 α -Hydroxy-6 α -methylpregna-1,4-diene-3,20-dione	none
3. 16 α ,17 α -Dihydroxy-6 α -methylpregn-4-ene-3,20-dione	none
4. 16 α ,17 α -Furfurylidenedioxy-6 α -methylpregn-4-ene-3,20-dione	none?
5. 6 α -Methylpregna-1,4-diene-3,20-dione	+
6. 16 α ,17 α -Isopropylidenedioxy-2,6 α -dimethylpregna-1,4-diene-3,20-dione	++ (+)
7. 16 α ,17 α - <i>p</i> -Fluorobenzylidenedioxy-6 α -methylpregn-4-ene-3,20-dione	+++
8. 16 α ,17 α -Isopropylidenedioxy-6-methylpregna-1,4,6-triene-3,20-dione	+++ (+)
9. 17 α ,21-Dihydroxypregn-4-ene-3,11,20-trione	+++
10. 16 α ,17 α -Ethylidenedioxy-6 α -methylpregna-1,4-diene-3,20-dione	+++
11. 6 α -Methyl-16 α ,17 α -(1-methylpropylidenedioxy)pregna-1,4-diene-3,20-dione	+++
12. 16 α ,17 α -Isopropylidenedioxy-6 α -methylpregn-4-ene-3,20-dione	+++ (+)
13. 16 α ,17 α -Isopropylidenedioxy-6 α -methylpregna-1,4-diene-3,20-dione (B.D.H. 3144)	++++
14. 11 β ,17 α ,21-Trihydroxypregna-1,4-diene-3,20-dione	++++ (+)

The control animals were given the vehicle only. For comparative purposes cortisone, hydrocortisone, prednisone and prednisolone were included in the studies. The per cent reduction in the weight of the granuloma capsule represents a measure of the anti-inflammatory activity. The results are recorded in Tables I and II.

DISCUSSION

6 α -Methylpregn-4-ene-3,20-dione (No. 1*) is one of the simplest structures to show anti-inflammatory activity, which may be demonstrated by direct injection of the steroid into the granuloma pouch (Glenn, Richardson and Bowman, 1959). It is inactive in our assay. The introduction of an unsaturated bond in the 1,2 position into 6 α -methylpregn-4-ene-3,20-dione, producing 6 α -methylpregna-1,4-diene-3,20-dione (No. 5), however is accompanied by the appearance of slight anti-inflammatory potency, which is destroyed by the introduction of an 17 α -hydroxyl group (No. 2). 16 α ,17 α -Dihydroxy-6 α -methylpregn-4-ene-3,20-dione (No. 3), the parent compound of the present series, is likewise without activity in the present assay. Conversion of the 16 α and 17 α hydroxyl groups into the acetonide group results in the production of a highly potent compound (No. 12),

* The numbers refer to Table II.

which is approximately twice as active as cortisone. A similar potentiation of anti-inflammatory activity through acetonide formation has been observed in the triamcinolone series (Bernstein, Heller, Littell, Stolar, Lenhard, Allen and Ringler, 1959). A further increase in anti-inflammatory activity is produced by introduction of an unsaturated bond in the 1,2 position into No. 12 to give B.D.H. 3144, which now approaches prednisolone in potency. Attempts to increase activity still further by methylation at the 2 position (No. 6), by increase of conjugation (No. 8), or by modification of the isopropylidene moiety (Nos. 4, 7, 10 and 11) resulted in all cases in loss of potency.

B.D.H. 3144, in common with other anti-inflammatory steroids, also possesses thymolytic activity and causes a loss in body and adrenal weights. This latter action, however, can be prevented by the simultaneous administration of ACTH. It has glucocorticoid activity as shown in the liver glycogen deposition test and it prolongs the survival time in adrenalectomised rats. It has some anti-anaphylactic, progestational and claudogenic activity (cf. Petrow, 1960), but no apparent androgenic, oestrogenic or mineralocorticoid properties. It is hoped to report these results in detail at a later date.

B.D.H. 3144 has been tested for topical activity in human volunteers using the patch test of Schlagel and Northam (1959). In this assay the irritant property of tetrahydrofurfuryl alcohol, when applied to the skin of the forearm, is balanced against the anti-inflammatory activity of the steroid which is itself dissolved in the irritant material. The lowest concentration of steroid required to prevent erythema of the skin completely, then furnishes a measure of its potency. B.D.H. 3144 proved equal to hydrocortisone in this assay, both compounds being tested at 1 per cent concentration.

The marked potency of B.D.H. 3144 on topical application shows that its anti-inflammatory activity is an inherent property of its unique structure and not the property of a metabolic (and more highly oxygenated) product formed from it *in vivo*. It differs markedly from current anti-inflammatory steroids, in the absence of an 11 β -hydroxyl group [c.f. prednisolone (No. 14)] which has hitherto been regarded as mandatory for significant activity of this type. B.D.H. 3144 consequently may be regarded as a new type of anti-inflammatory agent.

Experimental

Optical rotations were measured in a 1 dm. tube for chloroform solutions. Ultra-violet absorption spectra were kindly determined (for ethanol solutions) by Mr. M. T. Davies, B.Sc. B.D.H. chromatographic alumina was used.

16 α ,17 α -Dihydroxy-6 α -methylpregn-4-ene-3,20-dione (III; R = H).—A solution of potassium permanganate (9 g.) in aqueous actone (450 ml. of 85 per cent) was added during 30 min. to a stirred solution of 6 α -methylpregna-4,16-diene-3,20-dione (Burn and others, 1957) (14.7 g.) in a mixture of acetone (450 ml.) and acetic acid (4.2 ml.). After treatment with sulphur dioxide, the pale yellow solution was poured into water (3 l.), from which

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a crystalline product (7 g., m.p. 190–197°) separated during 45 min. Repeated crystallisation of this material first from aqueous ethanol and then from aqueous acetic acid gave the *diol*, needles, m.p. 232–235°, $[\alpha]_D^{21} + 82^\circ$ ($c=0.94$), λ_{\max} 240 m μ ($\log \epsilon$ 4.19) (Found: C, 73.5; H, 8.8. $C_{22}H_{32}O_4$ requires C, 73.3; H, 8.95 per cent).

16 α ,17 α -*Isopropylidenedioxy-6 α -methylpregn-4-ene-3,20-dione* (IV). A suspension of the foregoing compound (100 mg.) in acetone (10 ml.) was treated with 2 drops of perchloric acid (72 per cent w/w), and the mixture stirred for 15 min. The product obtained by the addition of water was purified from aqueous methanol to give the *acetone*, leaflets, m.p. 166–167°, $[\alpha]_D^{21} + 118^\circ$ ($c=0.82$), λ_{\max} 240 m μ ($\log \epsilon$ 4.18) (Found: C, 74.8; H, 8.85. $C_{25}H_{36}O_4$ requires C, 75.0; H, 9.1 per cent).

16 α ,17 α -*Isopropylidenedioxy-6 α -methylpregn-1,4-diene-3,20-dione* (V; R = R' = Me). A solution of the foregoing compound (3.5 g.) and 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (3.5 g.) in benzene (35 ml.) was heated under reflux for 22 hr. The mixture was poured into 10 per cent aqueous sodium hydroxide (100 ml.) and the product isolated with ether. Crystallised from aqueous methanol, the *dienedione* formed plates, m.p. 219–220°, $[\alpha]_D^{20} + 60^\circ$ ($c=0.82$), λ_{\max} 243.5 m μ ($\log \epsilon$ 4.17) (Found: C, 75.0; H, 8.5. $C_{25}H_{34}O_4$ requires C, 75.3; H, 8.6 per cent).

