Biochemistry. — *"The Spreading of Oxy-hemoglobin."* By E. GORTER and F. GRENDEL. (Communicated by Prof. P. EHRENFEST.)

(Communicated at the meeting of December 19, 1925.)

With the LANGMUIR [1])—ADAM [2]) apparatus we have examined the spreading of oxy-hemoglobin solutions, in the same way as these investigators have studied the spreading of simpler substances as fatty acids.

The oxy-hemoglobin was prepared according to HEIDELBERGER [3]). We started from horse's blood supplied by the abattoir, which was centrifuged. The red blood cells were then washed with chilled 0.85 percent sodium chloride solution. The blood corpuscles are then rinsed into a flask with a few cubic centimeters of water. To this toluene was added in amount equal to $^1/_7$ of the volume of the blood corpuscles used, cooled down to 0° C., and a mixture of 4 parts of carbon dioxide and 1 part of oxygen was introduced till a paste was obtained. This was continued for a few minutes with vigorous stirring, the flask was stoppered, and allowed to stand for 24—48 hours in the ice box. Then the oxy-hemoglobin has been crystallized out for the greater part. By centrifuging at low temperatures the crystals are separated from the thick paste. The crystal mass is drained in the ice box on a chilled porous plate, in which care is taken that desiccation at the surface is prevented by constantly renewing the surface layer. During this process a slow stream of carbon dioxide is directed over the surface of the plate. After this the oxy-hemoglobin is scraped into a chilled mortar, and ground to a smooth paste with sufficient ice-cold water to bring the final volume up to 3 to 3.5 times the weight in grams of the oxy-hemoglobin present in the original blood. Then this liquid is titrated with normal sodium carbonate solution to minimum turbidity, the whole mass again centrifuged in chilled tubes, and a stream of the carbon dioxide oxygen mixture passed into the chilled oxy-hemoglobin solution, until crystallisation begins. After having been kept in the ice-box for 24 to 48 hours, the oxy-hemoglobin is again crystallised out for the greater part, and a renewed re-crystallisation can take place in the same way as described above. The oxy-hemoglobin used by us was 5 $\times$ recrystallised in this way, and was used in a 12.5 % solution.

Experiments with fatty acids dissolved in alcohol or acetone had shown that almost equally good results could be obtained in the case of liquid fatty acids as with ether solutions, if only care is taken to bring the alcohol or acetone solution very carefully at the surface of the water in a small quantity. The result was, however, worse in the case of solid fatty acids: too small values were often obtained. Also the results obtained with

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[1]) LANGMUIR, I., J. Am. Chem. Soc., 1917, XXXIX, 1848.

[2]) ADAM, N. K., Proc. Roy. Soc. London, Series A, 1921, XCIX, 336; 1922, CI, 452, 516,

[3]) HEIDELBERGER, J. Biol. Chem., 1922, LIII 31.

emulsions of fatty acid in water, were much too low. Very remarkably the spreading from alcohol was often ½, from an emulsion often 1/3 of the spreading from ether.

When we now transferred the oxy-hemoglobin solution very carefully in a very small quantity on the water surface, we obtained at first very divergent results. We soon found that *time* had an important influence on the size of the surface occupied by the protein. This very clearly appears from the following curves (fig. 1 and 2).

When the protein is allowed to spread on water of a ph 7, a considerable time elapses before the spreading has reached its final value. Finally a constant value is found [1]), from which assuming a molecular weight of 16000, it can be calculated that the surface occupied by 1 molecule of oxy-hemoglobin amounts to :

