The Haemolytic Acid present in Horse Brain

2. EXAMINATION BY THE INSOLUBLE MONOLAYER TECHNIQUE

By E. D. GODDARD AND A. E. ALEXANDER
Department of Colloid Science, University of Cambridge

(Received 20 March 1950)

The results obtained in Part 1 of this investigation (Morton & Todd, 1950) have indicated as the structure of the haemolytic acid a straight chain monobasic fatty acid of 18 carbon atoms, containing one unsaturated bond of the *cis* form. Because of the small amount of the acid available, elucidation of its structure by degradative studies was rather difficult, and therefore some physical approach to the problem was sought. A compound of this type would be expected to lend itself readily to investigation by the insoluble monolayer technique and, as will be shown below, considerable information as to the structure of the acid has in fact been obtained by this means.

EXPERIMENTAL

The structures and melting points of the materials used are given in Table 1. All contain 18 carbon atoms.

The apparatus used for measuring the force-area (π -*A*) and surface potential (ΔV) characteristics of the various monolayers was of the standard type developed in this department, as described in numerous earlier publications. To reduce electrical interference, the film balance and the valve electrometer were enclosed in the same shielded box. The trough and its housing were held at a constant temperature by circulating water at 25°.

The unsaturated acids were spread from benzene, and the dihydroxy acids from a 1:4 (v/v) ethanol-benzene mixture.

RESULTS AND DISCUSSION

The haemolytic acid on 0.01*N*-HCl gave a liquid expanded film, becoming coherent at approx. 60 A.²/molecule and collapsing at approx. 30 A.² to an oil lens (see Fig. 1). The fact that the pressure increased slowly below the collapse area suggests the presence of some impurity in the compound. For purposes

of comparison the π -*A* and ΔV -*A* characteristics of three known *cis* acids were examined, and it is clear from Fig. 1 that these differ but little from one

Fig. 1. Force-area (π -*A*) and surface potential-area (ΔV -*A*) curves of *cis*-octadecenoic acids and the haemolytic acid on 0.01 *N*-HCl.

Acid	π - <i>A</i> curve	ΔV - <i>A</i> curve
<i>cis</i> -Octadec-6-enoic	A	E
<i>cis</i> -Octadec-9-enoic	B	F
<i>cis</i> -Octadec-11-enoic	C	G
Haemolytic	D	H

another and from the haemolytic acid. It is interesting to observe that the spreading pressures of the four acids are approximately equal: evidently,

Table 1. *Acids examined*

Acid	Structure	Melting point (°)
<i>cis</i> -Octadec-6-enoic (petroselinic)	CH ₃ ·(CH ₂) ₁₀ ·CH:CH·(CH ₂) ₄ ·CO ₂ H	30
<i>cis</i> -Octadec-9-enoic (oleic)	CH ₃ ·(CH ₂) ₇ ·CH:CH·(CH ₂) ₇ ·CO ₂ H	13
<i>trans</i> -Octadec-9-enoic (elaidic)	CH ₃ ·(CH ₂) ₇ ·CH:CH·(CH ₂) ₇ ·CO ₂ H	44
<i>cis</i> -Octadec-11-enoic	CH ₃ ·(CH ₂) ₅ ·CH:CH·(CH ₂) ₉ ·CO ₂ H	11
<i>trans</i> -Octadec-11-enoic (vaccenic)	CH ₃ ·(CH ₂) ₅ ·CH:CH·(CH ₂) ₉ ·CO ₂ H	39
Haemolytic	C ₁₇ H ₃₃ ·CO ₂ H	Approx. 9
<i>cis</i> -6:7-Dihydroxyoctadecanoic	CH ₃ ·(CH ₂) ₁₀ ·CHOH·CHOH·(CH ₂) ₄ ·CO ₂ H	122
<i>cis</i> -9:10-Dihydroxyoctadecanoic	CH ₃ ·(CH ₂) ₇ ·CHOH·CHOH·(CH ₂) ₇ ·CO ₂ H	131
<i>cis</i> -11:12-Dihydroxyoctadecanoic	CH ₃ ·(CH ₂) ₅ ·CHOH·CHOH·(CH ₂) ₉ ·CO ₂ H	127
Dihydroxyhaemolytic	C ₁₇ H ₃₅ O ₂ ·CO ₂ H	124.5

then, these pressures are independent of the position of the double bond in the hydrocarbon chain, and are determined by the work required to remove the polar group from the surface.