16 α ,17 α -*Isopropylidenedioxy-2 α ,6 α -dimethyl-pregn-4-ene-3,20-dione*. Sodium hydride (12 g. of a 50 per cent dispersion in oil) was added to 16 α ,17 α -isopropylidenedioxy-6 α -methylpregn-4-ene-3,20-dione (12 g.) in dry benzene (240 ml.) and redistilled diethyl oxalate (12 ml.), and the mixture set aside, under nitrogen, for 3 days. After careful addition of methanol (until effervescence ceased), treatment of the mixture with light petroleum (700 ml.) gave a yellow crude sodio-derivative which was collected, washed well with light petroleum and air-dried. This material (30 g.) was suspended, together with potassium carbonate (12 g.), in acetone (270 ml.) and methyl iodide (48 ml.), and the mixture heated under reflux for 48 hr. The product was isolated with ether and treated for 18 hr. at room temperature with sodium ethoxide (from 1.2 g. sodium) in ethanol (120 ml.). The product, isolated with ether, was chromatographed on alumina (160 g.), employing light petroleum and light petroleum-benzene mixture (9 : 1) as eluants, to give material which crystallised from aqueous ethanol. 16 α ,17 α -*Isopropylidenedioxy-2 α ,6 α -dimethyl-pregn-4-ene-3,20-dione* separated in blades, m.p. 209–210°, $[\alpha]_D^{20} + 120^\circ$ ($c=0.72$), λ_{\max} 238.5 m μ ($\log \epsilon$ 4.15) (Found: C, 75.6; H, 9.4. $C_{26}H_{38}O_4$ requires C, 75.3; H, 9.2 per cent).

Isopropylidenedioxy-2,6 α -dimethyl-pregn-1,4-diene-3,20-dione prepared from the foregoing compound, crystallised from aqueous methanol in rods, m.p. 205–206°, $[\alpha]_D^{21} + 89^\circ$ ($c=1.05$), λ_{\max} 243.5 m μ ($\log \epsilon$ 4.18) (Found: C, 75.8; H, 9.2. $C_{26}H_{38}O_4$ requires C, 75.7; H, 8.8 per cent).

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A FURFURAL REAGENT OF HIGH SPECIFICITY FOR THE DETECTION OF CARBAMATES ON PAPER CHROMATOGRAMS

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A spot reaction with high specificity for the detection of carbamates, and its application to paper chromatograms, is described. Chromatographic separation methods are given, together with results obtained on a number of currently used carbamate drugs. The incorporation of the reagent in a normal screening procedure for acidic and neutral drugs is suggested.

THE detection of meprobamate [2,2-di(carbamoyloxymethyl)pentane] on paper chromatograms has been described by both Curry in 1957 and Ludwig and Hoffman in 1959 in personal communications, and also by Bedson (1959) and Walkenstein, Knebel, Macmullen and Seiftner (1958). These workers caused chlorine to react with nitrogen-containing compounds to form a chloramino-derivative which could be located by spraying with a starch-iodide reagent. This reaction was reported by Rydon and Smith (1952) for the detection of peptides, proteins, diketopiperazines, and acylated amino-acids. Modified reagents have been used for the detection of other compounds. Thus, Pan and Dutcher (1956) used the reaction to detect acylated neomycins; Reindel and Hoppe (1954) used it for the detection of proteins, peptides and amino-acids; Jackson and Moss (1960) applied it to the detection of several different kinds of neutral drugs, and Grieg and Leaback (1960) to the detection of several sugar derivatives. It is apparent, therefore, that many different types of compound are detected by this procedure, and it was felt that a more specific reaction for carbamates was required, especially in view of the increased use of this type of drug.

Ludwig and Hoffmann (1957) have described a method for the colorimetric determination of meprobamate in biological fluids. They used the colour reaction which occurs when meprobamate is treated with furfural under the influence of antimony trichloride. They did not establish the chemical nature of the coloured compound, but postulated that it was due to a furylidene coupling through the amido-group of the carbamate, under the influence of a suitable dehydrating agent.

We have investigated this reaction as a spot test for drugs containing the carbamoyloxy group, and find that the procedure described can be simplified to the extent of merely treating with furfural in the presence of concentrated hydrochloric acid.

Procedure

The test solution of the carbamate spotted on to filter paper can be dipped into a mixture of furfural one part and concentrated hydrochloric

acid 4 parts, mixed immediately before use. Because of the very rapid deterioration of this mixture the following procedure is preferable, and gives less background colour.

The paper is very lightly sprayed with furfural, followed by spraying more heavily with concentrated hydrochloric acid. The fumes from this mixture are highly objectionable and should be contained in a fume cupboard. Carbamates appear as purple to intense blue-black spots within a few seconds. The colour intensifies for about half a minute and persists

TABLE I
DETECTION OF CARBAMATES ON PAPER CHROMATOGRAMS

Drug	R-NH-CO-O-R'		Solvent for standard	Acetic and oleic acids	Pentanol and ammonia	Ultra-violet light	Furfural and HCl
	R	R'					
Ethinamate (1-ethynyl-cyclohexyl carbamate)	H	$\begin{array}{c} \text{C} \equiv \text{CH} \\ \\ \text{Cyclohexyl} \end{array}$	Ethanol	51	93	—	+
Methyl pentynyl carbamate (3-carbamoyloxy-3-methylpent-1-yne)	H	$\begin{array}{c} \text{Me} \\ \\ \text{—C—C} \equiv \text{CH} \\ \\ \text{Et} \end{array}$	Ethanol	72	88	—	+
Mephesisin carbamate (1-carbamoyloxy-methyl-2- <i>o</i> -tolyl-oxyethanol)	H	—CH ₂ ·CH(OH)·CH ₂ ·O·C ₆ H ₄ ·Me	Acetone	64	83	+	+
Meprobamate [2',2-di(carbamoyloxy-methyl)pentane]	H	$\begin{array}{c} \text{CH}_2 \quad \text{Me} \\ \diagdown \quad / \\ \text{C} \\ / \quad \diagdown \\ \text{Pr} \quad \text{CH}_2 \cdot \text{O} \cdot \text{CO} \cdot \text{NH}_2 \end{array}$	Acetone	71	86	—	+
Styramate (β-hydroxyphenethyl carbamate)	H	—CH ₂ ·CH(OH)·C ₆ H ₅	Acetone	78	76	+	+
Carbachol (2-carbamoyloxyethyltrimethylammonium chloride)	H	$\left[\text{—[CH}_2\text{]}_2\text{—N—Me}_3 \right]^+ \text{Cl}^-$	Methanol	82	0	—	+
Urethane (ethyl carbamate)	H	—Et	Acetone	89	73	—	+*
Phenylurethane (ethylphenylcarbamate)	C ₆ H ₅	—Et	Acetone	36	94	+	—
Urea			Methanol	86	16	—	+

* Evaporates. Must be spotted immediately before solvent run.

for several months. The background is pale green or grey. Urea is the only commonly occurring interfering substance, which is readily distinguishable from the carbamates by its destruction by urease, by its relative insolubility in ether, and by its R_F value in paper chromatography (see Table I). A positive reaction is also given by phenazone (antipyrine; 2,3-dimethyl-1-phenylpyrazol-5-one) but only after a time lag of about half an hour. The mono- and di-substituted carbamates, phenylurethane and neostigmine, do not react, presumably because of their *N*-substituents. Used as a spot test the reaction will readily detect 5 μg. of most carbamates.

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No reaction was obtained with barbiturates, alkaloids, ureides, di-ketopiperdines, hydantoins, oxazolidines, purines, sympathomimetic amines, or local anaesthetics of the basic ester type.

Application to Biological Material

Spot tests for $\mu\text{g.}$ quantities of carbamates and other drugs are rarely successful when applied directly to biological material; a systematic extraction scheme is usually essential. Such isolation techniques are time-consuming. For routine testing or preliminary screening, therefore, our aim is to remedy this situation by chromatographic separation and sequential treatment with a series of reagents applied to the same chromatogram. It has been found possible to incorporate the furfural reagent in such an analytical scheme. Table I lists structures, R_F values and reactions of the carbamates examined.

