Studies on the Mode of Action of Excess of Vitamin A

9. PENETRATION OF LIPID MONOLAYERS BY COMPOUNDS IN THE VITAMIN A SERIES*

By A. D. BANGHAM
Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

J. T. DINGLE and J. A. LUCY
Strangeways Research Laboratory, Cambridge

(*Received* 14 *June* 1963)

Vitamin A_1 alcohol has been shown to haemolyse erythrocytes (Dingle & Lucy, 1962). It is also able to disintegrate the cell and nuclear membranes of fibroblasts and to cause their mitochondria to swell (Dingle, Glauert, Daniel & Lucy, 1962). Mitochondrial swelling has been observed by Jackson & Fell (1963) in a study of the epidermal fine structure of embryonic chick skin under the influence of vitamin A_1 alcohol *in vitro*. In investigations of isolated subcellular fractions, it has been found that vitamin A_1 alcohol liberates a proteolytic enzyme from isolated lysosomes (Dingle, 1961; Fell, Dingle & Webb, 1962) and causes isolated rat-liver mitochondria to swell rapidly (Lucy, Luscombe & Dingle, 1963). The action of vitamin A on erythrocytes and on isolated lysosomes and mitochondria has been shown to have a high degree of molecular specificity. Thus haemolytic activity is a property of vitamin A_1 alcohol and aldehyde that is possessed in only a small degree by closely related molecules. Since haemolytic activity is associated with a capacity to penetrate cholesterol films or to disperse protein films (Schulman & Rideal, 1937*a*, *b*), the ability of compounds in the vitamin A series to penetrate lipid films at an air–water interface has been investigated. The process of penetration by one molecular species into a partially compressed monolayer of another molecular species has been studied in the past but most of the compounds investigated were chosen more because of their known surface-active properties than for their biological importance. However, Skou (1961) showed that a relationship exists between the potency of a local anaesthetic and its ability to penetrate lipids of nerve tissue. More recently it has been demonstrated that there is a correlation between the prevention of carbon tetrachloride-induced necrosis in rat liver by antihistamines, and by cetyltrimethylammonium bromide, and the capacity of these compounds to penetrate a monolayer of lecithin and cholesterol (Bangham, Rees & Shotlander, 1962).

* Part 8: Lucy, Luscombe & Dingle (1963).

In the present experiments it has been found that vitamin A_1 alcohol penetrates and expands lipid films very readily, though many closely related compounds that are much less haemolytic are less surface-active. It is thought that this property of the vitamin is closely connected with the observation that the initial effect of vitamin A_1 alcohol on the erythrocyte is greatly to increase the surface area of the cell (Glauert, Daniel, Lucy & Dingle, 1963). It is suggested that the properties of the molecule of vitamin A_1 alcohol that allow it to penetrate lipid monolayers and lipoprotein membranes are also properties that enable it to fulfil its physiological functions.

EXPERIMENTAL

Materials. Ovolecithin, which was a gift from Dr R. M. C. Dawson, was isolated from mixed egg phospholipids by chromatography on alumina and silicic acid columns (Dawson, 1958). Cholesterol, vitamin A_1 alcohol and vitamin A_1 acetate were obtained from Roche Products Ltd., and all-*trans*-vitamin A_1 aldehyde and all-*trans*-vitamin A_1 acid from Distillation Products Industries (Kodak). Vitamin A_1 methyl ether, vitamin A_2 alcohol and β-ionylidene-ethanol were gifts from Dr P. L. Harris of the Biochemistry Laboratories, Distillation Products, Rochester, N.Y., U.S.A. Hydrogenated vitamin A_1 and anhydrovitamin A_1 were prepared as described by Fell *et al.* (1962).

The solution in the trough was unbuffered 0·145 M-NaCl prepared in glass-distilled water. The equimolar mixture of lecithin and cholesterol was dissolved in light petroleum (b.p. 40–60°) and the vitamin A derivatives were dissolved in ethanol.

Trough and surface-pressure measuring apparatus. Force–area measurements and experiments on the penetration of monolayers were made at an air–water interface at room temperature (22°) in a Fluon (polytetrafluoroethylene) trough, 20·5 cm. long × 4·5 cm. wide, of capacity 70 ml. Surface pressures were recorded continuously by means of a glass dipping plate (2·2 cm. × 2·2 cm.) suspended from an arm rigidly attached to a torsion wire. Also suspended from the arm was a gate which varied the amount of light falling on a selenium photocell according to the position of the dipping plate. The output from the photocell was fed directly into one channel of a d.c. recording potentiometer

(Honeywell). The dipping plate–recorder system was calibrated to read directly in dynes/cm. before each sequence of measurements (Bangham & Dawson, 1960).

Measurement of increase of area at constant pressure. An a.c. reversing motor, suitably geared down, was used to revolve a long lead-screw held horizontally above the trough. Two vertical pistons, driven by compressed air, were fixed to a nut travelling along the lead-screw; the pistons gripped the waxed barrier confining the lecithin–cholesterol monolayer. Any deviation from the chosen film pressure, e.g. a rise resulting from penetration, was detected by a pair of micro-switches actuated by cams attached to the pen recorder. The micro-switches controlled the direction of rotation of the reversing motor and hence the position of the barrier.

