acid, suramin, alginic acid and a number of other organic acids, the exact effect in each case varying with pH.

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Unimolecular Films of Polymyxins A, B, D and E at the Air-water Interface

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The polymyxins consist of a group of five water-soluble basic polypeptide antibiotics possessing a specific action against Gram-negative bacteria; they are produced by many strains of the organism *Bacillus polymyxa*. Benedict & Langlykke (1947) first reported the antibacterial properties of cultures of this organism.

Chemical analysis of the hydrolysates of the antibiotics showed that a fatty acid, identified by Wilkinson (1949) as D-6-methyloctan-1-oic acid, was present in all the polymyxins. Amino-acid analyses demonstrated that polymyxin A contained L-threonine, L- α -diaminobutyric acid and D-leucine (Catch, Jones & Wilkinson, 1948); polymyxin B contained the above amino-acids with D-phenylalanine in addition (Catch, Jones & Wilkinson 1949); polymyxin C differed from polymyxin B in not containing D-leucine; polymyxin D possessed the same amino-acids as polymyxin A but in addition D-serine (Jones, 1948); and polymyxin E had the same amino-acid composition as polymyxin A,

but the intact antibiotics could be readily differentiated by paper chromatography (Jones, 1948).

The chemotherapeutic and pharmacological properties of the five polypeptides were studied by Brownlee, Bushby & Short (1952) and showed that the polymyxins A, C and D all caused severe proteinuria when administered parenterally in animals and man, this occurring even with the purest samples of the antibiotics. In this respect polymyxins B and E differed considerably since they exhibited far less nephrotoxic action.

Relatively little information has accumulated concerning the physical properties of the antibiotics. Jones (1948, 1949) has carried out chromatographic analyses of the antibiotics and has also studied their electrometric titration curves. Bell *et al.* (1949) have determined the molecular weight of polymyxin D by an osmometric method.

In the present communication, the investigation is described of the properties of unimolecular films of four of the polymyxins when spread at the air-water

interface on concentrated salt solutions. These experiments would be expected to provide information concerning the structure and intermolecular packing of the polypeptides. In addition, in order to determine if the nephrotoxic polypeptides possessed significantly different molecular weights compared with the non-toxic compounds, measurements have been carried out at low surface pressures to obtain an estimate of the molecular weights of the antibiotics. This method was first introduced by Guastalla (1939, 1942, 1943) who found that when proteins, fats and fatty acids were spread as unimolecular films at the air-water interface, they behaved as non-ideal two-dimensional gases at low surface pressures. He was able to estimate the molecular weights of several proteins by the use of

weight increases, the surface pressures produced by the film molecules decreases, and the accuracy of their measurement decreases correspondingly.

EXPERIMENTAL

Apparatus. In the study of the surface characteristics of the polymyxins at high surface concentrations a standard Langmuir-Adam type surface balance was used with a sensitivity of 0.2 dyne/cm. Simultaneous recordings of the surface potential changes produced by the presence of the film molecules were made with the electrometer circuit described by Few & Pethica (1952). The surface potential changes (ΔV) have been plotted throughout as ΔVA against A , i.e. as the product of the surface potential in millivolts times the area of the unimolecular film in sq.m./mg. The function ΔVA is directly proportional to the vertical com-

Fig. 1. Diagram of the Allan type low-pressure surface balance.

a two-dimensional equation analogous to that employed for molecular weight determination from osmotic pressure data. This method has been subsequently extensively investigated by Bull (1945, 1946, 1947, 1950) and by Allan (1951) at the air-water interface, and at the liquid-liquid interface by Cheesman (1952). The method finds best application for substances having a 'surface molecular weight' not greater than 20 000, since as the molecular

ponent of the permanent dipole of the oriented monolayer molecules (Schulman & Hughes, 1932).

Of the two main methods available for the determination of surface pressures at high surface dilution, the Wilhelmy hanging-plate technique has been used by Bull (1945, 1946, 1947, 1950), Dieu & Bull (1949) and Cheesman (1952). However, Allan (1951) found that inconsistent results were obtained by this method, due mainly to contact angle changes at the Wilhelmy plate and to drifting of the zero reading caused by drainage and evaporation of the trough

solution from the plate. To overcome these difficulties a modified horizontal Alexander type (1947) film balance was developed by Allan (1951) and this balance has been used in the present investigation.

