Medicine. — *The spreading of* MACHEBOEUF*'s lipoprotein.* By E. GORTER and J. J. HERMANS.

(Communicated at the meeting of September 26, 1942.)

By repeated precipitation from horse serum MACHEBOEUF [1]) in 1929 succeeded in isolating a water soluble substance containing 59.3 % protein and 40.7 % lipid. After about seven precipitations the composition of this substance remained constant. Since MACHEBOEUF examined the ninth precipitate in particular, he designated this product as A9. The lipids present are lecithin and cholesterol. According to MACHEBOEUF the lecithin content is 22.6 ± 0.3 %. As regards the cholesterol content, it is not quite clear from his paper whether it amounts to 14.0 ± 0.3 % [2]) or 11.2 ± 0.5 % [3]). The lipid could only be separated from the protein by boiling with alcohol, thereby denaturing the protein. For several reasons MACHEBOEUF assumed this protein to be serum albumin, although no definite identification could be achieved.

The procedure followed by us was the one described by MACHEBOEUF. 300 cc of horse serum were mixed with 300 cc of saturated ammonium sulphate. The globulins were filtered off and the filtrate acidified with sulphuric acid to $p_H = 3.8$. The yellow precipitate obtained was filtered off from the colourless filtrate and dissolved in 75 cc of water to which ammonia was added to bring the final p_H up to 7.5 — 8. The clear solution was again acidified with sulphuric acid ($p_H = 3.8$), the precipitate separated in the centrifuge at 4000 revolutions per minute and dissolved at $p_H = 7.5 — 8$. This procedure was repeated eleven times. Usually 10 minutes at 4000 revolutions sufficed to obtain a clear centrifugate, except for the third and fourth precipitate which had to stand over-night in the ice-box before centrifuging. The eleventh precipitate was dissolved in dilute ammonia and dialysed against distilled water at 0° till it was free from ammonium ions.

By evaporating to dryness on a water-bath and finally drying in vacuo above sulphuric acid it was found that this solution contained 5.30 mg dry substance per cc. To determine the lipid content, the solution was boiled with alcohol, the precipitated protein filtered off and washed with alcohol and ether, the filtrate evaporated to dryness and the residue weighed. The lipid content was 2.10 mg per cc, i.e., 39.6 % of the dry substance.

Cholesterol was determined by a colorimetric method, using the reaction of LIEBERMANN–BURCHARD with the anhydride of acetic acid in chloroform. We found 69.4 mg % in the solution, or 13.1 % of the dry substance.

Finally, the nitrogen content was determined colorimetrically using NESSLER's reaction as described by DEMENIER [4]). We found 0.51 mg nitrogen per cc, which is 9.6 % of the dry substance, in good agreement with the nitrogen content calculated for MACHEBOEUF's A9. In fact, this substance should contain 10.0 % nitrogen: 0.6 times 16 for the protein and 0.23 times 1.8 for the lecithin.

The spreading technique was the one developed by GORTER and collaborators [5]).

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[1]) M. A. MACHEBOEUF, Bull. Soc. Chim. Biol. **11**, 268, 485 (1929); Bull. Soc. Chim. **45**, 663 (1929).

[2]) Reference 1, page 281.

[3]) Reference 1, p. 291 and p. 487.

[4]) G. M. DEMENIER, thesis, Bordeaux 1934.

[5]) E. GORTER and collaborators, Proc. Ned. Akad. v. Wetensch., Amsterdam, **37**, 788 (1934); **29**, 371 (1926).

With p_H below 2.6 the liquid in the tray was dilute HCl. For $p_H > 4$ we used acetate-veronal buffers 0.0033 molar as described by MICHAELIS [6]). With p_H between 2.6 and 3.3 dilute HCl was applied with the addition of NaCl to attain an ionic concentration of 0.0033 gmol/l. All p_H values were measured with the hydrogen electrode.