$$1400—1600 \times 10^{-16} \text{ cm}^2.$$

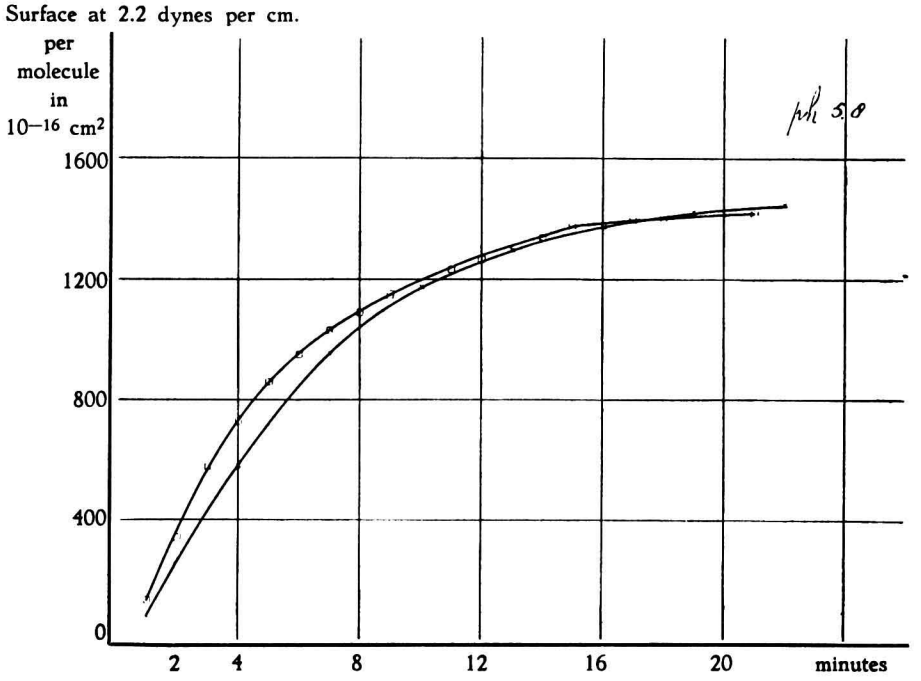


Fig. 1.

It now appeared that the time required for the spreading became very much smaller, when it was examined on a buffer mixture which reacted fairly acid. Thus the spreading was already almost immediately completed on a solution of 6.8 % acid potassium phthalate (ph 4), the experiment immediately giving the final result, when the ph of this buffer was reduced to 3 by the addition of HCl. (See curve 3.)

[1]) Where not stated otherwise, the size was determined at a pressure of 2.2 dynes per cm. (50 mgr.).

That not the ph of the liquid alone determined this velocity, appeared when we examined the spreading on hydrochloric acid solutions of different

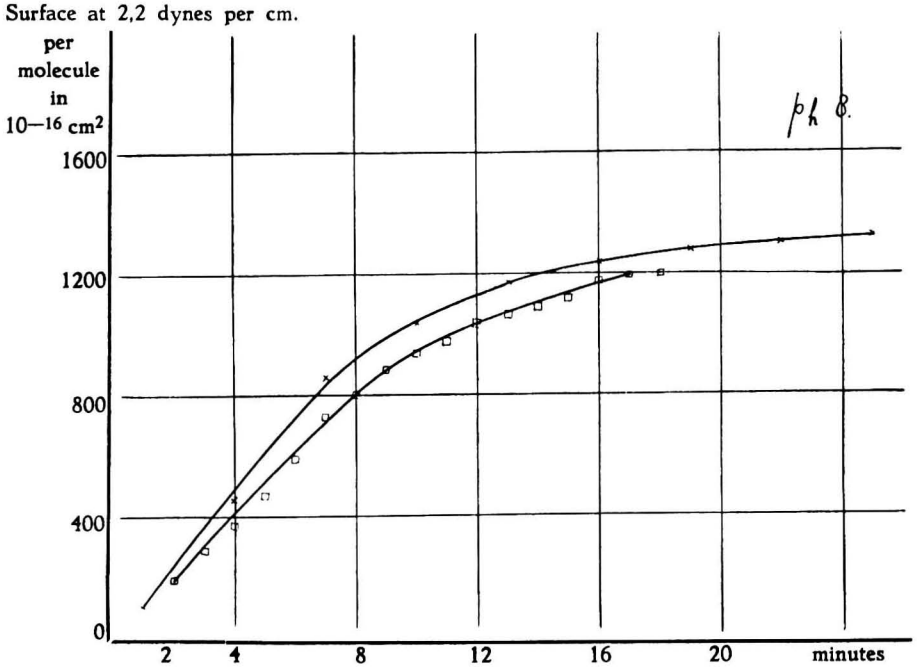


Fig. 2.

strengths. Only on a $^1/_{10}$ N. HCl-solution was the final value immediately reached, while even on a $^1/_{1000}$ N. HCl-solution with a time of observation of ½ hour hardly any increase of the initial spreading appeared to take place. (See fig. 3.) Probably the ph in the surface is the decisive factor, and this is much nearer the neutral point in the hydrochloric acid solution without buffer, because the protein and the carbonate in the hemoglobin solution bind hydrochloric acid.

This phenomenon could not be followed towards the alcaline side, because the blank values become too great there. Then the water is too sensitive to capillary active contaminations. On a phosphate mixture of a ph 8 the spreading was slow. (Curve 2).

