J. Pharm. Pharmac., 1969, **21**, *Suppl.*, 189 S–194 S *Received April* 28, 1969

The haemolytic activity of sols containing progesterone solubilized by phosphatidylcholine and lysophosphatidylcholine

I. W. KELLAWAY AND L. SAUNDERS

*Physical Chemistry Laboratories, The School of Pharmacy, Brunswick Square, London, W.C.*1, *England*

Lysis of rat erythrocytes by aqueous dispersions containing lysophosphatidylcholine (LPC), phosphatidylcholine (PC) and progesterone has been studied. The haemolytic activity of LPC was reduced by saturating it with progesterone; mixed LPC–PC micelles owed their lytic activity to the LPC alone. Cholesterol solubilized together with progesterone by LPC greatly reduced the lytic activity of the LPC, and when solubilized with progesterone by a 1:1 mixture of LPC and PC, prevented haemolysis. Triolein also inhibited the haemolytic properties of the sols.

The dispersion of steroids in aqueous solutions offers the possibility of producing alternative dosage forms for these compounds. In this work the co-dispersion of progesterone with phospholipids has been explored. Saunders, Perrin & Gammack (1962) reported that large concentrations of cholesterol can be co-dispersed with egg phosphatidylcholine (PC) to give clear and stable aqueous sols. Hoyes & Saunders (1966) and Kellaway & Saunders (1967) have also reported on the co-dispersion of other steroids with PC. In general, cholesterol-like steroids with only one polar group in the molecule are readily dispersed, whereas the hormones and other steroids with polar groups at opposite ends of the molecule are much less co-dispersed. Kellaway & Saunders have shown that the bipolar steroids can be dispersed up to a concentration greater than 2% in water by using mixed sols of PC and lysophosphatidylcholine (LPC). Use of LPC as a dispersing agent introduces undesirable lytic effects and the present work describes studies on the lysis of rat erythrocytes by these sols and of methods for eliminating this effect.

This work is a preliminary investigation on the haemolytic activity of LPC when co-dispersed with other lipids; no attempt has been made to produce a formulated product which would have entailed a more detailed study of haemolysis using human erythrocytes at 37° and rates of release of the steroid from the micelles.

The aqueous dispersion of progesterone

Although virtually insoluble in water at 20°, progesterone may be dispersed directly by ultrasonic irradiation to yield a colloidal or semicolloidal solution (Misek & Skauen, 1958a, b); the addition of surface-active materials facilitates the disruption of the progesterone aggregates (Misek & Skauen, 1958a) by displacing adsorbed air from around the solid surface thus allowing the wetting of the solid and reduction of the energy necessary for the disruption of the aggregates. When the concentration of the surfactant exceeds its critical micelle concentration, solubilization of progesterone occurs. Several workers (Ekwall & Sjöblom, 1950; Diczfalusy, Ekwall &

Sjöblom, 1952), have reported the preparation of colloidally dispersed progesterone in an aqueous medium using high concentrations of surface active materials. Concentrations of progesterone from 0·5–5·0 mg/ml were solubilized in aqueous solutions of sodium lauryl sulphate (10%) and polysorbate 20 (20%) (Diczfalusy & others, 1952). Biological activity was retained by these preparations.

The haemolytic activity of progesterone

Amongst the neutral steroids, progesterone is particularly haemolytic to human erythrocytes (Tateno & Kilbourne, 1954; Palmer, 1964). In concentrations of $7{\cdot}5 \times 10^{-4}$ to $1{\cdot}0 \times 10^{-3}$M it is among the most haemolytic of all steroids for rabbit erythrocytes (Weissmann & Keiser, 1965), although at lower concentrations its haemolytic activity is greatly reduced.

