Crystalline Lysoplasmalogen (Lysophosphatidal Choline): Preparation from Heart Muscle and Action on Erythrocytes and Spermatozoa

By E. F. HARTREE and T. MANN
A.R.C. Unit of Reproductive Physiology and Biochemistry, Molteno Institute and Department of Veterinary Clinical Studies, University of Cambridge

(*Received* 6 *October* 1959)

When mammalian spermatozoa are washed free from seminal plasma (and thus from fructose) and incubated as suspensions in Ringer solution they remain motile provided that oxygen is present. An explanation of this behaviour was put forward by Lardy & Phillips (1941*a*, *b*), who suggested that in the absence of the seminal sugar [later identified by Mann (1946) as fructose] the spermatozoa of bull and ram can derive energy for motility from the oxidative metabolism of intracellular phospholipids.

A study of the lipids present in freeze-dried spermatozoa provided no evidence for the presence of lecithin but showed that the phospholipid fraction consisted mostly of a choline-based plasmalogen (Lovern, Olley, Hartree & Mann, 1957). Subsequently investigations were made into the changes in lipid composition which occur when ram spermatozoa are freed from plasma and incubated as suspensions in Ringer solution (Hartree & Mann, 1958, 1959). The main change observed was a marked decrease in fatty acyl-ester bonds, and from this it was inferred that the reaction responsible for the decrease was the hydrolysis of the sperm plasmalogen to give lysoplasmalogen together with free fatty acid, which, through its oxidative metabolism, could provide the energy necessary to maintain motility.

Lysolecithin has already been shown to have a marked inhibitory action upon the respiration of ram spermatozoa (Dawson, Mann & White, 1957). Since choline lysoplasmalogen can be regarded as a possible product of intracellular-lipid metabolism of ram spermatozoa it was decided to prepare this compound and to compare its properties with those of lysolecithin. An ethanolamine lysoplasmalogen was obtained in crystalline form by Feulgen & Bersin (1939), who believed that they had isolated an unmodified muscle plasmalogen, and again by Rapport, Lerner, Alonzo & Franzl (1957), who established the relationship of the crystalline compound to the native ethanolamine plasmalogen of muscle. We have now obtained from ox-heart muscle the corresponding choline lysoplasmalogen in crystalline form and have studied its action upon erythrocytes and upon ram spermatozoa.

EXPERIMENTAL

Materials

Ram semen was collected and the spermatozoa were separated and washed as previously described (Hartree & Mann, 1959). As suspending media we have used (i) the calcium-free Ringer solution previously used by Mann (1946) for studies on the metabolism of spermatozoa ('sperm-Ringer'), and (ii) the same medium with the addition of phosphate buffer, pH 7·4, which was designated 'Ringer-phosphate' by Mann & White (1957). Suspensions of erythrocytes were prepared from defibrinated horse blood. The blood was centrifuged, the plasma sucked off and the cells were washed twice with Ringer-phosphate.

We are indebted to Dr R. M. C. Dawson for a sample of lysolecithin. This had been prepared from lecithin by the action of snake venom but had been crystallized from ethanol and so was presumably free from venom (see Marples & Thompson, 1958). The absence of venom was confirmed when it was found that the lytic action of the sample towards erythrocytes (see Analytical methods below) was unchanged by a second recrystallization from ethanol at −15°. Palmitaldehyde and its thiosemicarbazone were obtained as previously described (Hartree & Mann, 1959).

Ether was distilled from sodium and all other solvents were redistilled before use. All solvent mixtures are specified on a volume/volume (v/v) basis.

Analytical methods

Nitrogen was determined according to Chibnall, Rees & Williams (1943) and phosphorus according to King (1932). Estimations of aldehydogenic lipids (plasmalogen plus lysoplasmalogen) and of lipid acyl ester were made as previously described (Hartree & Mann, 1959). Choline was estimated by Glick's (1944) method. Fructose was determined according to Roe (1934), after deproteinization by the $Ba(OH)_2$–$ZnSO_4$ method of Somogyi (1945). Fructolysis was followed by the method of Mann (1948). Uptakes of O_2 were measured in Barcroft differential manometers at 37°; the centre wells of the flasks contained 2·5 N-NaOH and a roll of filter paper for absorption of CO_2. Lysis of erythrocytes was measured at 20° by the spectroscopic method of Keilin & Hartree (1946).

Chromatography on silicic acid columns was carried out at 20° according to Hanahan, Dittmer & Warashina's (1957) procedure for phospholipids. The adsorbent was a mixture of silicic acid (Mallinckrodt, 100 mesh, analytical reagent, 'suitable for chromatographic analysis') and

Hyflo Supercel (Johns–Manville Co. Ltd.). Ascending chromatography on silicic acid-impregnated papers was performed as described by Marinetti & Stotz (1956) except that smaller papers were used and the duration of the runs was curtailed. The papers (Whatman no. 1) were in the form of cylinders, 16 cm. × 5·5 cm. diam., and the chromatograms were developed at 24° in 2 lb. screw-top fruit-preserving jars. The developing solvent was di*iso*butylketone–acetic acid–water (8:5:1). Since lysoplasmalogen is known to be acid-labile the chromatograms were run for only 3 hr. The resolution under these conditions was less complete than with the larger papers and longer times as specified by Marinetti & Stotz (1956). The lipids on the developed chromatograms were made visible by treatment with rhodamine 6G and examination in u.v. light (Marinetti & Stotz, 1956). Aldehydes and aldehydogenic lipids on the papers were detected as follows. After the treatment with rhodamine 6G the papers were placed for 5 min. in the magenta–bisulphite reagent of Feulgen, Boguth & Andresen (1951), washed for 5 min. in running water, placed for 5 min. in 0·5% (w/v) sodium metabisulphite in 0·1 N-HCl, washed again with water and finally soaked for 1 min. in ethanol.