After we had observed this gradual spreading and had interpretated it as the disintegration of an associated layer of molecules, an article by CARY and RIDEAL [1]) came under our notice, in which the same phenomenon is described in fatty acids. If these are placed as such on a spot of the water surface, the spreading takes place according to a line similar to that observed by us for proteins.

Moreover the rapidity of the spreading of hemoglobin appeared to be dependent on the *temperature*. Thus on neutral water the rapidity of the

[1]) CARY und RIDEAL, Proc. Roy. Soc. London, Series A, October 1925.

spreading was increased by a rise of temperature, so that at 45° C. the final spreading is already reached after a few minutes.

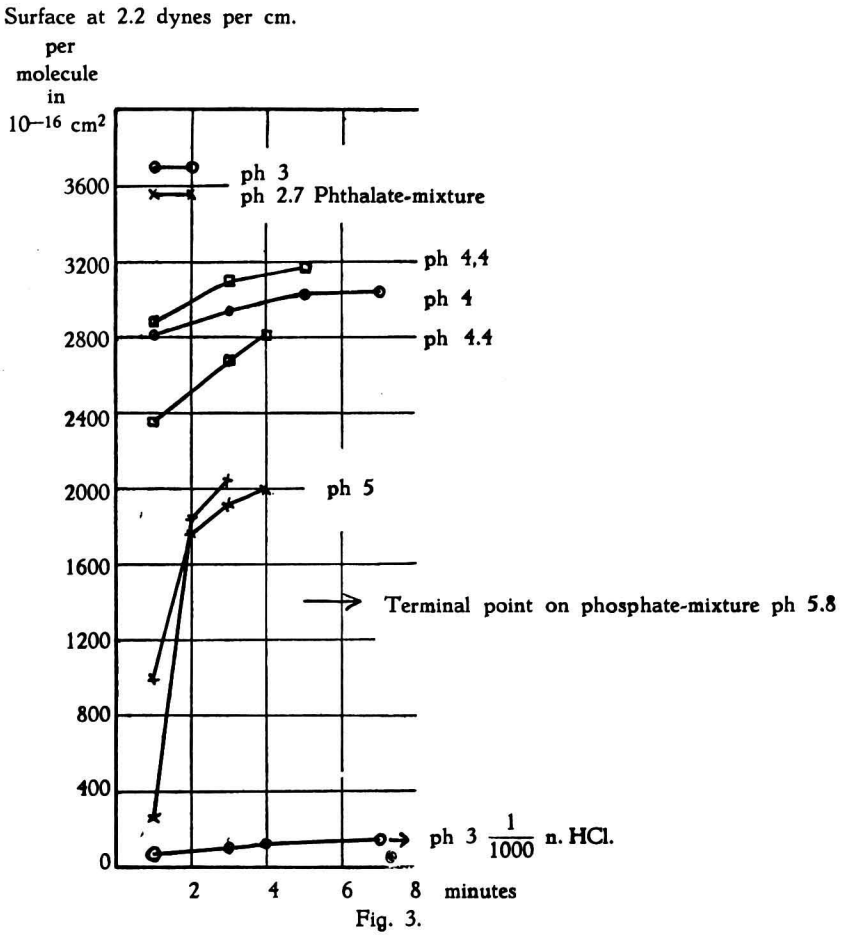


Fig. 3.

But not only the rapidity of the spreading appeared to vary, also the surface occupied by the final spreading by the hemoglobin was not always of the same size. On this, too, ph and temperature had a very marked influence. The greatest values were obtained on buffer mixtures with a ph < 3 when the experiment was made at a temperature of 15° C., the maximum spreading being already reached on buffer mixtures with greater ph at higher temperature. If for this maximum spreading the surface per molecule is calculated (assuming again 16000 for the mol. weight), 3200×10^{-16} c.m². is found. Very remarkably this is double the value found in the neighbourhood of the neutral point (ph 5.5—8). The non-buffered hydrochloric acid solutions exhibited the same phenomenon also in their influence on the extent of the final spreading: on a $^1/_{10}$ N. hydrochloric acid solution maximum spreading (3200×10^{-16} c.m².), on a $^1/_{100}$ N. hydrochloric acid solution half the maximum spreading, and on a $^1/_{1000}$ N. hydrochloric acid solution often scarcely more than

100×10^{-16} c.m^2. These results, too, were a function of temperature. The above data are valid for a temperature of 15° C.; higher temperature promotes the maximum spreading. The influence of the ph appears from the following curve.