In fig. 1 some of the pressure-area curves are recorded. It is seen that the nature of these curves is strongly dependent of p_H. With p_H below 3.5 the film shows the

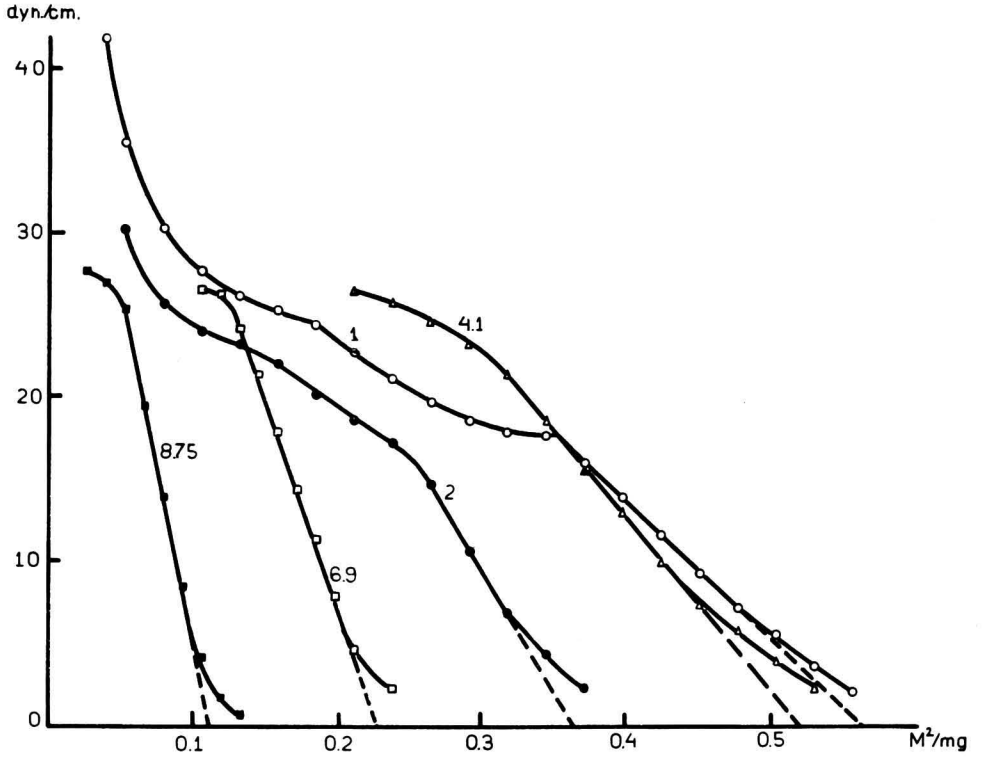

Fig. 1. Pressure-area curves at p_H = 1, 2, 4.1, 6.9 and 8.75.

characteristics of a lipid and may be exposed to high pressures without collapse. If $p_H > 4$, however, the film more and more assumes the characteristics of a protein. This implies that the curvature at pressures beyond about 25 dynes/cm has no definite physical meaning: at these high pressures the film is "folded up", and the pressure drops while compressing. The pressures indicated in this curved part could only be obtained by rapidly compressing the film, and would have been different at a different rate of compression.

The areas obtained when extrapolating the straight part of the curves to zero pressure are plotted against p_H in fig. 2. In this same figure the corresponding areas for serum albumin are recorded, with the only difference that the area is given in square meter per 0.6 mg on account of the fact that MACHEBOEUF's A9 contains about 60% protein. The striking resemblance between the two curves bears out MACHEBOEUF's assumption that the protein considered is identical with serum albumin.