Acidic Drugs

For biological samples or pharmaceutical preparations suspected of containing acidic drugs, for example, aspirin or barbiturates together with carbamates, the sample is acidified and extracted with ether. The residue obtained after evaporation of the ether is dissolved in a small quantity of ethanol and spotted on Whatman No. 1 chromatography paper. The chromatogram is developed with a monophasic solvent prepared by shaking 180 ml. of pentanol with 20 ml. of strong ammonia solution. The chromatogram should be developed with this solvent for at least 5 hr., by the ascending method. The paper is then air-dried and the following sequence of reagents used.

Ultra-violet light. (Hanovia Chromatolite) for fluorescent or absorbent drugs.

Cobalt chloride and ammonia (Jackson, 1958) for barbiturates.

Ferric chloride solution for salicylates and phenols.

Furfural and hydrochloric acid for carbamates.

Neutral Drugs

For biological samples and pharmaceutical preparations suspected of containing carbamates and neutral drugs only, reversed phase chromatography may be used with advantage. The drug is extracted as for acidic drugs and applied to chromatography paper prepared in the following way.

The paper is dipped in an acetone solution containing 40 per cent oleic acid, and blotted immediately. It is then allowed to dry in air at room temperature. Uniform impregnation of the paper is essential. The solution of the extracted material in ethanol is spotted on the paper and the chromatogram developed for at least 6 hr. by the ascending method with a 50 per cent solution of glacial acetic acid in water which has been equilibrated with a few drops of oleic acid. The paper is air-dried and the following sequence of reagents used.

Ultra-violet light. (Hanovia Chromatolite).

Chlorine and starch with potassium iodide (Jackson and Moss, 1960) for most "neutral" drugs.

Furfural and hydrochloric acid for carbamates.

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PHYTOCHEMICAL STUDIES ON THE GENUS *CROTALARIA*

PART I. A PHYTOCHEMICAL INVESTIGATION OF THE SEEDS OF *Crotalaria sericea* RETZ

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By chromatography of an unsaponifiable fraction of a petroleum extract, a saturated hydrocarbon triacontane, 22,23-dihydrostigmasterol and α -carotene were isolated. The identity of triacontane was confirmed by its melting point and elemental analysis. 22,23-Dihydrostigmasterol was identified by its melting point, mixed melting point, melting points of its acetate and benzoate and by its specific rotation in chloroform. α -Carotene was identified by its absorption maxima at 460 and 480 $m\mu$. From the ethanolic extracts a mixture of alkaloids was separated which on chromatography over alumina gave a crystalline alkaloid, monocrotaline, $C_{18}H_{23}NO_6$, m.p. 196–197° and a new crystalline base m.p. 180–181°, named sericine which has not been completely identified. It has the tentative empiric formula $C_{17}H_{24}NO_7$ and may be a new alkaloid. Presence of two more alkaloids was shown by paper chromatography.

Crotalaria sericea Retz. (Syn: *C. spectabilis*) of the family *Leguminosae* has been used in the indigenous system of medicine since very early times. This plant as well as other species of *crotalaria*, which are found all over the world, are toxic to livestock and have evoked much interest in the scientific field. Some of the toxic effects produced (Chopra, Badhwar and Gosh, 1949) are termed "crotalism" and "Missourie bottom" disease. The animals suffering from "crotalism" show inflammation and outgrowth of hoofs, and a general decline of bodily vigour, culminating in stupor, coma and death. A large number of animals are reported to die annually by consuming these plants.

Neal, Rusoff and Ahmann (1935) isolated an alkaloid, monocrotaline, from the Florida grown seeds of *C. spectabilis* and showed that this alkaloid had hypotensive properties. It lowers the blood pressure in dogs and decreases the rate and amplitude of terrapin heart. These authors isolated monocrotaline by extraction of the seeds with aqueous ammonia and gave it the tentative formula $C_{18}H_{23}NO_6$ by careful analysis of monocrotaline, its hydrochloride and methiodide. Structure and synthesis of monocrotaline and monocrotalic acid have also been established.

A study of literature revealed that no alkaloid other than monocrotaline had been isolated from *C. sericea*. As work on the non alkaloidal constituents of this plant has never been reported, a detailed phytochemical investigation was undertaken.

EXPERIMENTAL

The material used in this study was an authentic sample consisting of the seeds of *Crotalaria sericea* collected from Panjab University campus in

December, 1959. The dried seeds were coarsely powdered in a flour mill. By using suitable sieves the hard testa and horny endosperm could be effectively separated from the coarsely powdered embryo. The coarse powder representing the embryos was used in all subsequent studies.

Preliminary Chemical Examination

To obtain a general idea about the type of constituents of the seeds, tests for the presence of alkaloids, glycosides, tannins, phenols, steroids, flavonoids, reducing sugars, gums, resins and proteinous matter were made. Alkaloids, sterols, gums and proteins were detected. It was further observed by histochemical tests that the alkaloids were present chiefly in the embryo whereas the gums were present exclusively in the endosperm.

Preparation of Unsaponifiable Fraction

The powdered seeds (2.5 kg.) were extracted with light petroleum (b.p. 60–80°) in a soxhlet apparatus for 18 hr. After the removal of the solvent a yellowish brown oily substance (100 g.) was obtained. This, (50 g.) was processed for the preparation of unsaponifiable matter according to the B.P. (1958) method. The unsaponifiable fraction was obtained as a yellowish orange amorphous mass (7.6 g.) which gave positive tests for phytosterols and carotenoids. It was further fractionated by treating with cold light petroleum. In this way an orange coloured fraction, (2.6 g.), freely soluble in cold light petroleum and a yellowish white solid fraction (5 g.) sparingly soluble in cold light petroleum, were separated.

Isolation of Triacontane and 22, 23-Dihydrostigmasterol

The yellowish white solid residue (5 g.) obtained from the unsaponifiable matter was dissolved in light petroleum (b.p. 60–80°) (100 ml.). The solution, after cooling, was passed through a column containing Merck chromatographic alumina (125 g.). The column was eluted with 32 fractions each of 50 ml.; those numbered 1 and 2 with light petroleum; those 3 to 8 with benzene; those 9 to 10 with benzene: ethyl ether (19:1); those 11 to 13 with benzene: ethyl ether (4:1); those 14 to 29 with ethyl ether; and those numbered 30 to 32 with ethanol.

Triacontane. The product obtained from fractions 1 and 2 was white, waxy, and had a m.p. 63°. After two crystallizations from acetone and drying in a desiccator, colourless needles m.p. 64–65° were obtained. The elemental analysis was found to be C, 84.42: H, 14.08 per cent, (calculated for $C_{30}H_{62}$, C, 85.21: H, 14.69 per cent). Triacontane, $C_{30}H_{62}$ is reported to have m.p. 66°. No authentic sample was available.

22,23-Dihydrostigmasterol. The residues obtained from fractions 14–29 gave positive tests for sterols and had m.p. 134–137°. The combined residue on crystallisation from ethanol gave plate like crystals (4.2 g.) m.p. 136–137° (Shriner, Fuson and Curtin, 1956, reported 22,23-dihydrostigmasterol to have m.p. 137°). Its melting point with an authentic sample of 22,23-dihydrostigmasterol (Atal and Lamba, 1960) was undepressed. Its identity was further confirmed by determining its specific

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rotation in chloroform (found -36.65° : reported, Merk Index, 1952, -37°). Yield 0.17 per cent of the air dried seeds.

22,23-Dihydrostigmasterol acetate. 22,23-Dihydrostigmasterol (50 mg.) was treated with acetic anhydride (1 ml.) and pyridine (1.5 ml.) The solution was refluxed in a water bath for 2 hr. It was evaporated to a small volume and poured in water. The precipitate was washed and dried. Needle-like crystals were obtained m.p. $126-127^{\circ}$ (Shriner, Fuson and Curtin, 1956, give $126-127^{\circ}$).

Isolation of α -Carotene

The orange coloured fraction (2.69 g.), derived from the unsaponifiable matter, was redissolved in light petroleum (50 ml.) and passed through a column of Merck aluminium oxide (150 g.). The column was eluted with 30 fractions each of 50 ml.; those numbered 1 to 8 with light petroleum; those 9 to 12 with benzene; those 13 to 22 with benzene: ethanol (200:1). The fractions numbered 23 to 26 were eluted with benzene: ethanol (100:1) and those numbered 27 to 30 with ethanol.