Table 1. *Results of haemolyses by phospholipid sols*

Sol (ratios by weight)	Concentration of phospholipid % w/w	Time for 50% haemolysis (s)
PC	0·12	No haemolysis in 15 min
LPC	$5{\cdot}0 \times 10^{-4}$	66
	$6{\cdot}25 \times 10^{-4}$	34
	$7{\cdot}5 \times 10^{-4}$	26
	$8{\cdot}75 \times 10^{-4}$	20
	$12{\cdot}5 \times 10^{-4}$	15
LPC/PC 1 : 1	$12{\cdot}5 \times 10^{-4}$	61
	$18{\cdot}75 \times 10^{-4}$	34
	$25{\cdot}0 \times 10^{-4}$	30
	$31{\cdot}25 \times 10^{-4}$	27
PC saturated with progesterone	0·12	No haemolysis in 15 min
LPC saturated with progesterone	$5{\cdot}0 \times 10^{-4}$	46·5
	$6{\cdot}25 \times 10^{-4}$	37·0
	$7{\cdot}5 \times 10^{-4}$	28·5
	$10{\cdot}0 \times 10^{-4}$	25·5
LPC/PL 1 : 1 saturated with gesterone	$12{\cdot}5 \times 10^{-4}$	76
	$18{\cdot}75 \times 10^{-4}$	53
	$25{\cdot}0 \times 10^{-4}$	45
	$37{\cdot}5 \times 10^{-4}$	37
LPC/PC 1 : 1 saturated with cholesterol	0·12	No haemolysis in 15 min
LPC saturated with progesterone and cholesterol	$37{\cdot}5 \times 10^{-4}$	65
	$50{\cdot}0 \times 10^{-4}$	39·5
	$75{\cdot}0 \times 10^{-4}$	31·5
	$100{\cdot}0 \times 10^{-4}$	26·5
LPC/PC 1 : 1 saturated with progesterone and cholesterol	0·12	No haemolysis in 15 min
PC/triolein 1 : 1	0·12	No haemolysis in 15 min
LPC/PC/triolein	19×10^{-4}	200
	38×10^{-4}	82
	57×10^{-4}	65
	76×10^{-4}	55
	133×10^{-4}	50

The haemolytic activity of phospholipids

Investigating the haemolytic activity of LPC (containing some "lysocephalin") Gorter & Hermans (1943) showed that haemolysis was rapid over the first few minutes and that it then ceased. They found that a given amount of lysophosphatide is capable of haemolysing a fixed number of erythrocytes owing to adsorption of the LPC onto the cell membrane and it was estimated that haemolysis resulted when the equivalent of a unimolecular layer of LPC had become adsorbed. The rate of haemolysis is accelerated as the volume of the cell increases (Wilbur & Collier, 1943) and the quantity of LPC required to lyse a given number of cells varies with the concentration of the cell suspension (Collier, 1951–52; Nygaard, Dianzani & Bahr, 1954). The lysing action is temperature dependent, more 'lysin' being adsorbed on the cells at lower temperatures. This would suggest that an equilibrium exists between the LPC in solution and that absorbed by the cell. Collier (1951–52) on the basis of results obtained with rabbit erythrocytes has suggested that the LPC reacts to form a complex with the free cholesterol within the cell membrane. Sublytic concentrations of LPC produce alterations in the erythrocyte membrane: "sphering" occurs, hypotonic fragility decreases and the rate of thiourea penetration is greatly increased while calcium ions cause much shrinkage. The partial all-or-none characteristics of LPC haemolysis have been demonstrated by Feeney, MacDonnell & Fraenkel-Conrat, 1954) using rat and rabbit erythroctyes.

The action of PC in haemolysis is less well defined. Domonkos (1962) found that haemolysis by LPC was inhibited by phosphatides and Gjone (1961) observed this to be true for PC extracted from normal human serum. Using synthetic phospholipids and bovine erythroctyes, Reman & Van Deenen (1967) showed that certain diacyl phosphoglycerides possessed haemolytic activity, the didecanoyl PC being much more active than its lyso derivative.