Preparation of choline lysoplasmalogen (lysophosphatidal choline)

All evaporations were carried out under reduced pressure in a rotary evaporator.

A mixture of lecithin and choline plasmalogen was isolated from ox-heart muscle by Pangborn's method (see Lees, 1957). Purification was carried to the stage of treatment with $Ba(OH)_2$ and CO_2. Three hearts yielded 710 g. of acetone-dried powder from which 10·4 g. of lecithin–plasmalogen mixture was obtained. According to Rapport & Franzl (1957) this fraction contains 60% of choline plasmalogen and 40% of lecithin.

The lipid mixture was hydrolysed under conditions where only acyl-ester bonds are split (Dawson, 1954; Rapport & Lerner, 1958*a*), and treated in a manner similar to that described by Thannhauser, Boncoddo & Schmidt (1951). The lipid (5·2 g.) was dissolved in a mixture of 720 ml. of methanol and 90 ml. of water at 37° and treated with 200 ml. of warm methanolic N-NaOH. The mixture was held at 37° for 20 min. and then cooled to −50° in solid CO_2–acetone. To the cold mixture methanolic acetic acid was added dropwise, with thorough mixing until the pH fell to 6·5. About 200 ml. of normal acid was required. The solution was evaporated to a syrup, which was shaken with a mixture of 150 ml. of water, 40 g. of Na_2SO_4 and 140 ml. of chloroform–methanol (9:1). The cloudy lower layer was evaporated and the residue was dissolved in 40 ml. of the same chloroform–methanol mixture. This solution was treated with 10 vol. of acetone and left overnight at 4°. The resulting precipitate was shaken with 50 ml. of chloroform–methanol, a small insoluble residue was filtered off and the filtrate treated with 200 ml. of ether. After the mixture had been held at −15° for 4 hr. the precipitate was collected in a refrigerated centrifuge, washed with 100 ml. of cold ether and freed from residual solvent in a vacuum desiccator. The product was dissolved in 3 ml. of methanol, diluted with 10 vol. of propan-2-ol and stored overnight at −15°. A small yellow precipitate was centrifuged down in the cold and discarded. The supernatant solution was evaporated to give 614 mg. of lipid (containing 4·35% of phosphorus), which was dissolved in chloroform–methanol (1:1) to a volume of 10 ml. and stored at −15°.

Further purification was effected on a column, 28 mm. diam., containing a mixture of 36 g. of silicic acid and 18 g. of Hyflo Supercel which had been previously well washed with chloroform–methanol (1:1) and then poured into the column as a suspension in the same solvent mixture. Of the above-mentioned lipid solution, 8·9 ml. (containing 23·7 mg. of phosphorus) was added to the column, which was then developed by successive addition of the following solvents: 350 ml. of chloroform–methanol (1:1), 470 ml. of chloroform–methanol (2:5), 350 ml. of methanol, 350 ml. of methanol–water (9:1). A slight positive pressure ($> 0{\cdot}4$ atm.) of N_2 was applied to the column to maintain a flow rate of 4–5 ml./min. The outflowing solvent was collected as 9·4 ml. fractions. The results of this fractionation in terms of phosphorus (mg./fraction) are shown in Fig. 1. Fractions 12–20 were combined and evaporated to yield a mixture of yellow gum and white solid. From fractions 32–47 a similar but much smaller residue was obtained in which the solid predominated. These two fractions were combined to yield a chloroform-soluble gum (fraction A; 34·7 mg.) and an insoluble residue which was found to be silicic acid.

The main lipid component was present in fractions 51–76, which were combined and evaporated to yield a white waxy solid. This was dissolved in 6 ml. of chloroform–methanol (1:1) and a small residue of silicic acid was centrifuged down. The clear supernatant solution was treated with 40 ml. of ether and left overnight at 5°. The resulting white precipitate was centrifuged down in the cold and washed at 5° with ether–chloroform (10:1) and then with ether. The solid was dissolved in 1·5 ml. of methanol and ether was added, at the boiling point of the mixture, until a slight opalescence persisted. The mixture was cleared by addition of 1 drop of methanol and allowed to cool to 20°. The lipid crystallized in rosettes and bundles of fine needles (Plate 1). Crystallization was completed by cooling the