The few mg. orange coloured residues from fractions 9-12, 16-18, 20-21 and 24 gave tests for carotenoids (Strain, 1935). The residue from fraction 23 was dissolved in benzene and its absorption measured spectrophotometrically (Hilger). It gave absorption maxima at 460 and 488 $m\mu$ identical with that reported (Strain, 1935) for α -carotene. The other three carotenoid residues did not give clear maxima and may represent decomposition products of carotenes. Such decomposition is known to occur on alumina columns (Strain, 1945).

Preparation of Crude Alkaloidal Mixture

The powdered drug (2.5 kg.), after being successively extracted with light petroleum, benzene and chloroform, was extracted in a soxhlet apparatus for 24 hr. with ethanol (95 per cent). The ethanol was evaporated when a semisolid dark brown residue (160 g.) was obtained. It gave positive tests for alkaloids. The residue was dissolved in 0.5 N hydrochloric acid (500 ml.), the acid solution filtered and made alkaline with a dilute solution of ammonia and the alkaloids extracted by shaking with chloroform. On evaporation of the solvent a dirty-white crude alkaloid (16 g.) was obtained. Paper chromatography of the crude alkaloid, using butanol saturated with 5 per cent acetic acid as the mobile phase and modified Dragendorff's reagent, as the spray (Munier and Macheboeuf, 1957) showed the presence of four alkaloids (R_f 0.157, 0.224, 0.521 and 0.620). An attempt to separate the individual alkaloids from the mixture by fractional crystallisations did not succeed.

Isolation of Sericine and Monocrotaline

The alkaloidal mixture (8 g.) was dissolved in benzene (100 ml.) and added to a column containing chromatographic aluminium oxide (225 g.). The column was eluted with 42 fractions each of 50 ml.; those numbered 1 to 23 with benzene; those 24 to 38 with chloroform and those 39 to 42 with ethanol. Fractions 1-3 gave a light yellow waxy residue (0.060 g.)

which could not be crystallised. Fractions 4-6 gave a residue of large prismatic colourless crystals (1.833 g.) which gave positive tests for alkaloids m.p. 180-181°. The residue (4.723 g.) from fractions 7-42 was in the form of long fine needles, alkaloid positive, m.p. 195-197°, which was probably identical with monocrotaline.

Sericine. The crystalline residue from fraction 4-6 is regarded tentatively as a new alkaloid as evidenced by its crystal structure and melting point. To confirm this view and to prove that it was not an impure form of monocrotaline (fractions 7-42), the technique of mixed spotting on paper chromatograms was used. On a sheet of Whatman No. 1 filter paper, impregnated with pH 7.4 phosphate buffer, four spots were applied. Spot 1 was of residue from fraction 4-6, Spot 2 of residue from fraction 7-42, Spot 3 of a mixture of residues from fractions 4-6 and 7-42, and Spot 4 of an authentic sample of monocrotaline. Butanol saturated with 5 per cent acetic acid was used as the mobile phase. A comparison of these alkaloids on the chromatogram shows that the residue from fractions 7-42 is identical with monocrotaline (R_f 0.54) while residue from fraction 4-6 represents the new substance (R_f 0.63). The name sericine has been given to this crystalline base. The elemental analysis gave C, 58.8; H, 6.77; N, 3.96 per cent which corresponds to the tentative empirical formula $C_{17}H_{24}NO_7$. Yield of sericine was 0.07 per cent of the air dried seeds.

Sericine hydrochloride. The base (10 mg.) was dissolved in absolute ethanol (3 ml.) and neutralized to congo red by dilute hydrochloric acid. The solution was evaporated to dryness. The residue was taken up in 2 ml. hot methanol and ether added to turbidity. On cooling, the product was filtered and recrystallized from methanol-ether, m.p. 232-233°.

Sericine picrate. The base (20 mg.) was dissolved in absolute ethanol (2 ml.) and to it a saturated solution of picric acid in ethanol was added. The heavy crystalline precipitate so obtained was recrystallized from ethanol, m.p. 233-235°.

Sericine methiodide. The base (30 mg.) was dissolved in chloroform (1 ml.) and ethanol (0.1 ml.) and 0.2 ml. of methyl iodide added. On cooling an oily product separated which on crystallization from methanol-chloroform gave a crystalline addition compound, m.p. 242-245° (decomp.).

Monocrotaline. As shown above, the residues from fractions 7-42 were probably identical with monocrotaline, based on the melting point and R_f values (found m.p. 196-197°) (Rogers and Rogers, 1939, gave monocrotaline, 196-197°). The elemental analysis gave C, 59.16; H, 6.83 per cent (calculated for monocrotaline, $C_{16}H_{23}NO_6$, C, 59.07; H, 7.09 per cent). A mixed melting point with an authentic sample of monocrotaline was undepressed. The yield of monocrotaline was 0.5 per cent on the basis of air dried seeds.

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of sericine. Our thanks are also due to Shri K. C. Sahni of the Forest Research Institute, Dehradun, for the identification of the plant under investigation. We are also grateful to Dr. K. N. Gaind, Head of the Department, for his encouragement and valuable suggestions.

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CHROMATOGRAPHIC SEPARATION OF THE DEGRADATION PRODUCTS OF PRALIDOXIME IODIDE (PYRIDINE-2-ALDOXIME METHIODIDE)

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A satisfactory method for the separation and identification of *in vitro* breakdown products of pralidoxime iodide (PAM; pyridine-2-aldoxime methiodide; 2-hydroxyiminomethyl-*N*-methylpyridinium iodide) by paper chromatographic techniques is described.

STUDIES on the metabolic fate of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide salts require suitable methods for analysing the *in vivo* degradation products. The present study of the chromatographic separation and identification of the *in vitro* breakdown products of pralidoxime iodide (PAM; pyridine-2-aldoxime methiodide; 2-hydroxyiminomethyl-*N*-methylpyridinium iodide) represents a preliminary phase of the investigation.

Pralidoxime iodide breaks down *in vitro* to 2-formyl-*N*-methyl pyridinium iodide (PCAM; pyridine-2-aldehyde methiodide), 2-cyano-*N*-methylpyridinium iodide (PCNM; 2-cyanopyridine methiodide), 2-hydroxy-*N*-methylpyridine (NMAP; *N*-methyl- α -pyridone), 2-carboxy-*N*-methylpyridinium iodide (PCOM; pyridine-2-carboxylic acid methiodide) and possibly 2-carbamyl-*N*-methylpyridinium iodide (PCMM; pyridine-2-carboxamide methiodide) (Ellin, 1958).

In a recent study concerning the perfusion of ^{14}C -labelled pralidoxime iodide through liver, Way, Tong and Rabidean (1960) isolated a fraction which had chemical and spectral properties similar to those of PCNM. This investigation demonstrates that the aforementioned decomposition products can be qualitatively separated and identified by paper chromatographic methods. Furthermore, several distinct phenomena inherent in this experiment have been explained.

EXPERIMENTAL

All experiments were made with the descending technique on Whatman No. 1 paper chromatographic sheets, $18\frac{1}{4} \times 22\frac{1}{2}$ inches. A heavy walled Chromatocab (Research Equipment Corporation, Oakland, Calif.), was used for the chromatographic chamber. The samples were applied to the paper by 5 ml. self-filling pipettes and dried with a current of warm air. The paper was equilibrated for 4 hr. in the presence of both phases of the solvent system. Development required about 18 hr., in which time the solvent front travelled about 40 cm. After the sheets were air dried in a hood, the solvent front and the visible zones were marked under an ultra-violet lamp (Ultra-Violet Products, Inc., San Gabriel, Calif.). The sheets were then separately sprayed with the following reagents: (a) a

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modified Dragendorff spray reagent (Schriftman and Kondritzer, 1957); (b) platinum spray reagent prepared by mixing 2 ml. of 10 per cent platinum chloride solution ($\text{PtCl}_4 \cdot 2\text{HCl} \cdot 6\text{H}_2\text{O}$) with 1 g. of potassium iodide in 98 ml. of water (Goldbaum and Kazyak, 1956); (c) an aqueous 1 per cent palladium chloride solution; (d) Tollen's spray reagent composed of two separate solutions: 0.1N silver nitrate, and 0.5N ammonia; (e) 0.4 per cent 2,4-dinitrophenylhydrazine reagent in 2N hydrochloric acid followed by 10 per cent aqueous potassium hydroxide. In routine practice it was convenient to run a series of paper strips of each compound and develop each strip with a different reagent.