EXPERIMENTAL

Preparation of phosphatidylcholine (PC). The starting material was a Merck A.G. commercial sample of egg phospholipids. Alumina (Savory & Moore) was packed in chloroform and the crude phospholipid fraction (20 mg/g of alumina) dissolved in the minimum quantity of chloroform, was applied to the top of the column. Elution was accomplished by a chloroform–methanol mixture of 8:1 and 3:1 v/v, the latter extracting the PC fraction. All fractions extracted giving a positive ninhydrin reaction were rejected, the negative fractions extracted by the 3:1 solvent mixtures were bulked and evaporated below 40°. The dried residue of crude PC was further purified by dissolving it in the minimum quantity of chloroform and applying it to a column of silicic acid (Malinckrodt) mixed with celite (Koch-Light 545) 2:1 (Hanahan, Dittmer & Warashina, 1957) by weight and packed in chloroform. Elution was by chloroform–methanol 4:1 and 3:2 v/v, collecting 100 ml fractions. Fractions containing only PC were identified by thin-layer chromatography bulked and evaporated at 35°. The PC residue was recrystallized several times from warm methyl ethyl ketone and acetone and the final product stored under dry acetone at 5°. Yield 30–40%. Immediately before use, the PC was checked for purity by thin-layer chromatography and recrystallized if necessary, until a single spot resulted. Analytical data: found N 1·78, P 3·79%. N/P 1·04. Iodine value 71.

Preparation of lysophosphatidylcholine (LPC). This was prepared essentially by the method of Hanahan, Rodbell & Turner (1954). PC (10 g) in ether (750 ml) was

shaken with 5 ml of an aqueous solution (pH 7·4) containing Russell Viper Venom (10 mg), 1% NaCl and 0·005M $CaCl_2$. The solution was allowed to stand for several hours, the ether was evaporated and the residue taken up in the minimum quantity of chloroform: 2 ml aliquots were pipetted into centrifuge tubes and ether (23 ml) added. Refrigerated centrifugation for 10 min brought the light precipitate to the base of the tubes, enabling separation by decantation. This procedure was repeated several times to remove any unreacted PC and venom. The final product was obtained by recrystallizing three to four times from hot ethanol (Saunders, 1957) and was stored under dry acetone at 5°. Analytical data: found: N 2·7, P 5·9%. N/P 0·99.

Progesterone and cholesterol. Pure samples were obtained from BDH and Fluka respectively.

Triolein. This was purified by silica gel column chromatography using a mixed ether–light petroleum (b.p. 40–80°) solvent system and was a gift from M. C. R. Johnson.

Rat erythrocytes. Rat blood was heparinized, centrifuged and the erythrocytes washed five times with 0·9% NaCl to give a 50% haematocrit.

Preparation of the sols. The dispersion of progesterone and the separation of undissolved solids was according to Hoyes & Saunders (1966).

Examination of haemolysis. Saline (20 ml, 0·9%) was placed in the cell of a Unicam SP1400 Prism Absorptiometer, previously calibrated with reference to a neodymium filter ($\lambda = 537$ nm). 0·1 ml of the erythrocyte suspension was added to the cell and the transmission adjusted to read 10 at 625 nm. The phosphatide was added, the cell contents rapidly mixed and lysis (measured as an increase in transmission) recorded as a function of time.

Spectrophotometric determination of progesterone concentration. Progesterone was assayed by measuring the absorbance at 240 nm. Dilution of the sols by ethanol to give a measurable absorbance in 1 cm cells also dissolved the phospholipid micelles, thereby eliminating errors due to light scattering.

Haemolysis curves and treatment of results. From the experimental plot of transmission scale reading against time (Fig. 1), which gave haemolysis curves for lysophosphatidyl choline (concentration w/v), the time to produce 50% haemolysis was determined and these values are plotted against the concentration of lysin in Fig. 2.

DISCUSSION

The haemolytic activity of LPC towards rat erythrocytes is reduced by saturating it with progesterone. Assuming that the LPC monomers are a more active haemolytic species than the micelles, then the addition of progesterone will result in a shift to the right of the equilibrium,

$$n \text{ (monomers)} \rightleftharpoons \text{micelle},$$

thus reducing the overall haemolytic activity of the sol. Surface tension studies by Robinson & Saunders (1958) indicated a critical micelle concentration of 4 to 18 mg litre^{-1} for LPC in water at 25°. The mixed phospholipid micelle owes its haemolytic activity to the LPC alone, the PC slightly reduces the haemolysis by LPC. The sol with progesterone solubilized by the mixed phospholipids was less lytic than either the sol containing progesterone solubilized by LPC alone, or the mixed phospholipid sol in the absence of co-dispersed progesterone (Table 1).