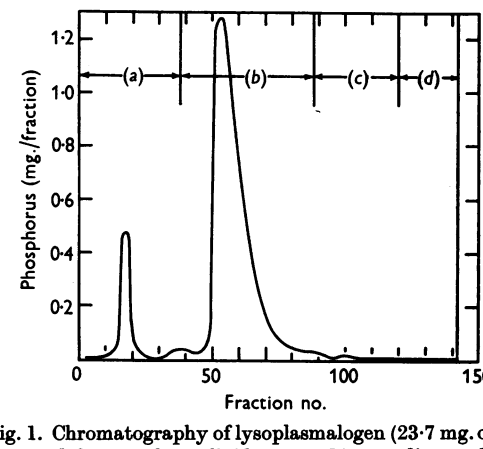
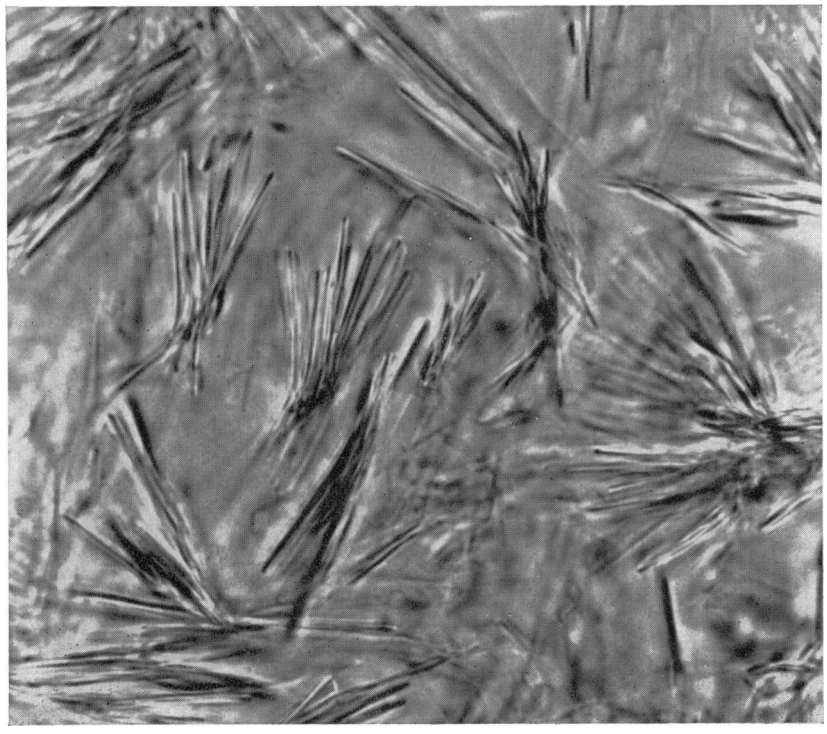


Fig. 1. Chromatography of lysoplasmalogen (23·7 mg. of P), prepared from ox-heart lipids, on an 18 mm. diam. column consisting of 36 g. of silicic acid and 18 g. of Hyflo Supercel. Solvents: (*a*) chloroform–methanol (1:1); (*b*) chloroform–methanol (2:5); (*c*) methanol; (*d*) methanol–water (9:1).



Crystalline choline lysoplasmalogen (× 1500) from methanol–ether.

mixture to $-10°$, centrifuging the mixture at the same temperature and washing the crystals with cold ether. The lipid was too hygroscopic to be obtained in the form of a powder and was therefore dissolved in dry methanol to a final volume of 10 ml. (fraction B; 190 mg.). In Table 1 are shown the analyses of fractions A and B together with theoretical values for stearal lysoplasmalogen.

Hydrogenation of lysoplasmalogen. According to Rapport *et al.* (1957), hydrogenation of plasmalogen under the conditions described by Mead & Howton (1950) results in the uptake of 1 mole of H_2, which adds on to the unsaturated ether grouping. The resulting saturated ether is acid-stable and is devoid of aldehydogenic properties. The procedure of Mead & Howton was followed except that uptake of H_2 was not measured. A solution of 38 mg. of lysoplasmalogen in 10 ml. of methanol was shaken for 2 hr. with 2 mg. of palladium–charcoal (5%, w/w) in an atmosphere of H_2. The catalyst was removed by centrifuging in the presence of a little Hyflo Supercel and the solution was cooled to $-15°$. The saturated ether was deposited as a white crystalline powder (25 mg.). From determinations of the ratio aldehyde:phosphorus it could be concluded that the lipid contained less than 1% of residual plasmalogen.

RESULTS

Comparison of the lytic activities towards erythrocytes of lysolecithin, lysoplasmalogen and hydrogenated lysoplasmalogen

The spectroscopic method for measuring rates of lysis (Keilin & Hartree, 1946) takes advantage of the fact that erythrocytes are impermeable to ferricyanide ions. Thus if ferricyanide is present in the iso-osmotic suspending medium the progress of lysis can be followed in terms of the appearance of methaemoglobin, which is formed instantaneously when the liberated oxyhaemoglobin comes into contact with ferricyanide. The ratio methaemoglobin:oxyhaemoglobin in the mixture can be determined continuously by visual spectroscopic comparison of the sample with an optical mixture of the two pigments contained separately in the compartments of a double-wedge trough.

Washed horse erythrocytes were suspended in Ringer-phosphate at a concentration of about 10^6 cells/ml., and solid $K_3Fe(CN)_6$ was added to give a concentration of 1·5 mM. This suspension was placed in both compartments of the double-wedge trough and lysolecithin in excess was added to one compartment to lyse the cells. A sufficient volume of the same suspension was placed in a flat-bottomed glass tube so that the depth of suspension equalled the optical width (2·3 cm.) of the double-wedge trough. To this end 4·05 ml. of suspension was added to a 15 mm. diam. tube. The lyso compound, dissolved in $\geq 0·2$ ml. of Ringer-phosphate, was stirred into the flat-bottomed tube at zero time and the time for 80% lysis was measured with a stop-watch. The results recorded in Table 2 indicate that the three lyso compounds under test have very similar activities and that in all cases a minimum of about 5×10^7 molecules of