The area increase of a penetrated monolayer was measured by converting the linear displacement of the waxed barrier along the trough into a d.c. potential; this was achieved by linking the lead-screw driving the barrier to a linear 10-turn helical potentiometer which divided a potential of 1·5 v. The divided potential was applied to the second channel of the pen recorder.

Force–area curves. After the aqueous surface of the trough had been swept clean with waxed-glass sweeps and the instrument calibrated, increasing quantities of vitamin A derivatives were carefully released on to the surface from an Agla syringe. As some of the compounds did not form stable films, pressures were arbitrarily read after 30 sec. (No attempt was made to sustain an atmosphere of nitrogen around the compound after it had been placed on the trough because of the difficulty of obtaining completely anaerobic conditions.)

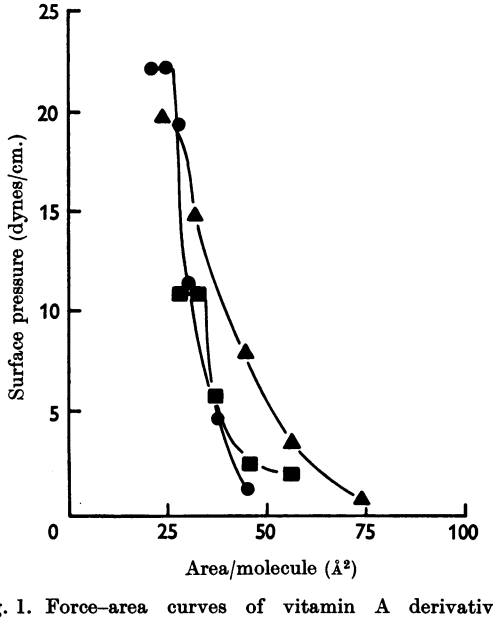

Fig. 1. Force–area curves of vitamin A derivatives. Increasing quantities of the vitamin A derivative were released on to the surface of 0·145 M-NaCl and the change in surface pressure was recorded as described in the text. The temperature was 22°. ●, Vitamin A_1 alcohol; ▲, vitamin A_2 alcohol; ■, vitamin A_1 acetate.

Penetration of lecithin–cholesterol monolayers at constant pressure. Equimolar solutions of ovolecithin and cholesterol were spread on to approximately half the area (45 cm.²) of the trough in quantities sufficient to produce a range of surface pressures of between 25 and 45 dynes/cm. After a short period for equilibration, the cam that activated the micro-switches was set at the equilibrium pressure and the pen recorder started. The vitamin A or other derivative was then injected carefully through the monolayer and the area-controlling mechanism started. Penetration was usually so rapid that the area-adjustment servo-mechanism did not keep pace with the rise in pressure. However, within a 5 min. period of adjustment the pressure had returned to its initial value and the expanded area of the monolayer had been recorded. In some experiments the area-compensating mechanism was allowed to continue operating after the initial equilibrium had been reached and any subsequent change in area of the monolayer was then studied as a function of time.

RESULTS

Force–area curves of compounds in the vitamin A series. Vitamin A_1 alcohol gives a liquid-expanded force–area curve with a collapse pressure of 22·5 dynes/cm. and a limiting area/molecule of 25 Å² (Figs. 1 and 2). All the other compounds in the vitamin A series that were studied were found to have collapse pressures of less than 22·5 dynes/cm. The force–area curves illustrated in the Figures were completed within 2–3 min. and the maximal loss of pressure from a monolayer near to its collapse was not more than 5 dynes/cm. in the

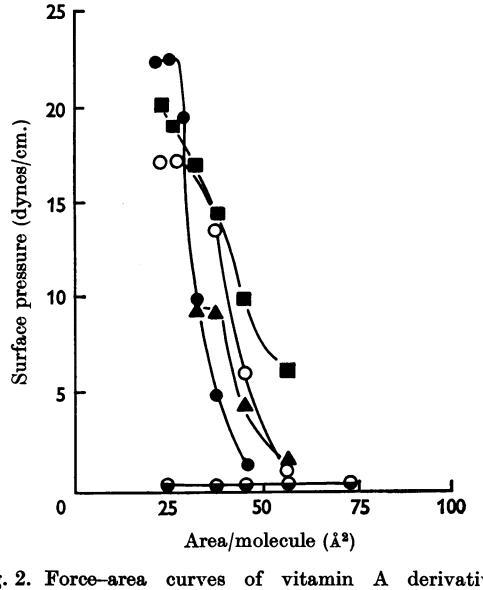

Fig. 2. Force–area curves of vitamin A derivatives. Details were as given for Fig. 1. ●, Vitamin A_1 alcohol; ■, hydrogenated vitamin A_1; ▲, vitamin A_1 methyl ether; ○, vitamin A_1 acid; ◒, anhydrovitamin A_1.

subsequent 10 min. As no attempt was made to exclude oxygen from either the substrate–saline or the gas phase, it was perhaps not surprising that the films of vitamin A_1 alcohol were unstable.