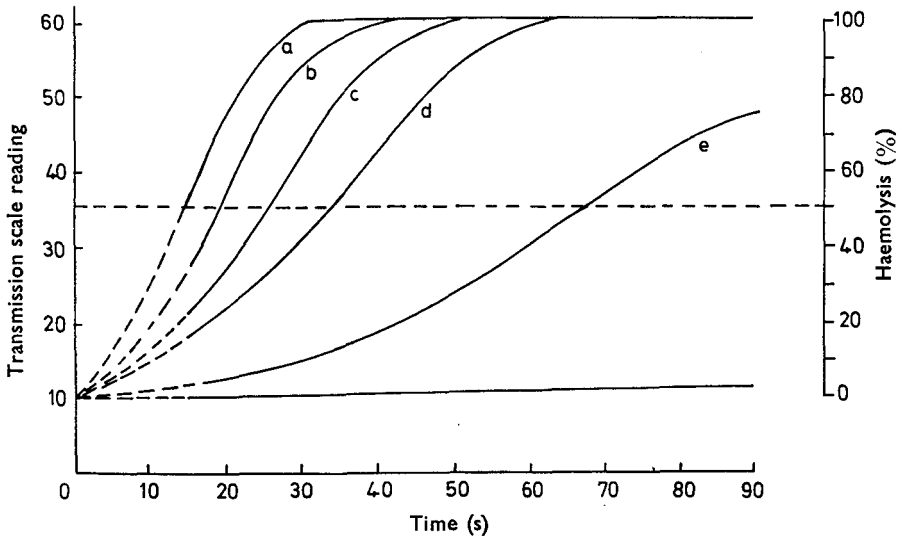

FIG. 1. Haemolysis curves for lysophosphatidylcholine concentrations in percentage w/w. a, $12{\cdot}5 \times 10^{-4}$. b, $8{\cdot}75 \times 10^{-4}$. c, $7{\cdot}5 \times 10^{-4}$. d, $6{\cdot}25 \times 10^{-4}$. e, $5{\cdot}0 \times 10^{-4}$. f, $3{\cdot}5 \times 10^{-4}$.

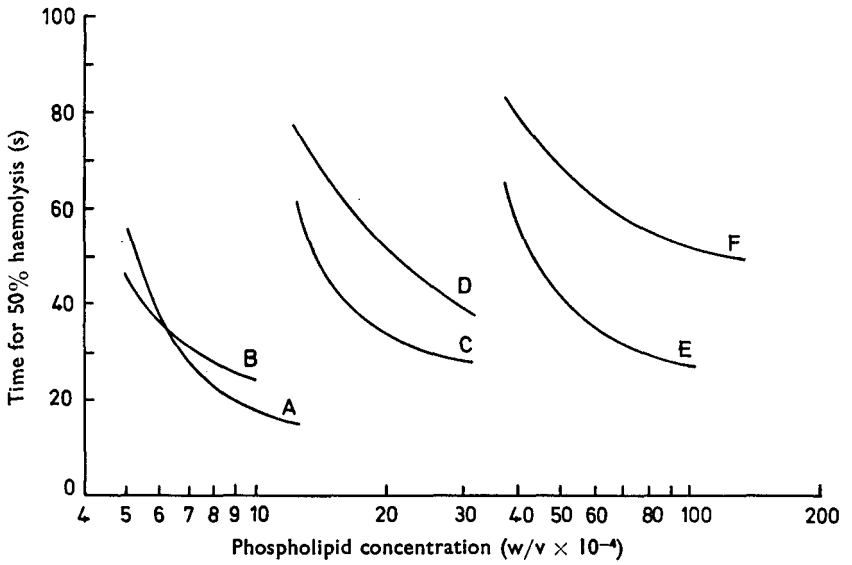

FIG. 2. Haemolysis–concentration graphs. A = LPC. B = LPC/progesterone sol. C = LPC/PC sol. D = LPC/PC/progesterone sol. E = LPC/progesterone/cholesterol sol. F = LPC/PC/triolein sol.