The fact that, on collapse, the haemolytic acid yielded a stable oil lens led us to suspect the presence of a *cis* double bond in the molecule. Marsden & Rideal (1938) observed that *trans* unsaturated long-chain acids never form oil lenses, but collapse to solid or unstable liquid films (see Fig. 2). It is to be

Fig. 2. Force-area (π - A) and surface potential-area (ΔV - A) curves of *trans*-octadecenoic acids and the haemolytic acid on 0.01 N-HCl.

Acid	π - A curve	ΔV - A curve
<i>trans</i> -Octadec-9-enoic	A	D
<i>trans</i> -Octadec-11-enoic	B	E
Haemolytic	C	F

expected that chain adlineation would be much easier in the case of the *trans* configuration than in the *cis*, where the hydrocarbon chain is bent (Marsden & Rideal, 1938). In agreement with these findings, elaidic and vaccenic acids, which also gave liquid-expanded monolayers, formed unstable liquid films at low areas. The high π and ΔV values shown by vaccenic acid may be attributable to the sample, which is thought to contain a small amount of stearic acid.

Striking differences between the *cis* and *trans* acids were found on an underlying solution 10^{-4} M with respect to Ca^{++} , pH 7.5 (see Fig. 3). As expected, the calcium ions condensed the films somewhat by binding together the ionized head groups. The *cis* acids and the haemolytic acid gave liquid expanded monolayers which collapsed below about $30 \text{ Å}^2/\text{molecule}$ to unstable liquid films. The *trans* acids behaved very differently, due, undoubtedly, to the

greater ease of packing of the chains, as pointed out above. Elaidic acid showed a transition to the condensed state at 34 Å^2 , and on further compression collapsed to a fragile solid. Vaccenic acid gave a more condensed and less stable monolayer than elaidic acid, and collapsed to an unstable liquid film.

Fig. 3. Force-area (π - A) curves of octadecenoic acids and the haemolytic acid on 10^{-4} M-Ca solution, pH 7.5.

Acid	π - A curve
<i>cis</i> -Octadec-9-enoic	A
<i>cis</i> -Octadec-11-enoic	B
<i>trans</i> -Octadec-9-enoic	C
<i>trans</i> -Octadec-11-enoic	D
Haemolytic	E

Fig. 4. Force-area (π - A) and surface moment-area (μ - A) curves of the dihydroxy derivatives of *cis*-octadecenoic acids and the haemolytic acid on 0.01 N-HCl.

Acid	π - A curve	μ - A curve
<i>cis</i> -6:7-Dihydroxyoctadecanoic	A	E
<i>cis</i> -9:10-Dihydroxyoctadecanoic	B	F
<i>cis</i> -11:12-Dihydroxyoctadecanoic	C	G
Dihydroxyhaemolytic	D	H

The experiments so far described clearly pointed to the double bond in the haemolytic acid having the

cis configuration. Some means of fixing the position of the double bond in the chain was therefore sought. (It is clear from Fig. 1 that it is not in the $\alpha\beta$ position, since Hughes & Rideal (1933) have shown that the surface moment of $\alpha\beta$ -*iso*-oleic acid is approximately double that of oleic acid. This confirms the results of the ultraviolet analysis referred to in Part 1). For this purpose an investigation of the dihydroxy derivatives of the various *cis* acids was undertaken. It was anticipated that the additional polar groups would tend to cause that section of the chain between them and the carboxyl group to lie in the surface, so that the π -A characteristics should depend upon the position of the double bond in the original unsaturated molecule.