Vitamin A_1 acid and vitamin A_2 alcohol were also unstable as monolayers and gave limiting areas/molecule of 27 Å^2 and 25 Å^2 respectively. Both substances gave a more expanded force–area curve than the vitamin A_1 alcohol. The effect of pH on the force–area curve of vitamin A_1 acid was not examined in detail; it should be remarked that the pH of the unbuffered solution of 0·145 M-NaCl was about 5·5.

The vitamin A_1 acetate and methyl ether derivatives have collapse pressures below 12 dynes/cm., both forming stable films. Anhydrovitamin A_1 behaves like a typical oil having no spreading pressure whatsoever.

Penetration of lecithin–cholesterol monolayers. (*a*) Increase in pressure at constant area. The collapse pressure of an equimolar lecithin–cholesterol monolayer is 50 dynes/cm. All the vitamin A derivatives examined caused an increase in surface pressure when injected beneath a monolayer of lecithin–cholesterol held at 30 dynes/cm. and at constant area. The magnitude of the changes is given in Table 1, and in detail for vitamin A_1 acid and alcohol in Fig. 3. In a control experiment, a volume of ethanol equal to that used for dissolving the vitamin A derivatives gave no change in surface pressure or area of the lecithin–cholesterol film. Table 1 shows that, of the compounds tested, vitamin A_1 methyl ether, vitamin A_1 acetate, hydrogenated vitamin A_1, anhydrovitamin A_1, β-ionylidene-ethanol and vitamin A_1 aldehyde all produced relatively little increase in surface pressure; the increases observed were all less than 10 dynes/cm. With the exception of vitamin A_1 aldehyde, these compounds have relatively little haemolytic action as compared with vitamin A_1 alcohol (Dingle & Lucy, 1962). Vitamin A_1 alcohol itself and two relatively weakly haemolytic derivatives, vitamin A_1 acid and vitamin A_2 alcohol, produced considerably larger increases in surface pressure.

(*b*) Increase in area at constant pressure (isopiezic expansion). The percentage increases in area resulting from the injection of various vitamin A derivatives beneath a lecithin–cholesterol film held at 30 dynes/cm. are listed in Table 1. This initial pressure was chosen since it is well above the collapse pressure of all the vitamin A compounds by themselves. At this pressure, 0·021 μmole of vitamin A_1 alcohol consistently gave a 60% increase in film area; this increase represented the incorporation into the monolayer of almost all of

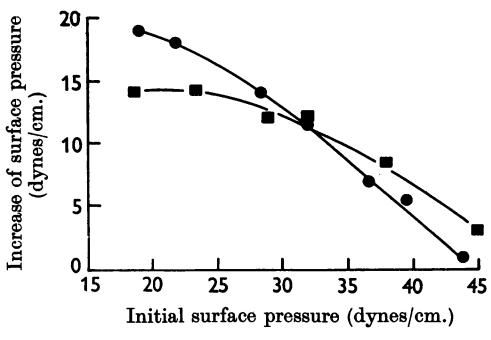

Fig. 3. Effect of vitamin A_1 alcohol and vitamin A_1 acid on the pressure of an equimolar lecithin–cholesterol film. Vitamin A_1 alcohol or vitamin A_1 acid (0·021 μmole) was injected under a film of lecithin–cholesterol. Sufficient lipid was added to give a film of 45 cm.^{2} of the initial pressure required over 0·145 M-NaCl. The increase in surface pressure of the film was measured as described in the text. The temperature was 22°. ●, Vitamin A_1 alcohol; ■, vitamin A_1 acid.

Table 1. *Summary of the properties of various vitamin A derivatives injected underneath a monolayer of lecithin–cholesterol*

Sufficient lecithin–cholesterol (approx. 0·017 μmole) was placed on an area of saline measuring 10 cm. × 4·5 cm. to give a surface pressure of 30 dynes/cm. Through this film 0·021 μmole of the vitamin A derivatives was injected in a total volume of 0·006 ml. of ethanol. Haemolytic action (Dingle & Lucy, 1962) was tested in a final concentration of 35 μM in 0·145 M-NaCl.

Vitamin A derivative	Initial change in pressure when area is kept constant (dynes/cm.)	Percentage increase in area at constant pressure (30 dynes/cm.)	Haemolytic action (% of that obtained with vitamin A_1 alcohol)
Vitamin A_1 alcohol	+13	61	100
Vitamin A_1 acid	+12	13	8
Vitamin A_1 methyl ether	+ 6	5	2
Vitamin A_1 acetate	+ 8	9	1
Vitamin A_2 alcohol	+14	8	9
Hydrogenated vitamin A_1	+ 4	5	10
Anhydrovitamin A_1	+ 5	0	3
β-Ionylidene-ethanol	+ 6	18	8
Vitamin A_1 aldehyde	+ 6	12	102

the vitamin A_1 alcohol molecules injected beneath the film. This calculation is based on the assumption that the molecules of vitamin A_1 alcohol in the monolayer are closely packed and occupy an area of 25 Å^2/molecule even when in the form of complexes with lecithin–cholesterol (see Fig. 1).

