SOME PROPERTIES OF MIXED SOLS OF LECITHIN AND LYSOLECITHIN

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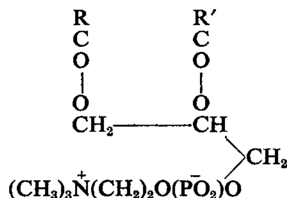
The preparations of pure crystalline lecithin and lysolecithin are described. Mixed sols of these phosphatides have been prepared and their viscosities, stabilities to salts, and haemolytic activities have been examined. The viscosity results indicate a marked interaction between the two compounds in the aqueous sols. The salt stability and haemolysis results are used to support a theory of cell membrane formation.

Lecithin no longer seems to have any direct application in pharmacy; the monograph on lecithin was removed from the last edition of the British Pharmaceutical Codex. As an emulsifying agent it is expensive and its therapeutic value is dubious. However, since the phosphatides, and lecithin in particular are important constituents of cell membranes their physical properties are of interest in elucidating the structure of such membranes. The soap-like structure of lecithin with its bi-ionic polar head group (I)



(I) (R,R′ are hydrocarbon chains, C_{13} up to C_{23} or higher)

renders it dispersible in water, and from the aqueous sols stable fatty films having some of the properties of simple cell membranes can be obtained at a boundary formed between the aqueous sol and a suitable salt solution[1].

The main difficulty in work with phosphatides is that of purifying the materials used. The physical chemistry of the lecithins was reviewed recently by Saunders and Elworthy[2] and it was pointed out that all the earlier work was carried out with samples of lecithin which contained varying amounts of lysolecithin (lysolecithin has the formula (I) with one of the fatty acid radicals RCO replaced by H). It was not until Lea and Rhodes developed their chromatographic purification on a silicic acid-Celite column in 1954[3] that complete removal of the lyso-compound from lecithin became possible. This removal causes a considerable change in the properties of aqueous lecithin sols particularly in their stability to salts and in the conditions necessary to form films from the sols[4]. The lyso-compound is invariably associated with lecithin from

natural sources and the present investigation has been undertaken to examine the interaction between the two phosphatides in water.

For this purpose pure synthetic materials would be ideal but the synthesis of quantities of lecithin[5] is difficult. The synthetic lecithins with saturated fatty acid groups also show considerable differences in their behaviour with water from the unsaturated material prepared from natural sources[6]. The synthetic compounds do not form stable sols.

The yolks of hen's eggs is an abundant source, containing up to 10 per cent dry weight of lecithin. But the extensive purification process results in a low yield. Lecithin so obtained has a mixture of fatty acid radicals in which palmitoyl, oleyl, and linoleyl radicals are the main components. In water, the phosphatides form large micelles in which all the fatty acid radicals are likely to be mixed in a random manner.

EXPERIMENTAL

Preparation of Lecithin

The yolks of twelve eggs were dried by extraction with 500 ml. of acetone, and the resulting solid filtered and extracted with 1 l. of warm ethanol in three successive portions. This extract was shaken with successive portions of alumina powder until it no longer gave a colouration with ninhydrin solution on warming. This treatment removed cephalins and other phosphatides containing primary amine groups. It was then evaporated to dryness at 40° under vacuum and the residue extracted with 150 ml. of ether. Crude lecithin was precipitated by adding to 500 ml. of cold acetone. The precipitate was dissolved in 40 ml. of 1:4-methanol-chloroform. The resulting solution was put on a column containing 75 g. of Mallinckrodt chromatographic silicic acid and 20 g. of Celite filter aid and the column developed with 1:4-methanol-chloroform. The first fraction contained some coloured compounds and a trace of ninhydrin reacting material which was rejected. The subsequent colourless effluent was collected until nearly all the lecithin had been eluted (about 1·5 l.), lysolecithin being firmly retained by the column, and was then centrifuged to remove any silicic acid and evaporated to dryness at 40°. The product was recrystallised three times from warm methyl-ethyl-ketone to give a white solid which showed parallel extinction under the polarising microscope. Finally it was dissolved in ethanol to give a 10 per cent solution which was stored in a nitrogen filled desiccator at −5°. We have found that although it is impossible to keep the solid in the dry state without decomposition, in ethanol solution it can be kept for many weeks without apparent change in its properties. The final yield was 5–6 g. of solid which had a nitrogen content of 1·74 per cent, a phosphorus content of 3·8 per cent and an iodine value of 73. The mean molecular weight calculated from the nitrogen and phosphorus contents was 810.