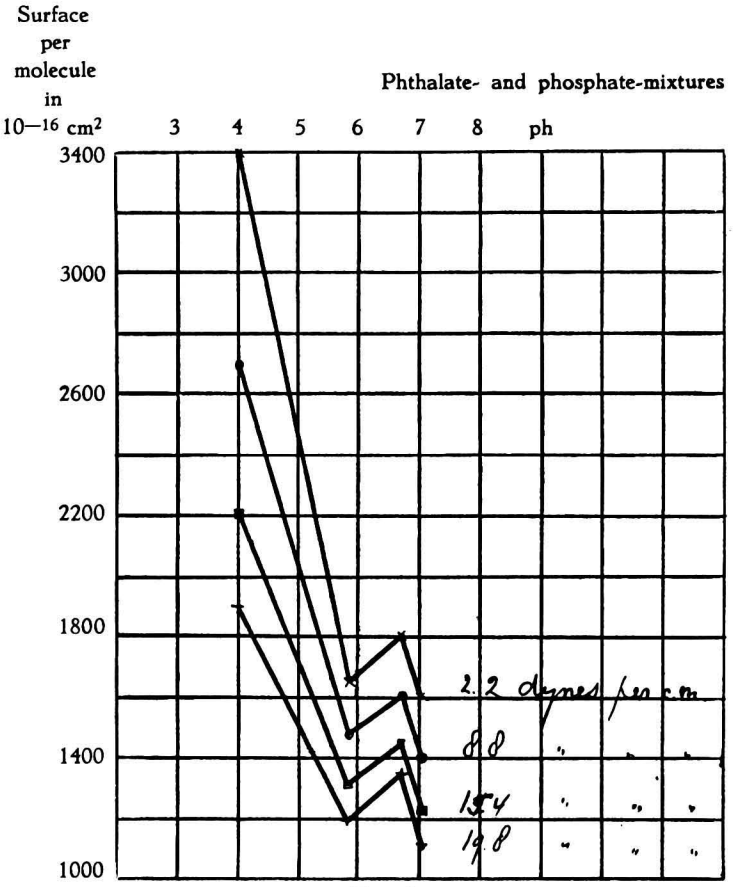


Fig. 4.

It is interesting to note that at the iso-electric point of the oxy-hemoglobin a greater value is constantly found on buffer mixtures than on mixtures with somewhat greater or smaller ph.

It is also noteworthy that the maximum final spreading, as it is reached on acid liquids, becomes little, if at all, greater by a rise of the temperature.

When the influence of the *pressure* on the protein film at the surface is studied, it appears to be greatly dependent both on temperature and on the ph of the water.

At laboratory temperature and on buffer mixtures of a ph between 5.8 and 7 the pressure surface curve is the same (Cf. curves 5, 6, and 7).

From a pressure from 4 tot 5 dynes per c.m. the line runs straight with the same slope. In comparison with the line of fatty acids (examined on acid) this slope is much less steep; i.e. the protein film has greater

compressibility. The force required to break the film lies at about 20—22 dynes. The absolute measure of the molecule being about the same in all

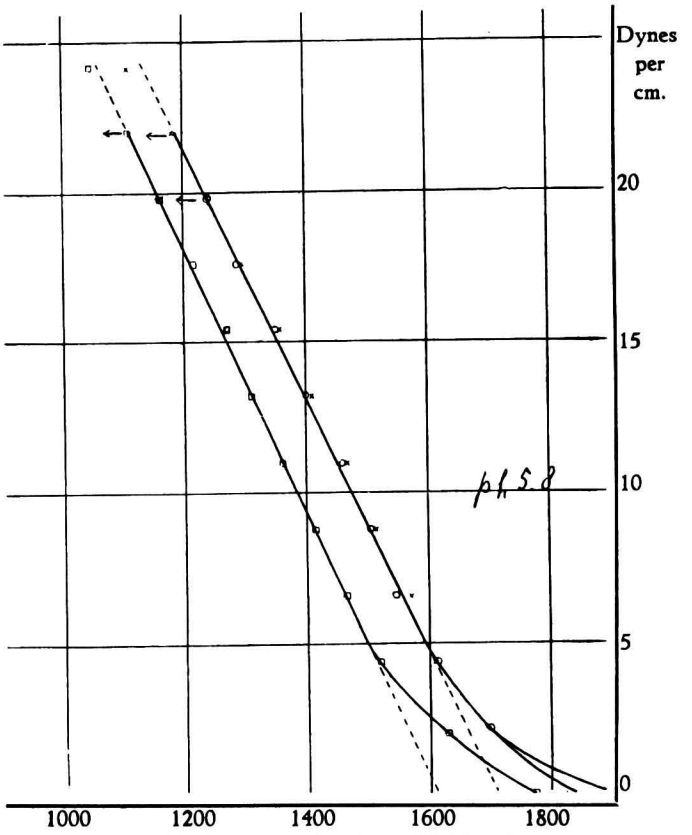


Fig. 5.