The surface characteristics of the various dihydroxy acids on 0.01N-HCl show the anticipated differences between the various isomers (Fig. 4). All gave vapour-expanded films at large areas, and, on compression, underwent a transition to the condensed state, where ultimately the chains must become close packed as the final areas are all around 20 A.²/molecule. From the above analysis the transition areas should be related to the limiting area at which all the molecules can lie in the surface thus,

Fig. 5.

and at smaller areas the dihydroxy groups will be lifted from the surface. Thus the area at the transition point should decrease as the double bond approaches the carboxyl group, and the values for the three known acids are in good agreement with this view.

Table 2. Transition areas of the dihydroxy-octadecanoic acids

Acid	Transition area (A. ²)	Difference in areas
Dihydroxy- <i>cis</i> -11:12-	104.5	12.5 A. ² for two CH ₂ groups
Dihydroxy- <i>cis</i> -9:10-	92	
Dihydroxy- <i>cis</i> -6:7-	74	18 A. ² for three CH ₂ groups
Dihydroxyhaemolytic	98	

It is interesting to note the figures in the last column of Table 2 which show that each additional CH₂ group between the carboxyl and carbinol groups increases the transition area by about 6 A.². This figure agrees reasonably well with the calculated area for a CH₂ group lying in the surface, namely 0.5 × 2.5 × 4.5 = 5.6 A.².

It would seem, from the value for its dihydroxy derivative in the above table, that haemolytic acid, if a pure compound, is the *cis*-10-ene-isomer. As pointed out earlier, the monolayer data on the acid

indicated quite clearly that it is not a pure compound. Later evidence (reported in Part 1), based on the isolation of the two compounds

from the oxidation products of dihydroxy haemolytic acid, suggested haemolytic acid to be a mixture of oleic and *cis*-octadec-11-enoic acids. This view is quite compatible with the present findings, as such a mixture would be expected to exhibit a behaviour intermediate between that of its two constituents.

It may be mentioned that the formation of a vapour-expanded film by the dihydroxy haemolytic acid is further confirmation of the *cis* configuration of the molecule, for Marsden & Rideal (1938), studying the dihydroxybehenic acids, found that, whereas the *cis* modification gave a vapour-expanded monolayer, the *trans* gave a condensed one.

The surface potential results shown in Fig. 4 are in general accord with the above conclusions. The surface moment (μ) remains fairly constant until the transition point is reached, after which it falls rapidly, due presumably to the removal of the two hydroxyl groups from the surface.

It will also be noticed in Fig. 4 that, in the expanded region, the μ values for the dihydroxy-oleic, -haemolytic and -*cis*-octadec-11-enoic acids are all approximately the same, namely just over 500 milli-Debyes (mD.), whereas that for the *cis*-petroselinic derivative is appreciably lower, about 450 mD. The former, after subtracting 180 mD. for the carboxyl group, leaves just over 300 mD. as the contribution from the two hydroxyl groups. This value for the surface moment contribution of two hydroxyl groups on vicinal carbons lying in the surface agrees with the value obtained by Marsden & Rideal (1938) in *cis*-dihydroxybehenic acid. The lower value for the dihydroxy-*cis*-petroselinic acid can be ascribed to steric effects arising from the proximity of hydroxyl and carboxyl groups. It is clear from a study of models that with only four CH₂ groups between carboxyl and carbinol groups it is impossible to obtain the full dipole contribution from all three polar groups, whereas with seven CH₂ groups the molecule possesses the necessary flexibility to make this possible.

A similar proximity effect was noticed by Fossbinder & Rideal (1933) in their study of γ -hydroxy-stearic acid, in which the carboxyl and carbinol are separated by two CH₂ groups. In the expanded region (50–60 A.²/molecule) this acid gave a μ value of 255 mD. Allowing 180 mD. for the carboxyl contribution, this leaves only 75 mD. for the hydroxyl. A further example of this proximity effect has come from a study of ester polymers by Wiltshire (1949). The normal contribution, per ester group (approx. 530 mD.), is obtained only when there are at least six CH₂ groups separating the ester groups (Table 3).