Preparation of Lysolecithin

This compound was prepared from the lecithin by a modification of Hanahan's method[7]. Five g. of lecithin was dissolved in 500 ml. of ether

and 5 ml. of an aqueous solution containing 10 mg. of Russell viper venom was added and the mixture shaken for a minute. After half an hour the liquid became turbid due to the hydrolysis of lecithin catalysed by the enzyme lecithinase A in the venom. This enzyme is a highly specific catalyst for the removal of one fatty acid radical from the lecithin molecule, to give lysolecithin which is insoluble in ether.

The lysolecithin was allowed to settle and the clear supernatant liquid was decanted. 50 ml. of acetone was added to granulate the slimy precipitate which was washed with acetone. The precipitate was dissolved in a minimum amount of chloroform and reprecipitated by adding it to 6 times its volume of ether. This procedure was repeated five times using a minimum volume of chloroform in each case. The final amorphous product was washed thoroughly with acetone, dried and dissolved in a small amount of warm ethanol. The solution was slightly cloudy owing to traces of proteinous matter from the venom and was centrifuged keeping the solution warm. The clear solution was then set aside for the lysolecithin to crystallise; the crystallisation was repeated twice. The author has added this crystallisation step to Hanahan's process which stopped at the amorphous material, in order to ensure removal of traces of venom from the product. The lysolecithin is a white solid of nitrogen content 2·8 per cent and phosphorus content 5·5 per cent, iodine value 4 (approx.), mean molecular weight from nitrogen and phosphorus 535, yield 2 g.

The low iodine value of the lysolecithin indicates that the venom removes the unsaturated fatty acid radicals preferentially from the lecithin; the molecular weight estimates indicate that the mean molecular weight of the acid removed was 275.

The lysolecithin was stored under ethanol.

Preparation of Aqueous Sols

Mixed sols of lecithin and lysolecithin were prepared by mixing ethanol solutions containing known amounts of the two phosphatides and evaporating to dryness, the residue being shaken with warm distilled water until dispersion was complete. Dispersion was checked by centrifugation and the sol was passed through an ion exchange column containing a mixture of the Amberlite resins IR-120 and IRA-400. These removed traces of small ions present as impurities, a fact checked by electrical conductivity measurements. The column was then washed with water and the combined effluent made up to the required volume. The loss of phosphatides in this step was small.

Properties of the Mixed Sols

Appearance. Pure lecithin sols although turbid in appearance were stable for long periods. Introduction of lysolecithin reduces the turbidity and at a weight fraction lysolecithin to total phosphatide of 0·35, the sols become optically clear.

Viscosity. The sols showed anomolous viscosities; for comparative purposes their times of flow through an Ostwald viscometer were divided

by the time of flow for a pure lecithin sol. The results for 0·5 per cent total phosphatide sols at 25° are shown in Table I.

Pure lecithin and lysolecithin sols both had viscosities similar to that of water. Introduction of lysolecithin into the lecithin sol caused a big rise in viscosity and at the maximum the sols were very thick liquids. These results indicate a very considerable interaction between the two compounds, in the mixed sols.

Stability to electrolytes. Film formation from a phosphatide sol is known to be related to the stability of the sol to electrolytes[4]. A salt solution of concentration such that it causes precipitation of the sol also gives films of measurable strength at a boundary formed between the sol and the salt solution.