The following solvent systems gave the best distribution and sharpest spots; (a) butanol:acetic acid:water (5:1:3), (b) water-saturated butanol; (c) 4 ml. acetic acid added to 100 ml. of butanol and the mixture saturated with water. Solvent systems were freshly prepared before use.

RESULTS AND DISCUSSION

All compounds synthesised for the experiments were purified by recrystallisation or distillation. However, when developed with solvent system (a), each of the compounds tested, with the exception of NMAP,

TABLE I
 R_F VALUES, AND TESTS TO DETECT PRALIDOXIME IODIDE AND ITS DEGRADATION PRODUCTS

Name	R_F^*	Dragendorff reagent	Platinum reagent	Palladium reagent†	Ultra-violet light
Pralidoxime iodide	0.51	+	+	+	+
NMAP	0.81	—	—	—	+
PCNM	0.42	+	+	+	+
PCAM	0.62	—	—	+	+
PCOM	0.38	—	—	+	+
PCMM	0.37	+	+	+	+
Hydrogen iodide ..	0.20	—	—	+	—

* The listed R_F values are for a butanol:acetic acid:water system (5:1:3). To separate PCMM from PCOM, a 4 per cent acetic acid in butanol solvent saturated with water was used. Their R_F values were 0.08 and 0.15 respectively.

† The positive reaction is due to reaction with iodide ion.

produced two spots on the chromatogram (Fig. 1). One of the spots has an R_F value of 0.20, gives a positive reaction with palladium spray reagent and is not visible under the ultra-violet lamp. The other spots, with variable R_F values, were visible under the ultra-violet lamp but did not give positive reactions with palladium reagent. When developing solvent (a) was replaced by solvent system (b), a single spot was obtained for each compound. The single spots gave positive reactions with palladium reagent and were detected by ultra-violet light. The results were resolved when the spot which had a R_F value of 0.20 in solvent (a) was identified as hydrogen iodide. Solvent system (a) had effected separation of the iodide from the protonated organic bases, resulting in formation of the corresponding quaternary acetate and hydrogen iodide. Palladium reagent does not give a reaction with acetate ion. Thus, the positive reaction given by the various quaternary iodides with palladium spray can be attributed to reaction of the iodide ion and not the quaternary

moiety. This result was further confirmed by spotting pralidoxime nitrate (pyridine-2-aldoxime methonitrate) on chromatographic paper and developing with the acid solvent system. No spot giving a positive reaction with palladium spray reagent was found.

An unusual result occurred with PCAM. Before development with solvent system (a), the PCAM gave a positive reaction for aldehyde with both Tollen's and 2,4-dinitrophenylhydrazine reagent sprays. After development, the resulting spot gave a positive reaction only with 2,4-dinitrophenylhydrazine. This would indicate that the PCAM had reacted with

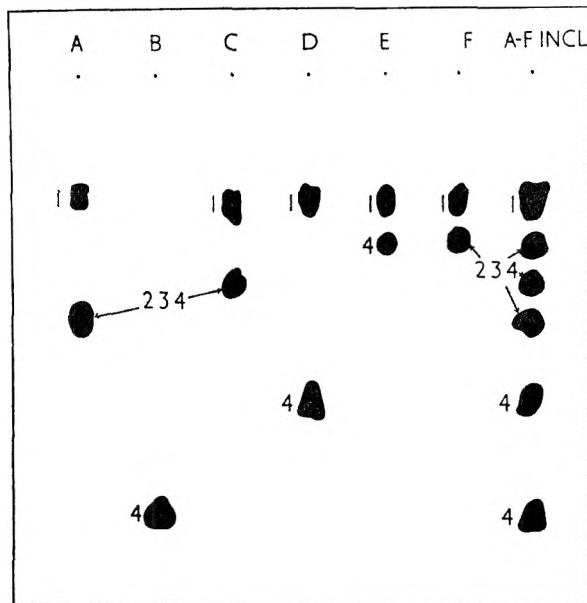


FIG. 1. Chromatogram of *in vitro* degradation products of pralidoxime iodide. A, Pralidoxime iodide; B, NMAP; C, PCNM; D, PCAM; E, PCOM; F, PCMM. A-F INCL, a mixture of the above compounds on a single spot. Positive reactions with: 1, palladium (ous)chloride spray; 2, Dragendorff spray; 3, platinum chloride spray; 4, ultra-violet light.

the alcohol in the solvent system to produce the corresponding hemiacetal or acetal.

In Table I are listed the various degradation products of pralidoxime iodide which have been studied. The R_f values given are the averages of 6 or more runs, using the descending method of development. The plus marks indicate those tests which are applicable for detecting the compounds. Variations in the R_f values of repeated runs were found to be less than ± 6 per cent.

When the five degradation compounds were spotted at the same point (Fig. 1, A-F INCL) on the chromatographic paper and developed with solvent system (a), a predictable separation was obtained. Two of the five compounds, PCOM and PCMM had similar R_f values and could not be separated by solvent system (a). The application of two dimensional

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chromatography using a second solvent system, solvent (c), effected a satisfactory separation. The two spots were identified under ultraviolet light, the top spot being PCMM and the lower, PCOM.

CONCLUSIONS

Mixtures of *in vitro* degradation products of PAM have been separated by descending paper chromatography. A single solvent system can be used for the resolution of any combination of the compounds, with the exception of one. The latter required two-dimensional chromatography techniques. A number of spray reagents were used; reliance on the most sensitive reagent, palladium chloride, for detecting quaternary compounds would have led to erroneous conclusions. The spot corresponding to PCAM was actually the corresponding hemiacetal or acetal, but, nevertheless was satisfactory for the identification of the aldehyde component initially present.

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THE EFFECT OF TRICHLOROACETIC ACID ON THE PAPER CHROMATOGRAPHY OF TISSUE AMINES

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The nature of the artifacts formed in the paper chromatography of solutions of catechol amines, histamine, and various tryptamine derivatives in trichloroacetic acid (TCA) has been considered. With TCA solutions of histamine the artifact is not the corresponding *N*-acyl derivative. It is suggested the production of these artifacts is assisted by the formation of molecular complexes between the above compounds, acting as electron donors, and the TCA acting as electron acceptor. A solvent is described which eliminates multiple spot formation when TCA solutions of the above type are subjected to paper chromatography.

WHEN solutions of histamine in trichloroacetic acid (TCA) are chromatographed in the solvent system butanol:acetic:water, two spots are obtained, one corresponding to the histamine base and the other to an artifact (West and Riley, 1954). These workers found that the formation of the artifact was not due to a pH effect, but depended on the concentration of the TCA, the concentration of histamine, and on the presence of the basic amino-acids arginine, lysine or ornithine. They suggested that the ability of TCA to extract histamine from tissues might in part be due "to its participation in a loose complex containing histamine (probably as the trichloracetate) and basic amino-acids", and we assume, therefore, that the artifact was thought to be such a complex.

Beckett, Beaven and Robinson (1960) have shown that TCA solutions of many sympathomimetic amines yield two spots on chromatography, the upper spot being closely associated with the TCA spot. It would seem likely that the histamine artifact spot arises in a similar manner, but its exact nature is not clear. Some further observations on the nature of the histamine artifact are now presented.

EXPERIMENTAL METHODS

General Method

Solutions of the amines were applied to Whatman No. 4 paper as indicated in Table I. Ascending chromatograms were run for 18 hr. during which time the solvent front moved about 25 cm. at 18–22°.