We further separated the protein from the lipids with the aid of boiling alcohol. Since the protein was denatured in the process, it did spread no longer. The protein was washed with alcohol and ether, the combined filtrates evaporated, and the residue solved

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[6]) L. MICHAELIS, Biochem. Z. **234**, 139 (1931).

in petroleum ether. This solution was spread on the tray; its concentration was determined by evaporating the petroleum ether and weighing. For all p_H values the area at zero

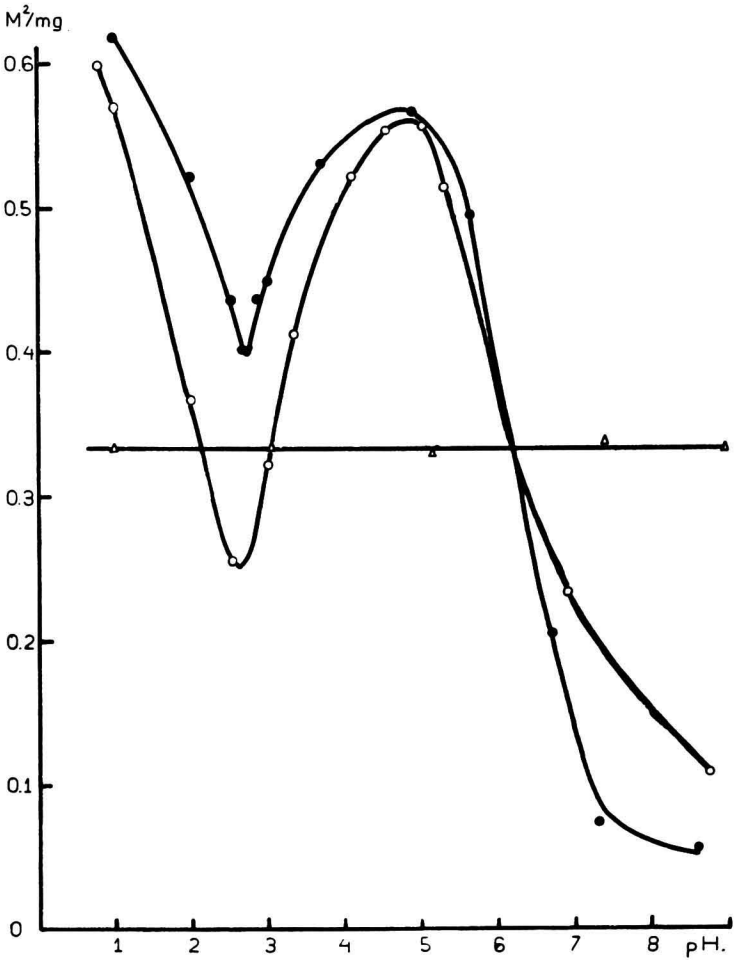

Fig. 2. ○ MACHEBOEUF's A9
● 60 % serum albumin
△ 40 % lipids

pressure is 0.82 m^2/mg. It is interesting to observe that lecithin has a spreading area of 0.86 m^2/mg, while that of cholesterol is 0.62 m^2/mg. Consequently, a mixture of 2/3 lecithin and 1/3 cholesterol would give 0.78 m^2/mg if the spreading values are simply additive. The area found by us thus appears to be quite compatible with MACHEBOEUF's analysis.

In view of the remarkably close resemblance between the area-p_H curves for A9 and serum albumin it would seem that neither the amino groups nor the carboxyl groups of the protein enter into relation with the lipids to any appreciable extent. Yet the existance of a compound is indisputable, since the solution of A9 in water is perfectly clear, whereas, if the lipids after being separated from the protein are shaken with water, a very unstable milky suspension is formed. On the other hand, if the lipids are stirred with a solution of serum albumin in water at $p_H = 8$, they are dissolved, forming a slightly turbid mixture. It was shown, however, by MACHEBOEUF that the lipid is easily

extracted from this mixture by cold ether, whereas the solution of A9 wants a treatment with boiling alcohol for the separation of protein from lipid to be complete. We confirmed this result, and in addition made some spreading experiments with the solution of the lipids in dilute serum albumin, taking care that the protein-lipid ratio was the same as in MACHEBOEUF's A9. It was found that the spreading areas were larger than those of A9. Moreover, the pressure-area curves were quite different. Beyond a certain pressure partial collapse occurred and the protein was squeezed out of the film. A similar behaviour was observed with compounds of oleic acid and ovalbumin which will be described in a later paper.