In another series of experiments (Fig. 4) the percentage increase in film area produced by three selected compounds in the vitamin A series was determined at a number of different initial pressures of the lecithin–cholesterol monolayer. The 'threshold' pressure for penetration by vitamin A_1 alcohol into the spread monolayer was much higher than that for any of the other derivatives studied. Above the threshold pressure, e.g. at 38 dynes/cm., vitamin A_1 alcohol penetrated the monolayer of lecithin–cholesterol but the initial pressure was easily restored by a small increase in area of the film. When the initial pressure was at or below the threshold pressure, restoration of the initial pressure after penetration required a large increase in the area of the film. The film continued to expand at constant surface pressure until a 1:1 complex of lecithin–cholesterol and vitamin A_1 alcohol was achieved.

Vitamin A_1 acid was able to raise the pressure at constant area but only slightly expanded the monolayer at a constant pressure of 30 dynes/cm. This is clearly seen by comparing the values obtained for the two parameters with vitamin A_1 acid and with vitamin A_1 alcohol (see Table 1). Vitamin A_2 alcohol, like vitamin A_1 acid, produced a large increase in surface pressure but only a very small increase in area at constant pressure. Vitamin A_1 alcohol was therefore the only compound of those studied which was able to cause both a large increase in surface pressure and a large increase in the area of the film at a constant pressure of 30–34 dynes/cm.

Films of lecithin–cholesterol that had been penetrated by vitamin A_1 alcohol were not stable at 22° or above. Usually within 1–2 min. of equilibration at the expanded area or at the raised pressure, the surface pressure of the film began to fall and continued to do so until either the original area or the original pressure of the film was reached. This process occurred over a period of about 10–20 min.

Penetration of vitamin A_1 alcohol into lecithin and into cholesterol. The threshold pressure for isopiezic expansion of a lecithin film by vitamin A_1 alcohol is greater than for expansion of either a mixed (equimolar) lecithin–cholesterol film or a simple cholesterol monolayer (Fig. 5). Indeed, the threshold pressure below which vitamin A_1 alcohol markedly expands the cholesterol film is close to the collapse pressure of vitamin A_1 alcohol itself. This suggests that there is relatively little interaction between cholesterol and vitamin A_1 alcohol. Conversely, there is a considerable expansion of a lecithin film or a lecithin–cholesterol film at pressures well in excess of the collapse pressure of vitamin A_1 alcohol itself. Vitamin A_1 alcohol and lecithin thus appear to interact more strongly than vitamin A_1 alcohol and cholesterol.

Penetration of vitamin A_1 alcohol into lecithin–

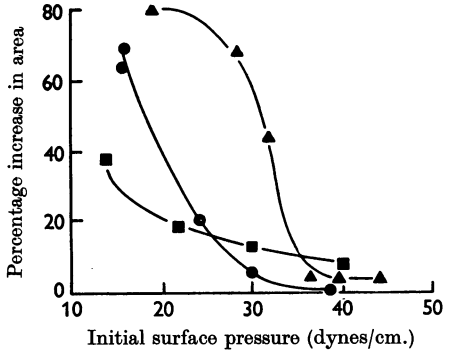

Fig. 4. Penetration of lecithin–cholesterol by vitamin A derivatives. The percentage increase in area was measured at room temperature (22°) at the addition of 0·021 μmole of vitamin A_1 alcohol, vitamin A_1 methyl ether or vitamin A_1 acid. Sufficient lecithin–cholesterol was used to give a film of 45 cm.^{2} at the various initial pressures required over 0·145 M-NaCl. ▲, Vitamin A_1 alcohol; ●, vitamin A_1 methyl ether; ■, vitamin A_1 acid.

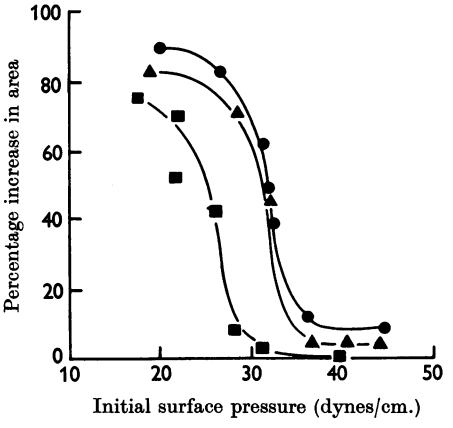

Fig. 5. Penetration of films of different composition by vitamin A_1 alcohol. The percentage increase in area was measured at room temperature (22°) with films of cholesterol, an equimolar mixture of lecithin–cholesterol and lecithin alone. Sufficient lipid was added to give a film of 45 cm.^{2} over 0·145 M-NaCl at the initial pressure required. Then 0·021 μmole of vitamin A_1 alcohol was added and the increase in area measured as indicated in the text. ●, Lecithin; ▲, lecithin–cholesterol; ■, cholesterol.