The low-pressure film balance is shown diagrammatically in Fig. 1. The torsion strip of the Alexander balance was removed and replaced by a horizontal steel strip, near the centre of which were cemented two microscope-slide coverslips. The movable suspension of the balance was fitted with two hardened steel screws ground to a point and adjusted to give a sensitive bearing, in the form of a balance bridge, when the suspension was placed in position on the glass coverslips. A paraffin-waxed mica float was made with two holes in it, through which passed freely two platinum wires attached to the balance bridge. This bridge had a mirror fitted in a horizontal position and two short arms each carrying small aluminium pans. Weights were added to these pans both for calibration and to adjust the bridge so that it rested horizontally with clean water in the trough. With this instrument the sensitivity was governed primarily by the threads attached to the mica float and used to confine the unimolecular film. Using Nylon monofilament threads of a diameter 0.125 mm., rendered hydrophobic by a thin film of petroleum jelly, and with an optical lever of 200 cm. to magnify the movement of the balance bridge, the instrument had a sensitivity between 0.3 and 0.4 cm./0.01 dyne/cm. surface pressure. The surface balance was contained in a wooden box with a removable Perspex front. In one side of the box a hole was made through which was fitted the barrel of an Agla micrometer syringe. The syringe was clamped rigidly in a horizontal position but was free to rotate about its cylindrical axis. A no. 6 serum hypodermic needle was bent at right angles and attached to the syringe; by rotating the syringe, the tip of the needle could be made just to touch the surface of the aqueous solution contained in the trough. Measured volumes of polypeptide solutions could thus be added to the aqueous surface with a minimum of disturbance of the instrument.

After thorough cleaning of the surface of the trough solution, the sensitivity of the balance was determined and the surface was again swept. Successive points on the force-area curves (F - A) were obtained at constant trough area by the addition of measured volumes of the polypeptide dissolved in a 0.05% (v/v) isopropanol-water solution. Two min. were allowed to elapse after each addition of the solution before recording the pressure. This time interval was sufficient to allow for the disappearance of the isopropanol-water solvent from the surface phase. Measurements were made at $20^\circ \pm 1$.

Calculation of molecular weights from low surface pressure measurements. The equation of state of an ideal two-dimensional gas can be written in the form: $FA = RTW/M$ (Adam, 1926; Mitchell, 1935), where F is the surface pressure in dynes/cm., A is the area in sq.cm., W = weight of film spread in g., M is the molecular weight, R is the two-dimensional gas constant = 8.31×10^7 erg/°K, and T the absolute temperature. With the assumption that this equation applies at zero surface pressure and when A is expressed in sq.m./mg. the molecular weight can be determined from

$$M = \frac{RT}{\lim_{F=0} \frac{FA}{F}} = \frac{2435}{\lim_{F=0} \frac{FA}{F}} \text{ at } 20^\circ.$$

The value $\lim_{F=0} FA$ is obtained by extrapolation of the FA - F plot to $F = \text{zero}$. This equation is analogous to the van't Hoff relation for osmotic pressure.

Materials. Polymyxins A, B and E were kindly supplied by the Wellcome Research Laboratories, Beckenham, Kent, and a sample of polymyxin D was supplied by the American Cyanamide Co., Stamford, Conn., U.S.A. These materials were used without further purification. Spreading solutions of the polypeptides were prepared in a 0.05% (v/v) isopropanol-water solvent at a concentration of about 0.2 mg./ml. Attempts to form stable monolayers of the polypeptides on 0.85% (w/v) NaCl at pH 7 and 10, and 15% (w/w) Na_2SO_4 at pH 7 were unsuccessful, as the polypeptides rapidly passed into the trough subphase on compression to a few dynes/cm. Stable monolayers were obtained by using 70% (w/v) $(\text{NH}_4)_2\text{SO}_4$, pH 4.5, as the trough solution and this subsolution has been used throughout the investigation. Surface active impurities in this solution, which was prepared using A.R. grade $(\text{NH}_4)_2\text{SO}_4$, were removed by the addition of 5 g. 'Karbac' activated carbon to 1 l. of solution, and after 3 hr. contact the carbon was removed by filtration. Three such treatments were found to give a solution free from surface active impurities.