these experiments, it follows from the parallelism of the lines that also the force required to reduce the film to half its size has the same value (36 dynes per cm.); hence the constant of compressibility has also the same value.

At laboratory temperature and a ph under 3 we find an entirely different course of the pressure-surface curve. The point where the line begins to run straight, lies at higher pressure (6.6—8.8 dynes), and the slope of the straight portion of the line is greater, even if a correction is applied for the double size of the molecule by the choice of another scale. This may be thus expressed in a figure, that with greater hydrogen ion concentration the film can be diminished to half its size more easily, i.e. by a smaller force. (Here 24 dynes per cm.). The constant of compressibility becomes therefore greater with increasing ph.

Here too the same thing holds mutatis mutandis for HCl-solutions as was already stated for the rapidity and extent of the spreading. (Curve 8).

Here the same influence of the increase of the temperature may again

be observed. (Curves 9 and 10). While e.g. with a ph 5.8 and at a temperature of 15° C. 36 dynes are required for the reduction to half its

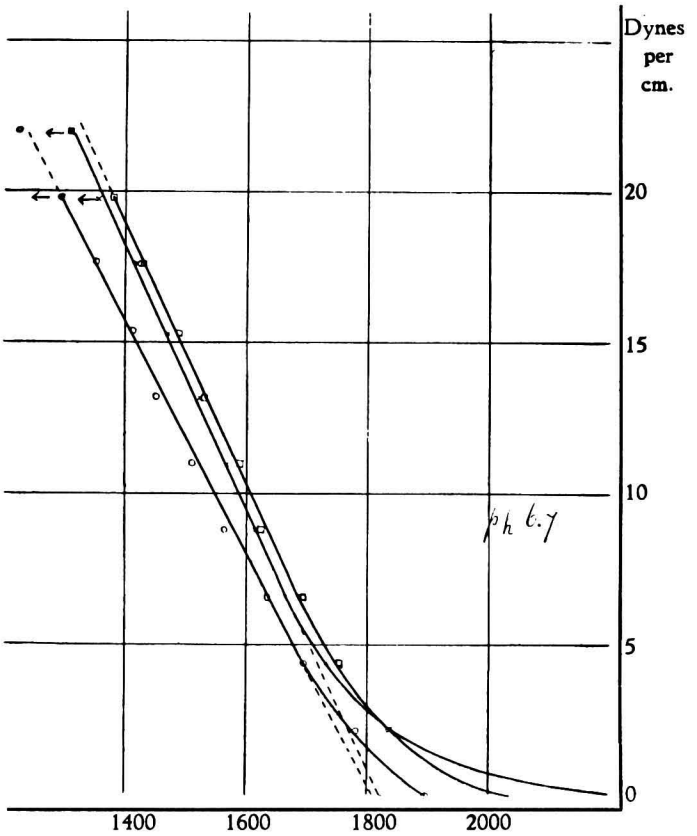


Fig. 6.

size (in the straight part of the line), 24 dynes are sufficient to obtain this result at 45° C.