Table 1. *Analysis of lipid-fractions A and B from silicic acid column and theoretical figures for stearal lysoplasmalogen*

	Fraction A (m-equiv./g. of lipid)	Fraction B (%)	Fraction B (m-equiv./g. of lipid)	Theoretical values for stearal lysoplasmalogen (m-equiv./g. of lipid)
Phosphorus	0·414	5·92	1·92	1·92
Nitrogen	0·447	2·73	1·95	1·92
Fatty aldehyde	0·256	—	1·91	1·92
Choline	0·116	—	1·93	1·92
Acyl ester	0·045	—	0·02	0

Table 2. *Lytic activities of lysolecithin, lysoplasmalogen and hydrogenated lysoplasmalogen towards horse erythrocytes* ($1·05 \times 10^6$/*ml.*) *at* 20°

Lysin	Concentration of lysin (μM)	$10^{-8} \times$ Molecules of lysin/cell	Time for 80% lysis (sec.)
Lysolecithin	24	1·36	18
	12	0·68	29
	6	0·34	No lysis in 25 min.
Lysoplasmalogen	24	1·36	20
	12	0·68	29
	6	0·34	No lysis in 25 min.
Hydrogenated lysoplasmalogen	24	1·36	20
	12	0·68	27
	6	0·34	40% lysis in 25 min. No further change at 50 min.

lysin/erythrocyte is necessary for lysis to occur at a significant rate.

Effect of lyso compounds upon the metabolism of ram spermatozoa

Effect of lysolecithin, lysoplasmalogen and hydrogenated lysoplasmalogen upon respiration. Manometer flasks received 2 ml. of suspension of washed spermatozoa ($\sim 10^9$ cells) in sperm-Ringer together with either 1 ml. of Ringer-phosphate or 1 ml. of a solution of lyso compound in the same medium. The flasks were equilibrated for 15 min. before the taps were closed.

The effects of the three lyso compounds upon the oxygen uptake of spermatozoa are shown in Fig. 2. Under the experimental conditions both lysoplasmalogen and its hydrogenated derivative were more inhibitory than lysolecithin. Thus the first two compounds were effective inhibitors at concentrations below 1 mM whereas lysolecithin had a slight stimulating effect at the same level and only became inhibitory at concentrations above 1 mM.

Effect of lysoplasmalogen on fructolysis. For this experiment 4 ml. of whole ram semen was diluted with 6 ml. of sperm-Ringer and 15 ml. of 0·5 % (w/v) fructose in Ringer-phosphate. Of this suspension, 10 ml. was diluted with 5 ml. of Ringer-phosphate and a second 10 ml. with a solution of 25 mg. of lysoplasmalogen in 5 ml. of Ringer-phosphate. Two Barcroft manometers each received 3 ml. of control suspension and a further two each received 3 ml. of suspension containing lysoplasmalogen (final concentration 3 mM). During a period of 100 min. an 82 % inhibition of oxygen uptake was observed. Anaerobic incubations at 37° for the same period were carried out by placing each suspension in a narrow stoppered test tube with the minimum air space. The fructose contents of the freshly made and the incubated suspensions are recorded in Table 3. Under anaerobic conditions fructolysis was inhibited by 70 %. In the presence of air, the effect of lysoplasmalogen was less marked.

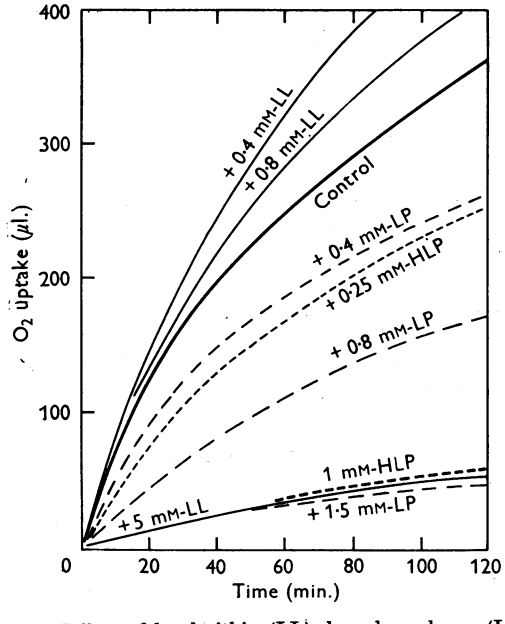


Fig. 2. Effect of lysolecithin (LL), lysoplasmalogen (LP) and hydrogenated lysoplasmalogen (HLP) on the respiration of washed ram spermatozoa (10^9 cells) suspended in a mixture of sperm-Ringer (2 ml.) and Ringer-phosphate (1 ml.).

Absence of fatty-aldehyde formation during incubation of spermatozoa with lysoplasmalogen. The lability of lysoplasmalogen in acid solution is such that paper chromatography under the conditions described by Marinetti & Stotz (1956) leads to quantitative hydrolysis and the appearance of a compact spot of free fatty aldehyde at the solvent front. Under the conditions specified for the present investigation there is considerable forward streaming of free aldehyde from the slow-moving lysoplasmalogen spot during the 3-hr. run, but no aldehyde reaches the solvent front (Fig. 3). It was thus possible to demonstrate that contact of lysoplasmalogen with ram spermatozoa does not lead to liberation of free aldehyde.