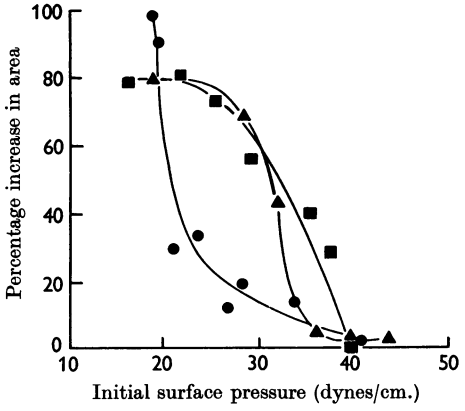

Fig. 6. Penetration of an equimolar lecithin–cholesterol monolayer by vitamin A_1 alcohol at various temperatures. The trough and measuring devices were placed in an insulated container. The temperature was controlled either by passing air, cooled over ice, or by thermostatically controlled heating. The percentage increase in area was measured at 13°, 22° and 42° after the addition of 0·021 μ-mole of vitamin A_1 alcohol dissolved in 0·006 ml. of ethanol. Sufficient lecithin–cholesterol was added to give an area of 45 cm.^{2} of the initial surface pressure required. ●, 13°; ▲, 22°; ■, 42°.

cholesterol monolayers at different temperatures. Fig. 6 shows the extent to which vitamin A_1 alcohol increases the area of films of lecithin–cholesterol at various temperatures. At and above 22°, marked isopiezic expansion occurs with initial surface pressures considerably in excess of the collapse pressure of vitamin A_1 alcohol. At 13°, however, penetration and expansion of the film were very poor. The penetrated films at 13° were stable for at least 15 min., unlike films of lecithin–cholesterol that were penetrated by vitamin A_1 alcohol at higher temperatures.

DISCUSSION

Apart from its function in vision the sites and mechanism of action of vitamin A have long been obscure. On the basis of recent studies made at the Strangeways Laboratory, it has been suggested that under certain conditions the vitamin may act initially on the lipoprotein membranes of cells and their organelles (Fell & Thomas, 1960; Lucy, Dingle & Fell, 1961; Dingle & Lucy, 1962; Lucy & Dingle, 1962). This hypothesis rests on observations of the action of vitamin A_1 alcohol on cartilage in organ culture, on intact mammalian fibroblasts, on isolated lysosomes and mitochondria, as well as on mammalian erythrocytes. To explain the action of the vitamin in such a variety of different systems it was necessary to postulate that it is able to penetrate or react with lipoprotein membranes. The work presented in the present paper shows that vitamin A_1 alcohol is a strongly surface-active agent and is able to penetrate lipid monolayers at pressures that are considerably above its own collapse pressure.

The molecules of vitamin A_1 alcohol, and of a number of other compounds in the vitamin A series, are able to orientate themselves at an air–water interface to produce a monolayer. In this respect, vitamin A derivatives appear to behave similarly to other amphipathic lipids such as cholesterol that have a hydrophilic hydroxyl group at one end of the molecule. Determinations have been made in the past of the collapse pressure and the minimum area/molecule for vitamin A_1 alcohol and for several related compounds by Weitzel, Fretzdorff & Heller (1952). In the present experiments, values of 22·5 dynes/cm. and 25 Å^2 were obtained for these two parameters with vitamin A_1 alcohol; these values are in reasonable agreement with those found by Weitzel *et al.* (1952) (29·4 dynes/cm. and 26 Å^2). Similar values to those given by these authors for certain other compounds in the vitamin A series were found and, in addition, it has been confirmed that some derivatives, e.g. anhydrovitamin A_1, are unable to form films but behave as non-spreading oils.

In the present study a film composed of equimolar quantities of lecithin and cholesterol has been used as a model for the lipid components of the plasma membrane. Since the lipids of rabbit erythrocytes are composed of cholesterol and mixed phospholipids in a molar ratio of approximately 1:1 (Burt & Rossiter, 1950), the behaviour of the artificial mixture used in these experiments might be expected to imitate that of the erythrocyte membrane in several respects. The ability of vitamin A_1 alcohol to penetrate a monolayer of lecithin–cholesterol at 12 dynes/cm. above the collapse pressure of vitamin A_1 alcohol indicates complex-formation with a component of the lipid monolayer. It would appear that lecithin is primarily concerned in the penetration by vitamin A_1 alcohol since the vitamin can cause expansion of a film of lecithin–cholesterol, or of lecithin, at pressures considerably above those at which it is able to expand a cholesterol film. Haemolysis of erythrocytes by vitamin A_1 alcohol may thus depend initially on penetration of the plasma membrane which results from a strong affinity between a phospholipid component of the membrane, i.e. the lecithin, and the added vitamin. This situation is the converse of that with certain other haemolytic compounds, e.g. saponin, which are haemolytic apparently as a result of their affinity for cholesterol (Schulman & Rideal, 1937*b*).

From recent experiments on the treatment of artificial mixtures of lecithin and cholesterol with

saponin (Bangham & Horne, 1962), and on treatment of cholesterol alone with saponin (Glauert, Dingle & Lucy, 1962), it would appear that the lipids of the erythrocyte membrane undergo remarkable reorientation when treated with this surface-active compound (Dourmashkin, Dougherty & Harris, 1962). Although the dimensions of the molecules of saponin and vitamin A are different, it is possible that similar modifications of membrane structures could be observed with the electron microscope as a result of the interaction of vitamin A with the phospholipids of membranes.