RESULTS

Measurements of low surface pressures. The results obtained for low surface pressure measurements using polymyxins A, B, D and E showed that the polypeptides could be divided into two groups. In Fig. 2 are shown the points for determinations on

Fig. 2. F - A curve for polymyxin A, $\bullet$ - $\bullet$, and polymyxin D, $\circ$ - $\circ$, spread upon 70% (w/v) $(\text{NH}_4)_2\text{SO}_4$, pH 4.5, at 20° . Insert: FA - F curve for polymyxins A and D, $\bullet$ - $\bullet$.

polymyxins A and D. These two polypeptides gave identical force-area (F - A) curves over a pressure range of 0.05–0.5 dyne/cm. and a surface concentration range of 35–2.5 sq.m./mg. The insert FA - F curve in Fig. 2 was calculated using values read from the smoothed F - A curve. Extrapolation of the linear low pressure portion of this curve to $F = 0$ gives $\lim_{F=0} FA = 2.0 \pm 0.1$ and a molecular weight of

1220 ± 60 for these polypeptides. The experimental error of $\pm 5\%$ in the value $\lim F/A$ is primarily due to an error of approximately $\pm 2\%$ in the pressure readings below 0.1 dyne/cm.; the area measurement was obtained with an error of less than $\pm 1\%$. Fig. 3 shows the experimental points obtained for both polymyxins B and E. Again, within experimental error the results can be represented by a single $F-A$ curve over a pressure range of 0.04–0.5 dyne/cm. and a surface concentration range of 31–2.0 sq.m./mg. The insert $FA-F$ curve in Fig. 3 was calculated as in the previous case. Extrapolation of the linear low pressure portion of this curve to $F=0$ gives $\lim F/A = 1.9 \pm 0.1$ and the molecular weight 1280 ± 70 . There is, therefore, no significant difference in the molecular weights of the four polypeptides as measured by this method.

Fig. 3. $F-A$ curve for polymyxin B, $\bullet-\bullet$, and polymyxin E, $\circ-\circ$, spread upon 70% (w/w) $(\text{NH}_4)_2\text{SO}_4$, pH 4.5, at 20° . Insert: $FA-F$ curve for polymyxins B and E, $\bullet-\bullet$.

Examination of the two $F-A$ curves of the polymyxins shown in Figs. 2 and 3 indicates that at pressures below 0.15 dyne/cm. they exhibit very similar properties, whilst above this pressure the $F-A$ curves become increasingly divergent. Thus at an area of 3 sq.m./mg. polymyxins A and D produce a pressure of 0.44 dyne/cm. and polymyxins B and E give only 0.31 dyne/cm.

A minimum occurs in each $FA-F$ curve at a pressure of between 0.15 and 0.20 dyne/cm. These correspond to that observed by Bull (1947) for zein films at the air-water interface, and by Cheesman (1952) for bovine serum albumin at the benzene-water interface. Bull has suggested that the minimum observed with zein films may not be due to attractive forces operating between the molecules in the surface phase, but to the displacement of small and soluble fragments of the film molecules into the aqueous subsolution as the film is compressed. As 6-methyloctanoic acid is contained in all the poly-

myxins, we suggest that the minimum observed for these polypeptides is not due to a disruption of the molecule but is caused by van der Waals' forces of attraction between the adjacent C_8 hydrocarbon chains of the film molecules, the effect of which becomes less as the surface concentration decreases.

Measurement of high surface pressures. The results obtained with surface films of these four polypeptides at high surface concentration are recorded in Fig. 4. Polymyxins A and D produce identical $F-A$ curves, characterized by a collapse pressure of 22.6 dynes/cm. and a minimum coefficient of com-

Fig. 4. $F-A$ curves for polymyxin A, $\circ-\circ$, polymyxin B, $\circ-\circ$, polymyxin D, $\bullet-\bullet$, and polymyxin E, $\bullet-\bullet$, spread on 70% (w/w) $(\text{NH}_4)_2\text{SO}_4$, pH 4.5, at 20° . Insert: $\Delta VA-A$ curves for the polymyxins A, B, D and E. The symbols have the same significance as in the $F-A$ curves.

pressibility ($-dA/AdF$) of 0.018 cm./dyne at an area of 0.85 sq.m./mg. Polymyxins B and E give significantly different $F-A$ curves when compared with those of polymyxins A and D. Whilst polymyxin B appears to occupy a slightly smaller area than polymyxin E at all pressures, the curves are in other respects closely similar. Thus both the polypeptides have a collapse pressure of 31.3 dynes/cm. and have the same minimum coefficient of compressibility of 0.013 cm./dyne at 0.70 and 0.75 sq.m./mg. for polymyxins B and E respectively. The slightly smaller area occupied by polymyxin B compared with polymyxin E may be due to the dissolution into the trough solution of traces of impurities of low molecular weight present in the sample used, although the close correspondence of the low pressure $F-A$ curve does not support this view.