Incubation of washed spermatozoa with 8 mM-lysoplasmalogen was carried out in duplicate manometers for 2 hr. as described in the previous section on respiration of spermatozoa (see Fig. 2). Two control manometers were run simultaneously. The effect of the lyso compound was to inhibit respiration by 90 %. At the end of the incubation the contents of the duplicate manometers were

Table 3. *Effect of lysoplasmalogen on fructolysis in ram semen*

The semen was diluted with 8·4 vol. of a mixture of sperm-Ringer and Ringer-phosphate containing 10 mM-fructose (see text). Lysoplasmalogen was present at a concentration of 3 mM. Aerobic experiments were carried out in Barcroft manometers; anaerobic conditions were achieved by placing the suspensions in stoppered tubes with the minimum air space. Temp., 37°.

	Fructose concentration (mM)	
	Without lysoplasmalogen	With lysoplasmalogen
Initial suspension	8·5	8·5
After aerobic incubation for 100 min.	6·2	7·3
After anaerobic incubation for 100 min.	4·8	7·3

combined to give two samples (with and without lysoplasmalogen) of 6 ml. To each was added, with shaking, 120 ml. of chloroform–methanol (2:1). The precipitate was filtered off and the filtrate shaken with 0·2 vol. of 3 mM-$MgCl_2$ (Folch, Lees & Sloane-Stanley, 1957). The lower layer was evaporated completely and the residue dissolved in 1·2 ml. of methanol. Of the two solutions thus obtained 20 μl. was spotted on to silicic acid-impregnated paper and the chromatogram was developed for 3 hr. A palmitaldehyde standard was run simultaneously.

The results obtained after staining the paper with Schiff's reagent are represented in Fig. 3. The nature of the components obtained from sperm lipid (I) has not been completely established but the spots are provisionally identified as follows:

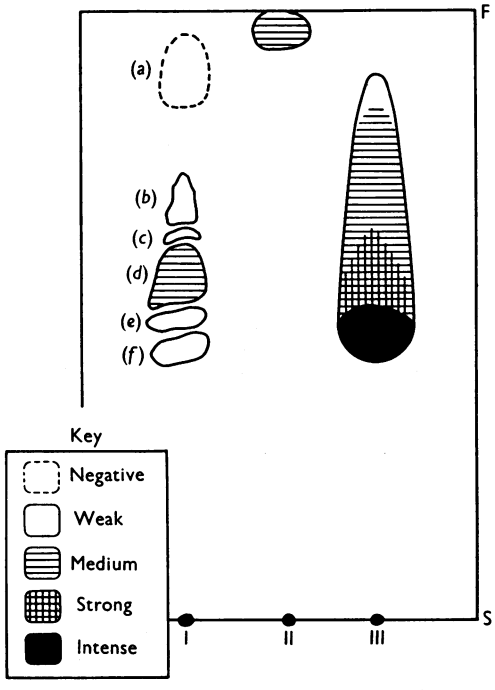


Fig. 3. Chromatograms on silicic acid-impregnated paper of palmitaldehyde and of lipids extracted from ram spermatozoa±lysoplasmalogen. Solvent: di*iso*butylketone–acetic acid–water (8:5:1), run for 3 hr. at 24°. The washed spermatozoa had previously been incubated aerobically for 2 hr. under the conditions of the experiments in Fig. 2. I, Lipids (≡30 μl. of semen) extracted from incubated spermatozoa; II, palmitaldehyde (0·08 μmole); III, lipids extracted from spermatozoa previously incubated in the presence of 8 mM-lysoplasmalogen (0·96 μmole of lysoplasmalogen present on the paper). S, Starting line; F, solvent front. Spots were made visible by staining with Rhodamine 6G and examination under ultraviolet light. The paper was subsequently treated with Schiff's reagent to give staining reactions indicated in the key.

(*a*) neutral lipids and fatty acids; (*b*) free aldehyde derived from labile aldehydogenic lipids; (*c*) ethanolamine plasmalogen; (*d*) choline plasmalogen (major component); (*e*) sphingolipid plus a plasmalogen-like lipid; (*f*) inositides plus lyso compounds (including any lysoplasmalogen). Authentic palmitaldehyde (II) travelled at the solvent front. Lipids derived from the mixture of spermatozoa and lysoplasmalogen (III) gave the staining pattern characteristic of lysoplasmalogen with considerable free aldehyde streaming ahead of the lipid spot. Chromatography of a mixture of palmitaldehyde and III produced a normal aldehyde spot at the solvent front. Thus the rate of movement of free fatty aldehyde was not influenced by the presence of an excess of other lipids. Since the molar ratio lysoplasmalogen in III/palmitaldehyde in II was 12 and since no Schiff's-positive reaction was obtained at the solvent front in III it can be deduced that no appreciable formation of free aldehyde occurred before the application of spot III to the paper.