Table 3. *Surface moments of ester polymers*

Ester	Contribution (μ) per ester group (mD.)
Polypropylene adipate (—CO.O.(CH ₂) ₃ .O.CO(CH ₂) ₄ —)	355
Polytetramethylene adipate (—CO.O.(CH ₂) ₄ .O.CO.(CH ₂) ₄ —)	420
Polyhexamethylene adipate (—CO.O.(CH ₂) ₆ .O.CO.(CH ₂) ₄ —)	530

The physical data presented here on the monolayer behaviour of the haemolytic acid and its dihydroxy-derivative, have provided excellent confirmation of the conclusions reached in Part 1 from organic degradation studies. The investigation represents an

interesting application of the monolayer technique to the structural elucidation of organic molecules.

SUMMARY

1. The monolayer properties of the haemolytic fatty acid have been determined and compared with those of *cis* and *trans* modifications of isomeric acids. The comparison has indicated that the acid has the *cis* configuration.

2. It has been concluded from an examination of the dihydroxy derivatives of the *cis* isomers that the haemolytic acid is a mixture of oleic acid and *cis*-octadec-11-enoic acid.

We are indebted to Dr I. D. Morton for the compounds used in these studies, and to the Elsie Ballot Trust for a scholarship which enabled one of us (E. D. G.) to take part in the investigation.

REFERENCES

- Fosbinder, R. J. & Rideal, E. K. (1933). *Proc. roy. Soc. A*, **143**, 64.
 Hughes, A. H. & Rideal, E. K. (1933). *Proc. roy. Soc. A*, **140**, 253.
 Marsden, J. & Rideal, E. K. (1938). *J. chem. Soc.* p. 1163.
 Morton, I. D. & Todd, A. R. (1950). *Biochem. J.* **47**, 327.
 Wiltshire, J. (1949). Private communication.

APPENDIX

X-Ray Examination of the Haemolytic Fatty Acid

By E. D. GODDARD AND M. F. PERUTZ

In this section is described an attempt to find the position and stereochemical nature of the double bond in the haemolytic acid by X-ray methods. Previous X-ray studies (e.g. Alexander, 1939) had shown the long spacing of the *cis* modification of an unsaturated fatty acid to differ considerably from that of its *trans* isomer; also for a series of *cis* isomers, that the long spacing varied according to the position of the double bond in the chain. It was to be expected, therefore, that measurement of the long spacings, especially of built-up multilayers, would throw light on the problem. Moreover, provided a sufficient number of orders of reflexion could be recorded, it seemed possible that the position of the double bond could be found directly by means of a Patterson synthesis.

A preliminary X-ray examination of an impure extract of the haemolytic compound was made by one of us (M. F. P.) in 1945. The sample examined had a melting point of 35° and undoubtedly contained a certain amount of saturated acid as impurity. An oriented preparation was made by allowing a drop of the molten substance to solidify on a coverslip. Oscillation photographs showed several reflexions corresponding to the first and

higher orders of a spacing of 38 Å. Odd orders were strong and even ones weak, indicating long-chain molecules with a polar end group. Orders between the 17th and 19th were too weak to observe. The 17th order had medium strength, the 19th was strong and the 21st absent.

From these data the degree of tilt of the chains relative to the normal to the layer plane could be estimated and hence the chain length deduced. This was found to be 23.2–23.8 Å., corresponding to a saturated fatty acid chain of either 17 or 18 carbon atoms, but as the former seemed improbable on account of the rare occurrence of acids with an odd number of carbon atoms, it was thought most likely that the haemolytic substance contained 18 carbon atoms. However, at the time, chemical analysis seemed to indicate a compound of the formula C₂₃H₄₂O₅ (Laser & Friedmann, 1945). Thus chemical and X-ray data could be reconciled only by assuming a branched chain. In retrospect it is interesting to note that the answer given by the preliminary X-ray analysis was close to the truth, even though the compound examined is now known to have been contaminated with saturated acid.