The curves of $\Delta V A$ against A in Fig. 4 also fall into the same groups as in the case of the high pressure F - A curves. The function $\Delta V A$ is directly proportional to the vertical component of the dipolar molecules forming the film, and is constant at film areas greater than 0.9 sq.m./mg. for all the polypeptides. The constant $\Delta V A$ for polymyxins A and D is greater than that exhibited by polymyxins B and E. These constant values obtained for $\Delta V A$ as the films are compressed from large areas indicate that no molecular reorientation occurs during this stage. This holds until the films are compressed to the areas giving minimum compressibility. On further compression below these critical areas the values of $\Delta V A$ begin to decrease, showing that reorientation of the film molecules has commenced. It is interesting to note that Bull (1947) has suggested, for protein films, that the area at minimum compressibility represents the smallest area to which a film can be compressed without partial collapse taking place; pressure readings taken at areas below this critical area become much more uncertain and dependent upon time. In the case of the polypeptides investigated here, whilst a reorientation of the film molecules undoubtedly takes place, pressure readings taken at areas smaller than those giving minimum compressibility were quite stable and reproducible. The collapse pressures of the polypeptides were similarly more readily reproducible than those of protein films, and this may be due to the considerable difference in molecular size of the two classes of compounds.

The structure of the polypeptide monolayers as revealed qualitatively by the observation of the motion of talc particles placed on the film surface showed that the films were liquid over the whole compression range. At high surface pressures no evidence for a 'gel' structure was observed, such as obtained with protein films (Hughes & Rideal, 1932); this might be due to a reduction of intermolecular hydrogen bonding in the presence of trough subsolution of low pH as discussed by Alexander (1942).

DISCUSSION

The results obtained for the molecular weights of polymyxins A, B, D and E based upon low surface pressure measurements with insoluble monolayers of the polypeptides show that they all possess the same surface molecular weight within the limits of experimental error. The mean molecular weight 1250 is in good agreement with the value 1150 found by Bell *et al.* (1949) for polymyxin D. It would indicate the presence in the four polypeptides of about nine amino-acid residues in addition to the fatty acid component as suggested by Bell *et al.* (1949) for polymyxin D. These authors also con-

cluded that the various biologically active materials related to polymyxin D were probably closely related in composition, as confirmed in this investigation.

Although there appears to be little significant difference in the molecular weights of the polymyxins, the characteristics of their films at high surface concentration show that the four polypeptides can be classified in two groups according to the compressibilities, collapse pressures and electrical properties of their monolayers. In these respects, polymyxins A and D have identical values, whilst films of polymyxin B appear to be slightly more closely packed than those of polymyxin E, but otherwise have identical properties. The polymyxins in these two groups have different amino-acid constitutions, but the monolayer results indicate that within both groups the intramolecular orientation of the amino-acids and fatty acid residues are closely similar. Moreover, under conditions of equal surface pressure the molecules of the polymyxins A and D are not capable of such close surface packing as those of polymyxins B and E. This, together with the greater compressibility of the polymyxins A and D monolayers, suggests that the intramolecular structures of these polypeptides are not as compact as those of polymyxins B and E.

It is interesting to note that polymyxins A and D, in addition to having identical monolayer properties, also possess similar pharmacological action since both these antibiotics produce severe proteinuria in animals, whereas polymyxins B and E produce only slight toxic effects (Brownlee *et al.* 1952).

SUMMARY

1. The properties of monomolecular films of polymyxins A, B, D and E have been investigated at air-water interface.
2. The molecular weights of the four polypeptides have been determined by application of a two-dimensional gas law equation to measurements carried out at low surface concentration. These results show that there is no significant difference in the molecular weights of the antibiotics.
3. Studies at high surface concentration have shown that the four polypeptides can be separated into two groups, polymyxins A and D in one group and polymyxins B and E in the other. The classification depends upon the compressibilities, collapse pressures and electrical properties of their monolayers. These results have been discussed with regard to the known pharmacological action of the polypeptides.

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