DISCUSSION

The exact chemical constitution of the crystalline lysoplasmalogen described in this paper remains uncertain. It is now well established that the aldehydogenic properties of plasmalogens and lysoplasmalogens derive from the presence of an acid-labile $\alpha\beta$ unsaturated ether group linking a non-polar fatty chain to a carbon atom of glycerol (Debuch, 1957; Rapport & Lerner, 1958*b*; Blietz, 1958). The ready cleavage of the unsaturated ether to yield fatty aldehyde is the basis of methods for estimation of plasmalogen. It is still a matter of dispute which hydroxyl group of glycerol is involved in ether formation (Klenk & Debuch, 1959). Before the unsaturated-ether nature of plasmalogens had been established it had been widely accepted that the acid-labile aldehydogenic group was linked at the α'-carbon atom (Klenk & Debuch, 1954). Whereas one school supports this contention by postulating an unsaturated ether at the α' position and an acyl ester at the β position of glycerol (Marinetti & Erbland, 1957; Marinetti, Erbland & Stotz, 1958*a*), other investigators adduce evidence for the reverse (Rapport *et al.* 1957; Gray, 1957). The problem has been rendered more complex by the findings (i) that at least in pig-heart muscle both isomeric plasmalogens may occur (Marinetti & Erbland, 1957; Marinetti *et al.* 1958*a*), and (ii) that the action of snake venom on lecithin can, contrary to general belief, give rise not only to β-lysolecithin but also to α'-lysolecithin (Marinetti, Erbland & Stotz, 1958*b*). It thus follows that the ability of venom to split fatty acid from plasmalogens (Rapport & Franzl, 1957; Hartree & Mann, 1959) is no longer

a satisfactory criterion for an α′-acyl-ester structure. In lysolecithin the transfer of acyl-ester linkage from the β to the α′ position takes place readily in dilute acid or in the presence of a specific enzyme (Uziel & Hanahan, 1957). Thus even when the structure of a plasmalogen has been fully established, the nature of a derived lysoplasmalogen will remain uncertain until the possibilities of β–α′ migration have been eliminated. We have found that paper chromatography of ram-sperm lipid indicates the presence in very small amount of a second plasmalogen-like component [Fig. 3 (*e*)]. It is thus possible that both plasmalogen isomers are present in ram spermatozoa.

If we are correct in suggesting (Hartree & Mann, 1959) that in the absence of fructose the plasmalogen in ram spermatozoa breaks down to provide fatty acids as a source of energy, then lysoplasmalogen should also be formed. It is remarkable that the three lyso compounds examined in the present study, despite their distinctive chemical properties, should be indistinguishable in terms of their power to lyse erythrocytes. Since lysoplasmalogen is highly active in this respect it appeared feasible to repeat with ram spermatozoa the experiments carried out by Maggio & Monroy (1955) on sea-urchin (*Arbacia lixula*) spermatozoa. It had been established earlier by Rothschild & Cleland (1952) that metabolism in sea water of spermatozoa of the sea urchin *Echinus esculentis* involves extensive breakdown of phospholipids. Maggio & Monroy (1955) found that when sea-urchin spermatozoa were suspended in sea water a substance diffused from the spermatozoa which had a lytic action upon erythrocytes. Furthermore, this lytic action became more pronounced if lipovetillin was added to the suspension. It thus seems likely that these spermatozoa contain a phospholipase A that can hydrolyse both intra- and extra-cellular phosphatides.

We carried out similar experiments with suspensions of washed ram spermatozoa in sperm-Ringer but in no case could any lytic agent be detected in the Ringer medium. This did not appear to be due to simultaneous production of a lysis inhibitor since lysolecithin and lysoplasmalogen, when added to such suspensions, exhibited their normal lytic activities. These negative results do not dismiss the possibility that lysoplasmalogen is formed when motile ram spermatozoa are stored in sugar-free media. It is known that mammalian spermatozoa are less permeable than sea-urchin spermatozoa (Mann, 1954): thus inward diffusion of lipids and outward diffusion of such metabolic products as lysophosphatides may not occur. This non-diffusibility may be due to a stronger lipid–protein binding, which, in itself, might modify or even suppress the inhibitory action of any intracellular lysoplasmalogen.

Although extracellular lysoplasmalogen is apparently not metabolized by ram spermatozoa it is still possible that the same lipid, if it is produced within the cell, may undergo further changes. It is hoped that new conditions for paper chromatography, under which lysoplasmalogen will be both stable and well separated from other lipids, will throw light on this problem. In the meantime it can be stated that there are significant decreases in the plasmalogen content of ram spermatozoa after incubation in sugar-free media (E. F. Hartree & T. Mann, unpublished results).

The toxic actions of snake and other venoms on living cells and on isolated enzymes and enzyme systems (Zeller, 1951; Braganca & Quastel, 1953) have in the main been ascribed to the presence of phospholipase A. Thus by heat-treatment of cobra and viper venoms Braganca & Quastel (1953) were able to inactivate all enzymes known to be present except phospholipase A. With such heated venoms they demonstrated that the types of enzyme system which were inhibited were those in which activity is linked with structural organization of component catalysts: soluble enzymes were mainly unaffected. The observed effects could be interpreted as an action of phospholipase A upon the lipoproteins which bind together the components of organized multi-enzyme systems.

Since lysolecithin is a product of phospholipase A activity and since it is highly surface-active it is not easy to distinguish between the effects upon cells or enzyme systems of phospholipase A, which hydrolyses lecithin, and of lysolecithin itself. Webster (1957) and Thompson & Webster (1957) associate the 'clearing action' of lysolecithin upon dispersions of brain and other tissues with its effect upon lipoproteins. This clearing action is independent of the presence of venom impurity (Habermann & Neumann, 1954) and is qualitatively indistinguishable from that of other surface-active agents.