Solvents. (1) A single phase mixture of n-butanol:acetic acid:water (12:3:5 v/v). (2) A solution of TCA (1 per cent w/v) in (1), and (3) A solution of TCA (5 per cent w/v) in (1).

After preparation the solvents were allowed to stand 4 hr. before use. Solvent (2) separated into two phases after 3 days, and could if necessary be used several times; solvent (3) separated after 30 hr., and so could be used only once.

Spray reagents. For catechol amines run in solvent (1): a 1 per cent w/v aqueous solution of potassium iodate followed by heating of the

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sprayed chromatogram at 100°. For catechol amines run in all solvents: an aqueous buffered solution of potassium ferricyanide (James, 1948) followed by treatment with ammonia, heating at 100° for 3 min. and irradiation with ultra-violet light. The use of this second spray for the detection of catechol amines was particularly necessary after using solvents (2) and (3) as there was appreciable lowering of the sensitivity of the potassium iodate reagent in these instances, presumably owing to the presence of TCA on the paper; the sensitivity of the potassium ferricyanide reagent was unaffected by this latter acid. For tryptamine and

TABLE I

SINGLE SPOT FORMATION BY SOLUTIONS OF AMINES IN 0.01 N HYDROCHLORIC ACID OR 10 PER CENT AQUEOUS TCA SOLUTION AFTER CHROMATOGRAPHY IN A SOLVENT CONTAINING TCA

Solution	Concentration (mg./10 ml.)	Amount of compound applied to paper (μg.)	R _F values in Solvent (3)
Histamine in A	10	100	0.61*
Histamine in B	50	100	0.62
4(5)-Methylimidazole in A	40	40	0.76
4(5)-Methylimidazole in B	40	40	0.76
Imidazole in A	40	120	0.68
Imidazole in B	40	120	0.72
5-Hydroxytryptamine in A	25	75	0.67
5-Hydroxytryptamine in B	25	75	0.67
Tryptamine in A	50	50	0.85
Tryptamine in B	50	50	0.87
Adrenaline in A	40	40	0.65
Adrenaline in B	40	40	0.64
Isoprenaline in A	40	40	0.77
Isoprenaline in B	40	40	0.77
Noradrenaline in A	40	100	0.57
Noradrenaline in B	40	100	0.58

A = 0.01 N hydrochloric acid. B = 10 per cent aqueous TCA.
 All R_F values are the average of readings from two chromatograms.
 * Some streaking.

5-hydroxytryptamine run in all solvents: a 0.5 per cent w/v solution of *p*-dimethylaminocinnamaldehyde in equal volumes of 5 N hydrochloric acid and absolute ethanol. For imidazole derivatives and 5-hydroxytryptamine run in all solvents: the Pauly diazotised sulphanilic acid reagent.

N-Trichloroacetylhistamine. A solution of histamine (150 mg.) in redistilled trichloroacetic anhydride (2 ml.), protected from moisture, was heated on a water bath at 80–90° for 3 hr. (longer heating or heating at higher temperatures led to gradual decomposition). The excess trichloroacetic anhydride was then removed under reduced pressure to leave a yellow oil which on trituration with dry ethanol crystallised as white prisms (206 mg.), m.p. 164–166° (with decomposition from 153°). Recrystallisation from dry ethanol gave white prisms, m.p. 165–167° (with decomposition from 153°) (Found: C, 33.3; H, 3.2; N, 16.0. C₇H₈Cl₃N₃O requires C, 32.9; H, 3.1; N, 16.4 per cent).

4(5)-*Methylimidazole.* This was prepared by the method of Bernhauer (1929).

Other compounds used were obtained commercially.

RESULTS AND DISCUSSION

The possibility that the histamine artifact spot might arise from the formation of *N*-trichloroacetylhistamine (N-TCAH) has now been investigated by preparing this compound and studying its chromatographic behaviour. When a solution of N-TCAH in 0.01 N hydrochloric acid was chromatographed in solvent (1), one spot (R_f 0.75) was obtained. After elution with 0.01 N hydrochloric acid and re-running in the same solvent, this again had R_f 0.75. Under identical conditions a solution of histamine in 10 per cent aqueous TCA gave two spots (R_f 0.18 and 0.65) corresponding to the base and the artifact, respectively. The latter spot, after elution and re-running as above, now showed R_f 0.18 corresponding to free histamine. The different R_f values and the behaviour on elution and re-running show that the artifact is not N-TCAH.

That the β -aminoethyl side-chain of histamine is not essential for the artifact formation was demonstrated by the observation that double spots were formed on chromatography in solvent (1) of 10 per cent aqueous TCA solutions of imidazole (R_f 0.44 and 0.69) and 4(5)-methylimidazole (R_f 0.55 and 0.73) from both of which the side-chain is absent; with 0.01 N hydrochloric acid solutions of these two compounds only single spots were obtained R_f 0.45 and 0.53 respectively. When the areas corresponding to the artifacts were eluted with 0.01 N hydrochloric acid, each eluate, re-run in the same solvent, gave a single spot characteristic of the compound in 0.01 N hydrochloric acid.

Among the conditions under which double spot formation may be expected to occur (Beckett and others, 1960) the possibility of complex formation has been considered. It is of interest that double spots are most clearly defined when TCA solutions of sympathomimetic amines, imidazoles and tryptamine derivatives (West, 1959) are chromatographed. This suggests that artifact production in such cases may be assisted by the formation of molecular complexes between these substances, acting as electron donors and the TCA acting as an electron acceptor by virtue of the marked electronegativity of the three chlorine atoms. These complexes would have structures similar to those formed between aromatic compounds and various electron acceptors as described by McGlynn (1958). Further evidence in support of this hypothesis comes from the following observations: (i) solutions of catechol amines, histamine, or 5-hydroxytryptamine in picric acid (a well-known electron acceptor in complex formation) and in trifluoroacetic acid (TFA) form double spots on chromatography in *n*-butanol:acetic acid: water (Beckett and others, 1960; Shepherd and West, 1952; West, 1959); (ii) artifact formation with sympathomimetic amines in various acid solutions increases in the order mono- < di < trichloroacetic acid (Beckett and others, 1960).

The above results further emphasise the care which must be taken when interpreting paper chromatograms of tissue extracts prepared in aqueous TCA, this acid being widely used owing to its coagulative effect on protein, which leads to clear extracts and so facilitates subsequent work.

Munier (1952) has suggested that artifact formation may be avoided by the use of the same acid in both the chromatography solvent and in

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the solution to be chromatographed. Accordingly we have found that solutions of histamine, imidazole, 4(5)-methylimidazole, tryptamine, 5-hydroxytryptamine, adrenaline, noradrenaline, and isoprenaline when chromatographed in solvent (1) to which TCA has been added, each give a single compact spot (Table I). When the chromatography solvent contained 1 per cent TCA (solvent 2), these substances gave single spots from either 0.01 N hydrochloric acid or 10 per cent TCA solutions, although, when the amount of histamine applied to the paper exceeded (25 μ g.), extensive streaking occurred in both cases. When the chromatography solvent contained 5 per cent TCA (solvent 3), histamine in amounts up to at least 100 μ g. applied from 10 per cent TCA solution gave a single compact spot, but when applied from 0.01 N hydrochloric acid solution in amounts exceeding 50 μ g., histamine still gave some streaking. Using solvent (3) the single spot produced by TCA solutions of all the above compounds had approximately the same R_f value as the corresponding artifact spot produced when they were chromatographed in solvent (1).

The use of solvent (3) provides a method for the chromatography of aqueous TCA extracts of tissues without the risk of misinterpreting the chromatograms owing to multiple spot formation by a single compound.