The shape of the curve for the isopiezic penetration of vitamin A_1 alcohol into lecithin–cholesterol indicates that the pressure below which massive incorporation of molecules of vitamin A_1 alcohol can take place is about 34 dynes/cm. Perhaps this critical pressure is not without significance when it is remembered that even short-chain alcohols, e.g. butanol, lyse erythrocytes and other cells at a bulk concentration of 0·39 M; at this concentration these alcohols are capable of producing a surface pressure at an air–water interface of 34 dynes/cm. (Pethica, 1958). Penetration of a monolayer of lecithin–cholesterol at a pressure of 34 dynes/cm. or less by butanol can therefore occur by non-specific absorption of the alcohol at the interface without the formation of any specific complex of butanol with either lecithin or cholesterol. This argument suggests that the lipids in the cell membrane may not be very tightly packed but are somewhat expanded and exert a lateral pressure equivalent to only about 34 dynes/cm. or even less. That mixed phospholipid bimolecular leaflets are stable in water in such an expanded state has been confirmed by Luzzati & Husson (1962), who showed by X-ray diffraction methods that the average area/hydrophilic group in such a system could be as high as 65 Å^2. This would correspond to a surface pressure of only 15 dynes/cm. for an equimolar lecithin–cholesterol mixture and of 36 dynes/cm. for a lecithin film. The average area/molecule of mixed lecithin–cholesterol films will naturally vary as the proportion of the two components in the film altered (Dervichian, 1958; van Deenen, Houtsmuller, de Haas & Mulder, 1962).

The molecule of vitamin A_1 alcohol has a bulky lipophilic ring system that is attached to a relatively long and rigid chain which is terminated by a hydrophilic end group. These three features are of considerable importance in determining the surface-active properties of the molecule. For instance, decreasing the length of the side chain results in a greatly decreased ability to penetrate the lipid monolayer at constant pressure or to increase the pressure of the lipid film at constant area; this change in molecular structure also decreases the haemolytic activity with mammalian erythrocytes. Hydrogenation of the unsaturated bonds produces a flexible side chain; modification of the molecule in this way also decreases its surface activity and its action on isolated subcellular particles, erythrocytes and tissues in organ culture (cf. Table 3 in Dingle & Lucy, 1962). Alteration of the hydrophilic end group by esterification, or by the formation of the methyl ether or the anhydro forms of the vitamin, is accompanied by decreases both in surface-active properties and in activity towards membrane systems. Conversely, the replacement of the hydroxyl group by a carboxyl group, which may increase the hydrophilic properties of the molecules, increases the ability of the molecule to penetrate a lecithin–cholesterol film at constant area but greatly diminishes its ability to increase the area of the film at constant pressure.

Vitamin A_1 aldehyde has much weaker surface-active properties in relation to the lecithin–cholesterol film than has vitamin A_1 alcohol. The aldehyde derivative therefore appears to be exceptional in that its haemolytic action is not paralleled by marked surface-active properties in the monolayer system studied above. Although vitamin A_1 aldehyde is at least as effective as, or even more effective than, vitamin A_1 alcohol in haemolysing erythrocytes (Dingle & Lucy, 1962), and is also able to increase the surface area of the erythrocyte (A. M. Glauert, J. T. Dingle & J. A. Lucy, unpublished work), the aldehyde is much less effective in causing swelling of isolated rat-liver mitochondria (Lucy *et al.* 1963). It would seem that other factors, as yet unknown, must play some part in the penetration of vitamin A_1 aldehyde into different membrane systems. It is possible that the binding of the aldehyde group to protein or to different phospholipids may be involved, or alternatively conversion of the all-*trans* form into a *cis* isomer may modify the penetration of vitamin A_1 aldehyde into certain lipoprotein membranes. Differences in activity between alcohol and aldehyde forms of vitamin A derivatives having modified chain lengths have also been observed in experiments on mitochondrial swelling; in each instance, the aldehyde form was less effective (Lucy *et al.* 1963).

Vitamin A_2 alcohol is relatively inactive in lysing erythrocytes and it does not produce a large increase in the area of the lecithin–cholesterol monolayer at constant pressure, despite the fact that it causes a surface-pressure change, when the area of the film is constant, that is at least as great as that produced by vitamin A_1 alcohol. The relationship between the change in surface activity and the introduction of the additional conjugated bond is not clear.