TABLE I

RELATIVE VISCOSITIES OF MIXED LECITHIN AND LYSOLECITHINS SOLS AT 25° RELATIVE TO A PURE LECITHIN SOL. ALL SOLS CONTAINED 0·5 PER CENT TOTAL PHOSPHATIDES

Weight fraction of lysolecithin	Relative viscosity	Weight fraction of lysolecithin	Relative viscosity
0·0	1·0	0·5	2·5
0·2	1·1	0·7	2·9
0·3	1·4	0·8	2·2
0·35	1·7	0·9	1·6
0·45	2·4	1·0	1·0

Sol stabilities were examined by setting up a series of tubes each containing 1 ml. of 0·5 per cent total phosphatide sol. To these, small volumes of salt solutions were added from an Agla microsyringe. The resulting mixtures were thoroughly stirred and set aside overnight. It was found that pure lysolecithin sols were stable to all the concentrations of sodium and calcium chlorides examined. It was not until the lysolecithin weight fraction of a mixed sol was reduced below 0·4 that precipitation by calcium chloride was observed. At a weight fraction of 0·33 lysolecithin the phosphatide was completely precipitated by 10^{-4}M calcium chloride but not by 0·1M sodium or potassium chlorides. Pure lecithin sols were precipitated by 2×10^{-5}M calcium chloride and also by 0·01M sodium and potassium chlorides. Addition of the lyso compound to lecithin sols can produce any required degree of stability to salts.

Haemolytic properties. Lysolecithin owes its name to its ability to dissolve the membranes of erythrocytes, releasing their contents into the surrounding medium. If the theory of cell membrane formation given in the discussion is correct it is to be expected that the addition of lecithin to lysolecithin would remove the lysing action of the latter. To test this the effect of introducing lecithin into the lyso-sols was examined using rabbit's blood containing sodium citrate as an anticoagulant.

Blood, 0·5 ml., was mixed in a centrifuge tube with 0·1 ml. of 0·1M sodium chloride solution in which lysolecithin was dissolved to give a 0·5 per cent sol, in addition varying quantities of lecithin were present in this sol. Each final mixture therefore contained a lysolecithin concentration of 0·08 per cent.

After mixing, the tubes were centrifuged for one minute and the extent of haemolysis estimated from the colour of the supernatant liquid. Table II shows the results, the amount of lecithin being shown as a weight fraction of total phosphatides.

It is seen that at a lecithin weight fraction between 0·53 and 0·68, that is a lysolecithin weight fraction between 0·47 and 0·32, the lysing action of the sol is lost.

TABLE II

HAEMOLYTIC EFFECTS OF MIXED SOLS. 0·5 ML. OF RABBITS BLOOD WITH 0·1 ML. OF SOL, 0·1M WITH RESPECT TO NaCl AND 0·5 PER CENT WITH RESPECT TO LYSOLECITHIN

Lysolecithin in final mixture per cent	Lecithin weight fraction	Lysolecithin weight fraction	Appearance of supernatant liquid
0·00	0	1	No colour
0·08	0	1	Deep red, complete lysis
0·08	0·37	0·63	Deep red, complete lysis
0·08	0·53	0·47	Pink, slight lysis
0·08	0·68	0·32	No colour

DISCUSSION

The pronounced interaction between lecithin and lysolecithin in aqueous sols, shown by the viscosity results, has not previously been reported. At a lysolecithin weight fraction of 0·35 to 0·4, important changes in the properties of the sols occur; below this range, the sols are precipitated by salts and any required degree of stability to salt solutions can be obtained by adjusting the proportion of the lyso-compound in the sol. Below the 0·35 to 0·4 range, the sols lose their lysing effect on erythrocyte membranes. It should be noted that a lysolecithin weight fraction of 0·39 corresponds to an equimolecular mixture of the two phosphatides.