Oxidative phosphorylation in mitochondria and in tissue dispersions is completely abolished by very low concentrations of lysolecithin (Habermann, 1954; Witter, Morrison & Shepardson, 1957); similarly the complete reaction chain for oxidation of succinate or β-oxybutyrate by oxygen is sensitive to lysolecithin but the component parts (e.g. succinic dehydrogenase, cytochrome oxidase) are not. These results suggest that the effect of lysolecithin is upon the binding structures of the complex of enzymes making up the respiratory chain rather than upon individual enzymes. Comparison of these results with those of Braganca & Quastel suggest that the action of venoms is due essentially to the lysolecithin that is released by phospholipase A action.

Whereas both snake venom and lysolecithin have powerful inhibitory actions upon respiration and motility of spermatozoa, their effects upon sperm morphology are quite distinct. Treatment of ram spermatozoa with venom in the manner previously described (Hartree & Mann, 1959) gives rise to a loosening and fragmentation of the outer cell structure and also to a separation of some of the heads from midpiece–tails. Dawson *et al.* (1957) reported that lysolecithin treatment of ram spermatozoa brings about a detachment of heads. However, with lower concentrations, we have observed neither detachment nor cellular disruption when spermatozoa are incubated at 37° with 5 mM-lysolecithin or lysoplasmalogen for as long as 2 hr. Under the same conditions, however, the inhibitory effect upon motility and respiration is apparent after very brief contact (Fig. 2).

Since both respiration and fructolysis are sensitive to lysolipids it may be assumed that the action of such compounds is not restricted to a particular enzyme or enzyme complex. It is possible that their action is primarily upon the lipoprotein of the outer cell structure, leading to impairment of selective permeability, with secondary actions upon the internal organization of the cell. The slight activating effect of lysolecithin at certain concentrations (Fig. 2) may be a resultant of the characteristic inhibitory effect of a lysolipid and the non-specific protective effect of phospholipids upon respiration and motility of spermatozoa.

SUMMARY

1. A choline-based lysoplasmalogen has been prepared in crystalline form from an alkaline hydrolysate of the lecithin fraction from ox heart. The ratio phosphorus:choline:fatty aldehyde is 1:1:1.

2. Lysoplasmalogen, hydrogenated lysoplasmalogen and lysolecithin are quantitatively equivalent as lysins for horse erythrocytes.

3. The respiration of ram-sperm suspensions (3×10^8 cells/ml.) is inhibited by lysolecithin only at concentrations greater than 1 mM. The other two lyso compounds are effective inhibitors at lower concentrations. Fructolysis in ram semen is also inhibited by lysoplasmalogen.

4. No free fatty aldehyde is formed when lysoplasmalogen is incubated with ram spermatozoa.

One of the authors (E. F. H.) wishes to express his thanks to Dr J. Hanahan and to Dr G. V. Marinetti for the facilities provided in their Laboratories for study of lipid chromatography.

REFERENCES

Blietz, R. J. (1958). *Hoppe-Seyl. Z.* **310**, 120.
Braganca, B. M. & Quastel, J. H. (1953). *Biochem. J.* **53**, 88.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Dawson, R. M. C. (1954). *Biochim. biophys. Acta*, **14**, 374.
Dawson, R. M. C., Mann, T. & White, I. G. (1957). *Biochem. J.* **65**, 627.
Debuch, H. (1957). *Biochem. J.* **67**, 27P.
Feulgen, R. & Bersin, T. (1939). *Hoppe-Seyl. Z.* **260**, 217.
Feulgen, R., Boguth, W. & Andresen, G. (1951). *Hoppe-Seyl. Z.* **287**, 90.
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
Glick, D. (1944). *J. biol. Chem.* **156**, 643.
Gray, G. M. (1957). *Biochem. J.* **67**, 26P.
Habermann, E. (1954). *Naturwissenschaften*, **41**, 429.
Habermann, E. & Neumann, W. (1954). *Hoppe-Seyl. Z.* **297**, 179.
Hanahan, D. J., Dittmer, J. C. & Warashina, E. (1957). *J. biol. Chem.* **228**, 685.
Hartree, E. F. & Mann, T. (1958). *Biochem. J.* **69**, 50P.
Hartree, E. F. & Mann, T. (1959). *Biochem. J.* **71**, 423.
Keilin, D. & Hartree, E. F. (1946). *Nature, Lond.*, **157**, 210.
King, E. J. (1932). *Biochem. J.* **26**, 292.
Klenk, E. & Debuch, H. (1954). *Hoppe-Seyl. Z.* **296**, 179.
Klenk, E. & Debuch, H. (1959). *Annu. Rev. Biochem.* **28**, 39.
Lardy, H. A. & Phillips, P. H. (1941*a*). *Amer. J. Physiol.* **133**, 602.
Lardy, H. A. & Phillips, P. H. (1941*b*). *Amer. J. Physiol.* **134**, 542.
Lees, M. B. (1957). *Methods in Enzymology*, vol. 3, p. 334. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Lovern, J. A., Olley, J., Hartree, E. F. & Mann, T. (1957). *Biochem. J.* **67**, 630.
Maggio, R. & Monroy, A. (1955). *Exp. Cell Res.* **8**, 240.
Mann, T. (1946). *Biochem. J.* **40**, 481.
Mann, T. (1948). *J. agric. Sci.* **38**, 323.
Mann, T. (1954). *The Biochemistry of Semen.* London: Methuen.
Mann, T. & White, I. G. (1957). *Biochem. J.* **65**, 634.
Marinetti, G. V. & Erbland, J. (1957). *Biochim. biophys. Acta*, **26**, 429.
Marinetti, G. V., Erbland, J. & Stotz, E. (1958*a*). *J. Amer. chem. Soc.* **80**, 1624.
Marinetti, G. V., Erbland, J. & Stotz, E. (1958*b*). *Biochim. biophys. Acta*, **33**, 403.
Marinetti, G. V. & Stotz, E. (1956). *Biochim. biophys. Acta*, **30**, 187.
Marples, E. A. & Thompson, R. H. S. (1958). *Biochim. biophys. Acta*, **30**, 187.
Mead, J. F. & Howton, D. R. (1950). *Analyt. Chem.* **22**, 1204.
Rapport, M. L. & Franzl, R. E. (1957). *J. biol. Chem.* **225**, 851.
Rapport, M. M. & Lerner, B. (1958*a*). *J. biol. Chem.* **232**, 63.
Rapport, M. M. & Lerner, B. (1958*b*). *Biochim. biophys. Acta*, **33**, 319.
Rapport, M. M., Lerner, B., Alonzo, N. & Franzl, R. E. (1957). *J. biol. Chem.* **225**, 859.

Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
Rothschild (Lord) & Cleland, K. W. (1952). *J. exp. Biol.* **29**, 66.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Thannhauser, S. J., Boncoddo, N. F. & Schmidt, G. (1951). *J. biol. Chem.* **188**, 417.
Thompson, R. H. S. & Webster, G. R. (1957). *Biochem. J.* **67**, 31P.
Uziel, M. & Hanahan, D. J. (1957). *J. biol. Chem.* **226**, 789.
Webster, G. R. (1957). *Nature, Lond.*, **180**, 660.
Witter, R. F., Morrison, A. & Shepardson, G. R. (1957). *Biochim. biophys. Acta*, **26**, 121.
Zeller, E. A. (1951). In *The Enzymes*, 1, part 2, p. 987. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.

Biochem. J. (1960) **75**, 258

Lactic Dehydrogenase and Cytochrome b_2 of Baker's Yeast

THE DEOXYRIBOSE POLYNUCLEOTIDE COMPONENT AND THE PHYSICOCHEMICAL PROPERTIES OF THE CRYSTALLINE ENZYME

BY C. A. APPLEBY* AND R. K. MORTON†
Department of Biochemistry, University of Melbourne, Australia

(*Received* 2 *July* 1959)

The absorption spectrum of reduced and oxidized crystalline cytochrome b_2 (Appleby & Morton, 1954, 1959*a*, *b*) shows a pronounced peak at about 265 mμ. It has been found that this absorption band is mainly due to a deoxyribose polynucleotide which is intimately associated with the enzyme as purified by the method of Appleby & Morton (1959*a*). This paper describes the chemical composition of this component and the nature of its association with the crystalline enzyme.

Electrophoresis and sedimentation studies of the crystalline cytochrome b_2 revealed only one protein component. The separation of nucleotide-depleted enzyme from intact enzyme by electrophoresis is described. The results confirm that yeast lactic dehydrogenase is identical with cytochrome b_2 and is a protein with both haem and flavin prosthetic groups (Appleby & Morton, 1954).

This work was reported by us at the meeting of the Australian and New Zealand Association for the Advancement of Science in Melbourne, 1955. It has been described by Appleby (1957), and brief accounts of some aspects have been published (Appleby & Morton, 1954; Morton, 1955, 1958).

MATERIALS

Crystalline yeast lactic dehydrogenase (cytochrome b_2*).* This was prepared as described by Appleby & Morton (1959*a*). Unless otherwise stated, the enzyme was recrystallized several times from a solution of the combined first crystals from a number of preparations.

Deoxyribonucleic acid and deoxyribonucleotides. Polymerized calf-thymus deoxyribonucleic acid (DNA) containing 8·2% of P was kindly supplied by Mr G. L. Ada, and chromatographically pure 5′-deoxyribonucleotides were obtained from the California Foundation for Biochemical Research.

Ribonucleic acid and ribonucleotides. Yeast ribonucleic acid (RNA) (British Drug Houses Ltd.) and chromatographically pure 5′-ribonucleotides (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used.

Alkaline phosphomonoesterase. The purified enzyme from calf intestinal mucosa (Morton, 1954) was used.

Deoxyribonuclease and ribonuclease. Crystalline preparations from pancreas were obtained from Worthington Biochemical Corp., U.S.A.

Bovine serum albumin. Crystalline protein (The Armour Laboratories, U.S.A.) was used.

METHODS

Estimation of enzymic activity. The units of activity and the assay procedures are given by Appleby & Morton (1959*a*).

Chemical determinations

Phosphate. For inorganic phosphate, the procedure of Weil-Malherbe & Green (1951) was modified so that one-third of the amounts of all reagents were used in a final volume of 3·1 ml. With these volumes it was found necessary to shake for 1 min. instead of 15 sec. *E* was read at 730 mμ.

For total phosphate the sample was digested for 10 min. with 0·12 ml. of 10N-H_2SO_4 and 0·05 ml. of 72% (v/v) perchloric acid, the digest was diluted to 1·5 ml. with water, hydrolysed for 10 min. at 100° and cooled. The inorganic phosphate was then estimated, acid being omitted from the molybdate reagent.

* Present address: Division of Plant Industry, C.S.I.R.O., Canberra.
† Present address: Department of Agricultural Chemistry, The Waite Agricultural Research Institute, The University of Adelaide.