The concept of vitamin A_1 alcohol as a surface-active molecule provides an explanation for the initial effects of the vitamin when added in excess to systems containing lipoprotein membranes. Previous observations that excess of vitamin A_1 alcohol disorganizes the membranes of different types of cells and of a number of intracellular particles indicates that the vitamin interacts with structural components that are common to these membranes (Dingle *et al.* 1962; Lucy & Dingle, 1962), and in accordance with the conclusions of Rideal & Taylor (1957, 1958) for a variety of ionic and non-ionic detergents. This concept is further supported by the observations by Blough (1963) on morphological changes induced in certain viruses by vitamin A. The observations described in the present paper show that, unlike many closely related derivatives, the lipophilic and hydrophilic moieties of the molecule of vitamin A_1 alcohol appear to be balanced in such a way as to produce a highly surface-active compound. Penetration of lipoprotein membranes by the vitamin may be only the first step in the action of the vitamin in biological systems. Both the action of vitamin A_1 alcohol on membrane systems, e.g. on erythrocytes, lysosomes and mitochondria, and the penetration of vitamin A into a monolayer of lecithin–cholesterol, occur much less readily below 15° than at 37°. After the initial penetration of vitamin A_1 alcohol into a membrane system, the subsequent complex effects of the vitamin might be expected to be governed by the chemical constitution of the membrane concerned, and the contents and function of the affected cells or intracellular particles. Thus the presence of vitamin E in a membrane may modify the disruptive action of vitamin A, since the rapid haemolytic action of vitamin A_1 alcohol is largely inhibited by α-tocopherol acetate (Dingle & Lucy, 1963). Further, the presence of α-tocopherol acetate has an important stabilizing effect on a monolayer of lecithin–cholesterol after penetration of the film by vitamin A_1 alcohol (A. D. Bangham, J. T. Dingle & J. A Lucy, unpublished work). The molecular specificity seen in the model system used in the present work would appear to go a considerable way towards providing an explanation for the molecular specificity of vitamin A observed in its action in animals, in organ culture, on isolated erythrocytes and fibroblasts, and on isolated intracellular particles.

SUMMARY

1. Vitamin A_1 alcohol forms a monomolecular film that has a collapse pressure of 22·5 dynes/cm. at an air–water interface. This collapse pressure is greater than that of the films formed by a number of closely related derivatives.

2. Compounds in the vitamin A series are able to penetrate a monolayer of lecithin–cholesterol held at 30 dynes/cm. and constant area. Vitamin A_1 alcohol, vitamin A_1 acid and vitamin A_2 alcohol cause the greatest increases in surface pressure on penetration of the lipid film.

3. Vitamin A_1 alcohol is the only compound of those studied that is able to cause both a large increase in surface pressure at constant area and also a large increase in area of a lecithin–cholesterol film at a constant surface pressure of 30 dynes/cm.

4. The effect of temperature on penetration of a lecithin–cholesterol film by vitamin A_1 alcohol has been investigated.

5. Vitamin A_1 alcohol interacts more strongly with lecithin than with cholesterol.

6. The penetration of a film of lecithin–cholesterol by vitamin A_1 alcohol indicates that penetration of lipoprotein membranes may be an initial step in certain actions of the vitamin; the molecular specificity observed in the experiments with lipid films may be the basis of the molecular specificity observed in many of the functions of vitamin A.

We thank Dr Honor B. Fell, F.R.S., for her encouragement and interest in this work. We are most grateful to Dr R. M. C. Dawson, and to Dr P. L. Harris, for gifts of chemicals. We are indebted to Mr N. Miller for skilful technical assistance. J.T.D. and J.A.L. are members of the Medical Research Council External Staff.

REFERENCES

Bangham, A. D. & Dawson, R. M. C. (1960). *Biochem. J.* **75**, 133.

Bangham, A. D. & Horne, R. W. (1962). *Nature, Lond.*, **196**, 952.

Bangham, A. D., Rees, K. R. & Shotlander, V. (1962). *Nature, Lond.*, **193**, 754.

Blough, H. A. (1963). *Virology*, **19**, 349.

Burt, N. S. & Rossiter, R. J. (1950). *Biochem. J.* **46**, 569.

Dawson, R. M. C. (1958). *Biochem. J.* **68**, 352.

Dervichian, D. G. (1958). In *Surface Phenomena in Chemistry and Biology*, p. 70. Ed. by Danielli, J. F., Pankhurst, K. G. A. & Riddiford, A. C. London: Pergamon Press Ltd.

Dingle, J. T. (1961). *Biochem. J.* **79**, 509.

Dingle, J. T., Glauert, A. M., Daniel, M. & Lucy, J. A. (1962). *Biochem. J.* **84**, 76P.

Dingle, J. T. & Lucy, J. A. (1962). *Biochem. J.* **84**, 611.

Dingle, J. T. & Lucy, J. A. (1963). *Biochem. J.* **86**, 15P.

Dourmashkin, R. R., Dougherty, R. M. & Harris, R. J. C. (1962). *Nature, Lond.*, **194**, 1116.

Fell, H. B., Dingle, J. T. & Webb, M. (1962). *Biochem. J.* **83**, 63.

Fell, H. B. & Thomas, L. C. (1960). *J. exp. Med.* **111**, 719.

Glauert, A. M., Daniel, M., Lucy, J. A. & Dingle, J. T. (1963). *J. Cell Biol.* **17**, 111.

Glauert, A. M., Dingle, J. T. & Lucy, J. A. (1962). *Nature, Lond.*, **196**, 953.