Pure lecithin sols are unstable to 0·01M sodium and potassium chlorides and so would be precipitated by salt solutions equivalent to the electrolyte composition of erythrocyte contents (mainly alkali metal chlorides total concentration $>$0·1M). Lysolecithin invariably occurs in association with lecithin from natural sources and this is to be expected since the former is the first decomposition product of the latter, and the specific enzyme for the conversion, lecithinase, is widely distributed in living systems. If sufficient lysolecithin is present in the internal fluid of a cell, it can stabilise the lecithin to the monovalent metal salts present, but can still permit precipitation of a phosphatide membrane when this fluid meets a solution containing divalent metal ions such as plasma (calcium ion concentration $>$0·001M). This membrane would be stable since the lysolecithin in the cell contents would not be lytic at the weight fraction necessary to give precipitation. In addition, the mixed phosphatide sol would have a high viscosity even without the presence of other colloidal material and so could be formed into units which would cohere until fatty membranes were formed around them.

In the development of a cell membrane the many other components present in the fluids will of course play a part, but it is suggested that this phosphatide balance in the cell contents is the important factor in the

primary formation of the cell membrane which is then probably strengthened by adsorption of proteins and modified by other lipids.

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DISCUSSION

The paper was presented by the AUTHOR.

The CHAIRMAN. The author was nearly dealing with the stoichiometric properties of lecithin; hydrolysis, as was suggested, took place from the ester group on the primary carbon atom, but it was also possible to break off the fatty acid grouping from the secondary carbon atom as well. How widely was lecithinase A distributed in the body?

MR. H. J. BRAGG (Folkestone). Had lecithin from soya bean been tried? There might be some natural antoxidants in soya bean which would help stabilise the lecithin. Chloroform might be a better volatile solvent than ethanol. The sols, although turbid, were said to be stable for a long period. For how long were they examined?

DR. J. B. STENLAKE (Glasgow). The viscosity measurements were made at 25° and he presumed that the other work was done at that temperature. Were the sols stable at 37° and at higher temperatures? What was the effect of other non-ionic substances on the stability of the sols towards electrolytes?

DR. G. E. FOSTER (Dartford). Were the lecithin and lysolecithin single substances? Was the hydroxyl group in the lysolecithin in the α or β position, or was it a mixture of the α and β esters?

MR. N. J. VAN ABBÉ (Loughborough). Was it known that lysolecithin was present *in vivo* with lecithin? Could such substances as rutin and hesperidin affect cell permeability by preventing the formation of lysolecithin in some way?

MISS A. E. ROBINSON (London). Did lysolecithin dissolve, disperse or solubilise the membrane? The erythrocyte membrane was thought to be a mosaic structure. It was stated in the paper that the phosphatides form large micelles in water, and the term "dissolve" seemed a vague one to use. Had the work of Rideal and Taylor on permeability of the cell been considered?

DR. SAUNDERS replied. A solvent which preserved the material for a reasonable period met his needs; ether free from peroxides was probably the best. The completely dispersed sol did not settle out for about a

week so long as it was protected from decomposition. At temperatures higher than 25° the sols in contact with air took up oxygen more quickly. Non-ionic surface-active materials like ether oxides increased the stability to electrolytes. Lecithin and lysolecithin were not single substances. All preparations of phosphatides from natural material were fractions. Single substances could be obtained only by synthesis. This was difficult. He believed Hanahan had found the OH group in lysolecithin to be in the α position. There was no analytical procedure to prove lysolecithin was present with lecithin *in vivo*, but it was the first decomposition product when lecithin was hydrolysed by lecithinase in the tissues. Lecithinase A occurred in a large number of body tissues. Viper venom was used because it was a concentrated source. Lysolecithin dissolved the fatty part of the erythrocyte membrane, which then broke up. It was behaving as a soap in its haemolytic effect. An inconclusive attempt had been made to estimate micelle size in the sols by diffusion studies.