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NEW APPARATUS

AN AUTOMATIC SYRINGE FOR USE WITH ISOLATED ORGAN BATHS*

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SINCE the introduction of Post Office selector switches and relays by Schild (1945) for controlling cyclical operations in isolated organ bath techniques, many variations and extensions of the original invention have been devised. The addition of doses of drug in the form of the ultimate dilutions required to affect the isolated organ, as used in some types of apparatus, presents difficulties; for example if the responses markedly

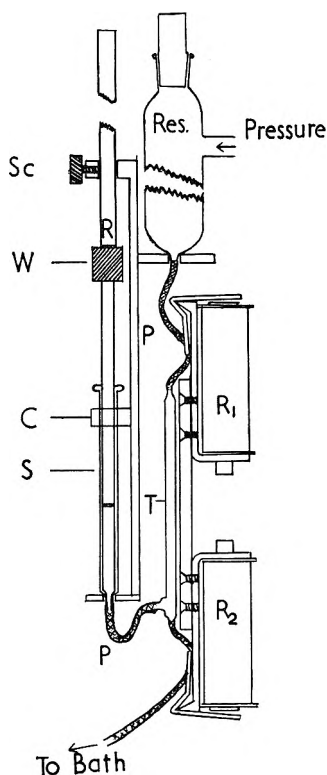


FIG. 1. The automatic syringe

Res., Drug reservoir; W, 15 g. weight recessed and cemented to plunger; S, 1 ml. glass syringe graduated in hundredths; T, Glass t-piece; R₁ and R₂, P.O. relays; P, P.V.C. tubing; C, Perspex clip to retain syringe; R, Adjustable rod to limit filling of syringe; Sc, Set screw to fix rod at required position.

* Demonstrated before the British Pharmacological Society, January, 1955.

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change to the first set of doses, time may be lost in preparing further sets of dilutions. This and other difficulties were noted by Burn and Dale (1922), long before Schild's invention was announced.

The automatic syringe described below has been found useful in that doses of concentrated drug delivered automatically to the bath may be rapidly changed at will. Reference to Fig. 1 shows the working of the device.

The body is constructed of Perspex. Gas pressure, at about 5 lb./in.², conveniently taken from the supply to the organ bath, is applied to the surface of the drug solution in the reservoir.

On opening relay R_1 , drug solution passes down the t-piece T, and drives the plunger up to the level limited by the rod R, held in place by the set screw Sc. R_1 is then closed, and at the appropriate time in the

TABLE I

THE VOLUMES (DETERMINED BY WEIGHT) OF SUCCESSIVE DELIVERIES FROM ONE SYRINGE WORKING AUTOMATICALLY

Dose number	Approximate setting in ml.		
	1.0	0.5	0.25
1	1.006	0.5156	0.2603
2	1.008	0.5150	0.2603
3	1.0083	0.5130	0.2609
4	1.0078	0.5156	0.2612
5	1.0076	0.5036	0.2632
6	1.008	0.5093	0.2571
7	1.0076	0.5132	0.2623
8	1.0073	0.5038	0.2606
9	1.0075	0.5141	0.2605
10	1.007	0.5134	0.2606
Means	1.00751	0.51166	0.26070
Standard deviation	0.0002	0.0011	0.0038

* Demonstrated before the British Pharmacological Society, January, 1955.

cycle relay R_2 is opened; the drug solution then passes into the bath, driven by the weight W. R_2 then closes. Springs, applied to the relay to keep the tubing closed when the relays are not energised, are not shown for the sake of clarity. The syringe may conveniently be filled by drawing the top out of the clip C. The plunger may then be withdrawn after opening R_1 .

Table I shows the constancy of repeated doses delivered from a single syringe.

In the present arrangement 5 syringe units are incorporated and driven from an electronically controlled device similar to that of Bourra, Mongar and Schild (1954). Switch arrangements are included so that any one syringe may be used repetitively, or any number in a preselected order.

The apparatus has proved to be consistent and reliable over the course of several years.

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BOOK REVIEW

SMOKING. ITS INFLUENCE ON THE INDIVIDUAL AND ITS ROLE IN SOCIAL MEDICINE. By C. Van Proosdij. Pp. viii + 319 (including Index). D. Van Nostrand Company Ltd., London, 1960. 57s. 6d.

It is difficult to write without prejudice about smoking, and it requires the skills of experimental psychology, sociology, chemistry, physics and statistics as well as of pathology and pharmacology to assess properly what is evidence and what is wishful thinking about this remarkable habit. Dr. Van Proosdij is a practising clinician, and although traces of his strong feelings are frequently detectable, he appears to this reviewer to have made a reasonably balanced review of the extensive available literature. The eight chapters deal with factors leading to smoking, psychological and pharmacological aspects, methods of investigation, damage done to the human organism sociopsychological aspects, prospects, and a summary. No one knows exactly why some people smoke much more than others, but there is abundant evidence that smoking is correlated with various diseases, especially lung cancer, bronchitis and amblyopia and less strongly with coronary artery disease and peptic ulceration among others. In some of these diseases there is also evidence of causality, and Dr. Van Proosdij rightly points out the extensive evidence that smoking and lung cancers are causally connected and not only separate consequences of a common genetic background. This book is well written and produced, and is an invaluable and well documented source of information about smoking. The attitude of individual readers to it is likely to be correlated with their own smoking habits.

MILES WEATHERALL.

LETTERS TO THE EDITOR

Hypoglycaemic Agents from Indian Indigenous Plants

SIR,—A hypoglycaemic agent from *Allium cepa* Linn. (the domestic onion) has already been reported by Brahmachari and Augusti (1961). The present communication describes two more orally effective hypoglycaemic principles from Indian indigenous plants.

The therapeutic use of the seeds of *Eugenia jambolana* Lam. and the bark of *Ficus bengalensis* Linn. in the treatment of diabetes mellitus, has been reported by Kirtikar and Basu (1933). Kramer (1918) reported an extract of the seeds of *E. jambolana* to alleviate glycosuria, and a hypoglycaemic action of extracts of these seeds has been claimed by Kaufmann (1928), Mercier and Bonnafous (1940) Mukerji (1953), Vaish and Kehar (1954), and Sepala and Bose (1956).

An attempt has therefore been made to isolate orally-effective hypoglycaemic principles from the sun dried seeds of *E. jambolana* and the bark of *F. bengalensis*. These were powdered and thoroughly extracted separately with different solvents in Soxhlets. Ethanolic (95 per cent) extracts were found to be hypoglycaemic on oral administration to groups of normal male albino rabbits weighing 2 kg. with fasting 18 hr. blood sugar levels of 100–125 mg./100 ml. Aqueous extracts also showed slight hypoglycaemic activity under similar conditions.

The viscous residues left after the evaporation of ethanol from these extracts were vacuum dried and separately assayed against tolbutamide (0.5 g./rabbit) as standard, and a dose-effect curve for each of these products was obtained for different doses following a similar procedure to that of Marks (1936). Blood-sugar was determined by the micro method of Folin and Malmros (1929).

The dose-effect relations of the two products within the dosage range of 0.5 g. to 3 g. per rabbit is shown in Fig. 1. These preliminary results establish the fact that both the seeds of *E. jambolana* and the bark of *F. bengalensis* contain some ethanol soluble orally effective hypoglycaemic principles.

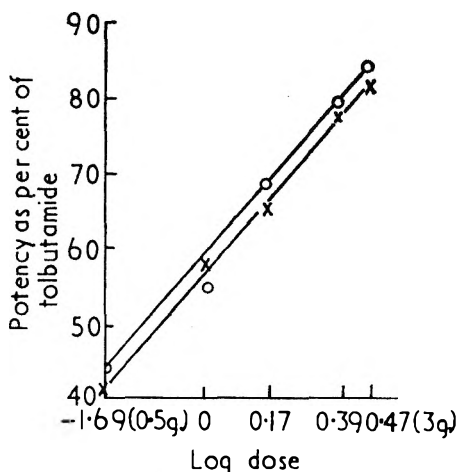


FIG. 1. The dose effect curves for a 95 per cent ethanolic extract from *Ficus bengalensis* (O—O), and *Eugenia jambolana* (X—X). Each curve is a mean of 6 rabbits.

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The separation and purification of these hypoglycaemic principles is in progress.