Jackson, S. F. & Fell, H. B. (1963). *Developmental Biol.* **7**, 394.
Lucy, J. A. & Dingle, J. T. (1962). *Biochem. J.* **84**, 76P.
Lucy, J. A., Dingle, J. T. & Fell, H. B. (1961). *Biochem. J.* **79**, 500.
Lucy, J. A., Luscombe, M. & Dingle, J. T. (1963). *Biochem. J.* **89**, 419.
Luzzati, V. & Husson, F. (1962). *J. Cell Biol.* **12**, 207.
Pethica, B. A. (1958). *J. gen. Microbiol.* **18**, 473.
Rideal, E. K. & Taylor, F. H. (1957). *Proc. Roy. Soc.* B, **146**, 225.
Rideal, E. K. & Taylor, F. H. (1958). *Proc. Roy. Soc.* B, **148**, 450.
Schulman, J. H. & Rideal, E. K. (1937*a*). *Proc. Roy. Soc.* B, **122**, 29.
Schulman, J. H. & Rideal, E. K. (1937*b*). *Proc. Roy. Soc.* B, **122**, 46.
Skou, J. C. (1961). *J. Pharm., Lond.*, **13**, 204.
van Deenen, L. L. M., Houtsmuller, U. M. T., de Haas, G. H. & Mulder, E. (1962). *J. Pharm., Lond.*, **14**, 429.
Weitzel, G., Fretzdorff, A. & Heller, S. (1952). *Hoppe-Seyl. Z.* **290**, 32.

Biochem. J. (1964) **90**, 140

The Analysis of the Fatty Acids of Normal Human Depot Fat by Gas–Liquid Chromatography

BY K. J. KINGSBURY AND D. M. MORGAN
Surgical Unit, St Mary's Hospital, London, W. 2

AND T. D. HEYES
Unilever Research Division, Port Sunlight, Cheshire

(*Received* 19 *February* 1963)

Kingsbury, Paul, Crossley & Morgan (1961) reported the use of gas–liquid chromatography, alkaline isomerization and chemical methods in a pilot study of the composition of human fat. These methods, however, were unable to analyse the variety of trace components present. Since that time analytical methods have improved (James, 1960; Landowne & Lipsky, 1961; Lipsky & Landowne, 1958; Lovelock, 1958; Hornstein, Elliott & Crowe, 1959; Farquhar, Insull, Rosen, Stoffel & Ahrens, 1959; Holman & Hayes, 1958), and this paper reports a procedure for the complete analysis of the C_{10}–C_{24} fatty acids of human depot fat by gas–liquid chromatography.

EXPERIMENTAL

All solvents were of analytical reagent grade.

Sampling. The samples of human fat were obtained by surgical incision from patients undergoing operations, or by a biopsy procedure (Kingsbury *et al.* 1962*a*) from out-patients and normal subjects.

Extraction of lipids. This was done by the methods described by Kingsbury *et al.* (1962*a*).

Saponifications were performed by the method advised by the Analytical Methods Committee (1959), since this was found to cause less fatty acid changes than the 'weak'-alkali method of Bottcher, Woodford, Boelsma-Van Houte & van Gent (1959). The propan-2-ol solutions of the fat were added to the reaction mixture.

Hydrogenation was carried out at room temperature, with hydrogen at atmospheric pressure and palladium as catalyst. The methyl esters of the fatty acids were prepared according to Schlenk & Gellerman (1960). Ether and diazomethane were removed by evaporation in a jet of nitrogen; the last traces of water and methanol were removed by evaporation several times with chloroform by using the nitrogen jet. The samples of methyl esters were warmed to 50° and run on to the columns as a liquid from micro-pipettes. In our hands methylation with diazomethane did not produce the volatile by-products, giving rise to peaks on the chromatogram in the C_{12} region, which were reported by Morrison, Lawrie & Blades (1961).

Gas–liquid chromatography. Two types of instrument were used. (1) A catharometer instrument equipped with a hot-platinum-wire catharometer detector, maintained at room temperature. The column used was 366 cm. long, 0·4 cm. diam. and consisted of 20% ethylene glycol–glutarate polyester supported on 100–120 mesh acid- and alkali-washed Celite, at a temperature of 185°. The column eluent was nitrogen. (2) A Pye argon chromatograph equipped with a radium D detector and the standard 4 ft. columns. Two stationary phases, 5% polyvinyl acetate and 5% Apiezon L grease (non-polar), supported on acid- and alkali-washed Celite, 100–120 mesh, were used. Analyses on the polyvinyl acetate column were carried out at 175–180° at an argon flow rate of 40 ml./min., and at 195° and 40 ml./min. on the Apiezon L column.

In the catharometer instrument all components were detected as carbon dioxide; hence the instrument was intrinsically linear but not of extreme sensitivity, and thus unsuitable for the analysis of components of chain length greater than C_{18}.

Because of its thermal stability at the high temperatures necessary for the elution of long-chain components (Hornstein, *et al.* 1959), polyvinyl acetate was used in the Pye argon chromatograph, which was much more sensitive, although its response was not as linear as the