Cholesterol co-dispersed with progesterone by LPC, greatly inhibited the lytic activity of the LPC, and with the mixed phospholipid sol was sufficient to prevent haemolysis of the rat erythrocytes. The ability of cholesterol to inhibit the haemolysing activity of LPC was first reported by Minz (1908), who believed the effect to be due to molecular fixation of cholesterol by LPC. A complex is formed between cholesterol and LPC having no haemolytic activity (Delezenne & Fourneau, 1914), but no compound formation takes place.

The LPC/PC/triolein system (equal parts of each by weight) showed a marked reduction in haemolytic activity over the mixed phospholipid system, thus indicating a steric inhibiting capacity of the triolein within the micelle, or a marked reduction in the number of LPC monomers present in the sols.

Kinetic equations were applied to the haemolysis of red blood cells by LPC, but neither first order, two stage first order nor second order kinetics gave satisfactory solutions to the problem.

Acknowledgement

I.W.K. thanks the Medical Research Council for a research studentship.

REFERENCES

COLLIER, H. B. (1951–52). *J. gen. Physiol.*, **35**, 617–628.
DELEZENNE, C. & FOURNEAU, E. (1914). *Bull. Soc. chim. Fr.*, **15**, 421–434.
DICZFALUSY, E., EKWALL, P. & SJÖBLOM, L. (1952). *Acta endocr.*, **9**, 373–389.
DOMONKOS, J. (1962). *Hoppe-Seyler's Z. physiol. Chem.*, **327**, 278–279.
EKWALL, P. & SJÖBLOM, L. (1950). *Acta endocr.*, **4**, 179–191.
FEENEY, R. E., MACDONNELL, L. R., & FRAENKEL-CONRAT, H. (1954). *Archs. Biochem. Biophys.*, **48**, 130–140.
GJONE, E. (1962). *Scand. J. clin. Lab. Invest.*, **13**, 369–373.
GORTER, E. & HERMANS, J. J. (1943). *Recl Trav. chim. Pays-Bas. Belg.*, **62**, 681–686.
HANAHAN, D. J., RODBELL, M. & TURNER, L. D. (1954). *J. biol. Chem.*, **206**, 431–441.
HANAHAN, D. J., DITTMER, J. C. & WARASHINA, E. (1957). *Ibid.*, **228**, 685–700.
HOYES, S. D. & SAUNDERS, L. (1966). *Biochim. biophys. Acta*, **116**, 184–185.
KELLAWAY, I. W. & SAUNDERS, L. (1967). *Ibid.*, **144**, 145–148.
MINZ, A. (1908). *Biochem. Z.*, **9**, 357–381.
MISEK, B. & SKAUEN, D. M. (1958a). *J. Am. pharm. Ass.* (*Sci. Edn*), **47**, 32–36.
MISEK, B. & SKAUEN, D. M. (1958b). *Ibid.*, **47**, 36–39.
NYGAARD, A. P., DIANZANI, M. U. & BAHR, G. F. (1954). *Expl Cell Res.*, **6**, 453–458.
PALMER, R. H. (1964). *Nature, Lond.*, **201**, 1134–1135.
REMAN, F. C. & VAN DEENEN, L. L. M. (1967). *Biochim. biophys. Acta*, **137**, 592–594.
ROBINSON, N. & SAUNDERS, L. (1958). *J. Pharm. Pharmac.*, **10**, 755–761.
SAUNDERS, L. (1957). *Ibid.*, **9**, 834–840.
SAUNDERS, L., PERRIN, J. H. & GAMMACK, D. B. (1962). *Ibid.*, **14**, 567–572.
TATENO, I. & KILBOURNE, E. D. (1954). *Proc. Soc. exp. Biol. Med.*, **86**, 168–170.
WEISSMANN, G. & KEISER, H. (1965). *Biochem. Pharmac.*, **14**, 537–546.
WILBUR, K. M. & COLLIER, H. B. (1943). *J. cell. comp. Physiol.*, **22**, 233–249.