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5-Hydroxytryptamine in Pineapples

SIR,—In view of the conflicting reports concerning the presence of 5-hydroxytryptamine (5-HT) in pineapples (Bruce, 1960; Foy and Parratt, 1960; West, 1960; Bruce, 1961), we have obtained the following results which we think will resolve the differences between Bruce, West and ourselves.

(a) Acetone extracts of the pulp of both unripe and ripe fruits contain a substance indistinguishable from 5-HT when assayed on the isolated rat uterus and rat colon preparations in the presence of atropine (10^{-7}). The contractions produced by both extract and standard 5-HT were antagonised by a specific anti-5-HT substance, bromolysergic acid diethylamide in a concentration of 2×10^{-8} – 10^{-7} . There appeared to be more in unripe fruit (50 and 60 $\mu\text{g./g.}$) than in ripe fruit (19 $\mu\text{g./g.}$ —an average of six estimations). A trace was also detected in stalk (0.2 $\mu\text{g./g.}$) but none in the leaves of the crown ($<0.08 \mu\text{g./g.}$) or base ($<0.08 \mu\text{g./g.}$).

(b) Juice squeezed from the pulp of ripe fruits contained 5-HT in a concentration of 13–22 $\mu\text{g./ml.}$, an average of eleven estimations being 16 $\mu\text{g./ml.}$

(c) Canned pineapple juice contained much less than this, and certainly nothing like the amounts quoted by Dr. Bruce (Bruce, 1960; 1961). One brand of Australian juice contained 3.5, 4.6 and 8 $\mu\text{g./ml.}$ and a Nigerian brand even less (1.3–4.2 $\mu\text{g./ml.}$ —an average of 2.8 $\mu\text{g./ml.}$ from six estimations).

There are three possible reasons for our previous failure to detect 5-HT in pineapple extracts (Foy and Parratt, 1960) and for the continuing discrepancy in the values for canned juice.

(i) There may be an enzyme in pineapple which destroys 5-HT, a situation already known to occur in the nettle plant (Collier and Chesher, 1956). But we have been unable to detect such an enzyme using a modification of the method used by Collier and Chesher.

(ii) Both fresh and canned pineapple juices are acid (pH 3.6–3.9). 5-HT is unstable in acid solution (Erspamer, 1940; Parratt, 1958), particularly when heated (Erspamer, 1940; Amin, Crawford and Gaddum, 1954). This would decrease the stability of 5-HT in acetone extracts of acid pulp and explain the lower values for canned juice compared with fresh juice.

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(iii) Our figures for 5-HT are calculated as base. Dr. Bruce mentions "serotonin" (Bruce, 1960) and "indole derivatives" (Bruce, 1961). If his results are calculated using 5-HT creatinine sulphate as his standard then his published results would need to be halved. This would bring them more in line with the above results.

The likelihood of an erroneous diagnosis of a carcinoid tumour by pineapple ingestion is, in our opinion, small. It would surely be much more likely to occur in countries where the staple diet contains 5-HT, as in parts of West Africa where plantains (containing large amounts of this substance) are ingested regularly (Foy and Parratt, 1960).

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The Effects of Gallamine, Carbachol, Nicotine, Ryanodine and Protoveratrine A and B upon Flux of Calcium-47 in Frog Skeletal Muscle

SIR,—We recently reported the effects of tubocurarine, decamethonium, suxamethonium, edrophonium and neostigmine on $^{47}\text{Ca}^{++}$ -uptake and release in frog sartorius muscle, and compared these with their influence upon uptake and release of $^{42}\text{K}^{+}$ and on $^{24}\text{Na}^{+}$ -uptake (Ahmad and Lewis, 1961). Using similar techniques, we have extended our observations to make a preliminary study of the effects of gallamine, carbachol, nicotine, ryanodine and protoveratrine A and B.

From 1 to 8 mg./ml. of gallamine did not significantly alter $^{47}\text{Ca}^{++}$ -uptake ($P = 0.4 < 0.3$) or release. At similar dose levels there was no significant change in $^{42}\text{K}^{+}$ -uptake ($P = 0.2 < 0.1$) or release, or in $^{24}\text{Na}^{+}$ -uptake ($P = 0.7 < 0.6$).

Carbachol (5 mg./ml.), did not significantly alter uptake of $^{47}\text{Ca}^{++}$ ($P = 0.3 < 0.2$) but its effects upon release were variable. It caused depression of $^{42}\text{K}^{+}$ -uptake ($P = 0.1 < 0.05$) with a slight increase in its release but there was no change in $^{24}\text{Na}^{+}$ -uptake ($P = 0.8 < 0.7$).

The effects of nicotine were more striking than those of gallamine or carbachol. 1 mg./ml. of nicotine caused a very significant increase in $^{47}\text{Ca}^{++}$ -uptake ($P = > 0.001$), $^{47}\text{Ca}^{++}$ -release and $^{42}\text{K}^{+}$ -release. It also markedly decreased $^{42}\text{K}^{+}$ -uptake ($P = > 0.001$) and significantly increased $^{24}\text{Na}^{+}$ -uptake ($P = > 0.02$).

Ryanodine at bath concentrations of 10 to 100 $\mu\text{g./ml.}$ increased markedly both release, and uptake ($P = 0.01 < 0.001$) of $^{47}\text{Ca}^{++}$. With 50 $\mu\text{g./ml.}$ of

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ryanodine, $^{42}\text{K}^+$ -uptake was markedly depressed ($P = >0.001$), there was a marked release of $^{42}\text{K}^+$, and an increase in $^{24}\text{Na}^+$ -uptake ($P = >0.001$).

Protoveratrine A and B had qualitatively similar effects, but protoveratrine A appeared to be more potent. At doses of 100 $\mu\text{g.}$ to 0.5 mg./ml. both depressed $^{47}\text{Ca}^{++}$ -uptake (protoveratrine A, $P = 0.05 < 0.02$; protoveratrine B, $P = 0.05 < 0.02$) and caused an increased release of $^{47}\text{Ca}^{++}$. This confirms the findings of Lister and Lewis (1959) using protoveratrine A. $^{42}\text{K}^+$ -uptake was not significantly (protoveratrine A, $P = 0.4 < 0.3$; protoveratrine B, $P = 0.2 < 0.1$) but $^{24}\text{Na}^+$ -uptake was markedly increased (protoveratrine A, $P = >0.001$; protoveratrine B, $P = 0.2 < 0.1$). In some experiments a slightly increased release of $^{42}\text{K}^+$ occurred.

These preliminary findings show that compounds capable of causing contracture of the isolated frog sartorius muscle increased both the release of calcium and potassium, and at the same time increased the uptake of calcium and sodium but depressed that of potassium. The more striking effects of ryanodine and nicotine are probably associated with their ability to cause a marked contractural response in skeletal muscle. The lesser effects of carbachol can perhaps be attributed to its low contracture-producing potency at the dose levels used, while protoveratrine does not cause its characteristic response in non-stimulated muscle.

Our results with ryanodine and nicotine are not unlike those of Bianchi and Shanes (1959) and Shanes and Bianchi (1960) who demonstrated that potassium depolarisation and electrical stimulation of the frog sartorius muscle were associated with an increase in both inward and outward fluxes of calcium. When ryanodine and nicotine are used, calcium may become more mobile and is perhaps displaced from combination with a carrier or receptor. This may result in the breakdown of barriers which retain potassium within the cell and sodium outside.

Shanes and his colleagues (1959) from experiments on the voltage-clamped squid axon, suggested that at lower levels of depolarisation, multivalent ions may react with sites on the cell membrane to reduce the number of these available for the passage through of monovalent ions. At higher levels of depolarisation more sites become available due to the displacement of the calcium ions. Our findings appear to be compatible with this view.

We hope to report our results more fully at a later date.

We are grateful to Dr. M. M. Bluhm for his help and advice, to Dr. G. Hoyle for a sample of ryanodine, to Dr. R. A. Ellis (Sandoz Ltd.) for supplies of protoveratrine A and B.

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