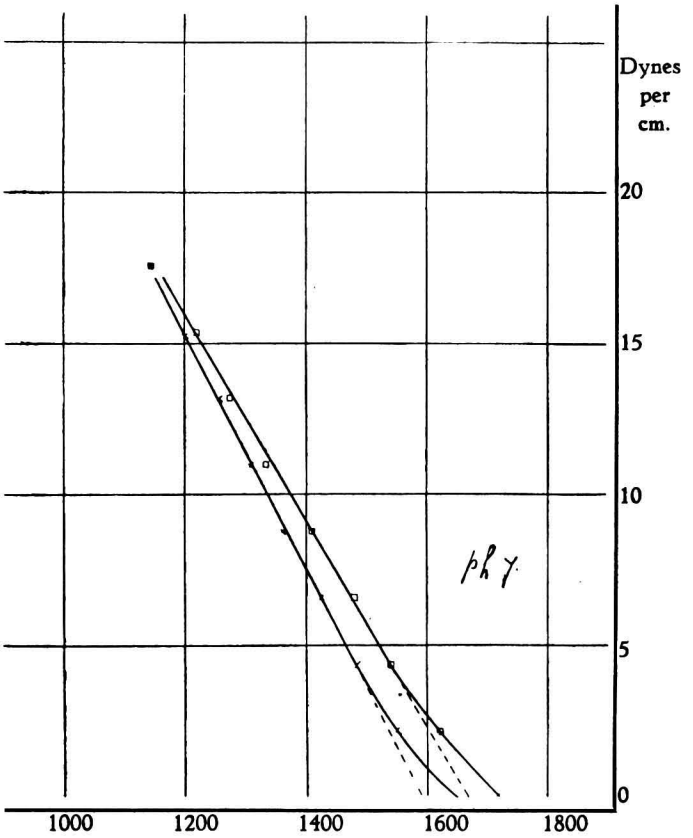
This result might have been expected, because also on the size of the spread film rise of temperature and increase of hydrogen ion concentration had the same influence.

By a suitable choice of a ph and temperature the two forms of compressibility may sometimes be seen to pass into each other in the same experiment (Cf. curve 11).

The influences of pressure and temperature on the extent of the spreading are *reversible*, unless the film has been broken.

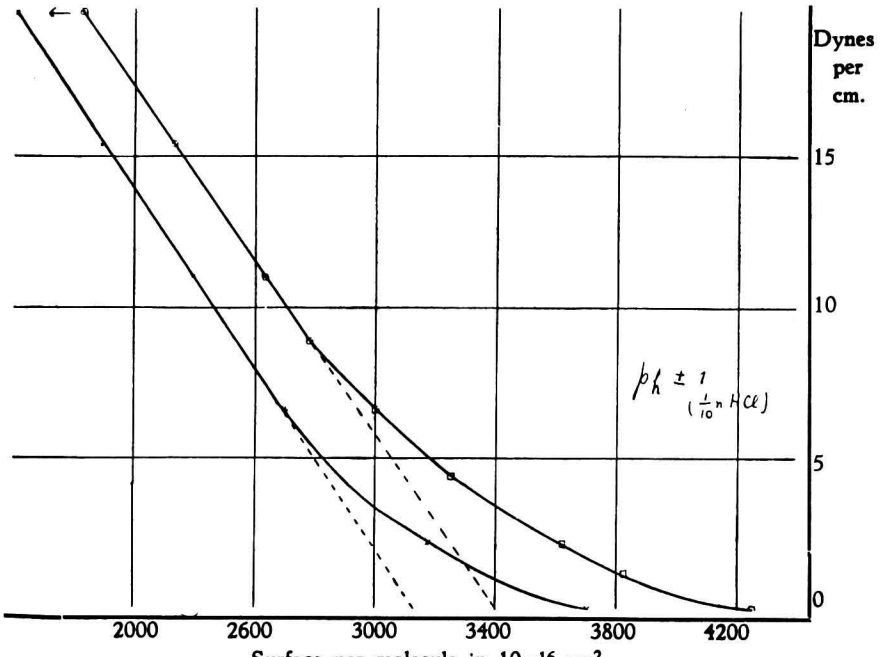
The first part of the line up to the point where it begins to run straight, has not yet been discussed. Already MARCELIN [1]) has pointed out that in fatty acids the corresponding line follows the law of BOYLE. Under small pressure the molecules at the surface behave as in a diluted solution or a gas. Also our measurements on proteins are in harmony with this view.

[1]) MARCELIN, Société de chimie physique, Décembre 1924.



Surface per molecule in 10^{-16} cm^2.

Fig. 7.



Surface per molecule in 10^{-16} cm^2.

Fig. 8.

When we now would try to give an explanation of the phenomena observed, we must first of all remember the structure of the proteins, in which amino acids are mutually bound, and CO—NH-groups are formed. These are polar with regard to water. It can be easily calculated [1]) that of the latter and of free NH_2-groups there are $\pm$ 180 per molecule present in oxy-hemoglobin. From this it may be calculated that starting from the greatest number found by us for the spreading, every CO—NH group occupies a space of about $\frac{3200}{180} = \pm 18 \times 10^{-16}$ cm^2. on the surface. When the extent of the spreading of a substance as a fatty acid or an amine is compared with this, which spreads 20×10^{-16} cm^2. due to the

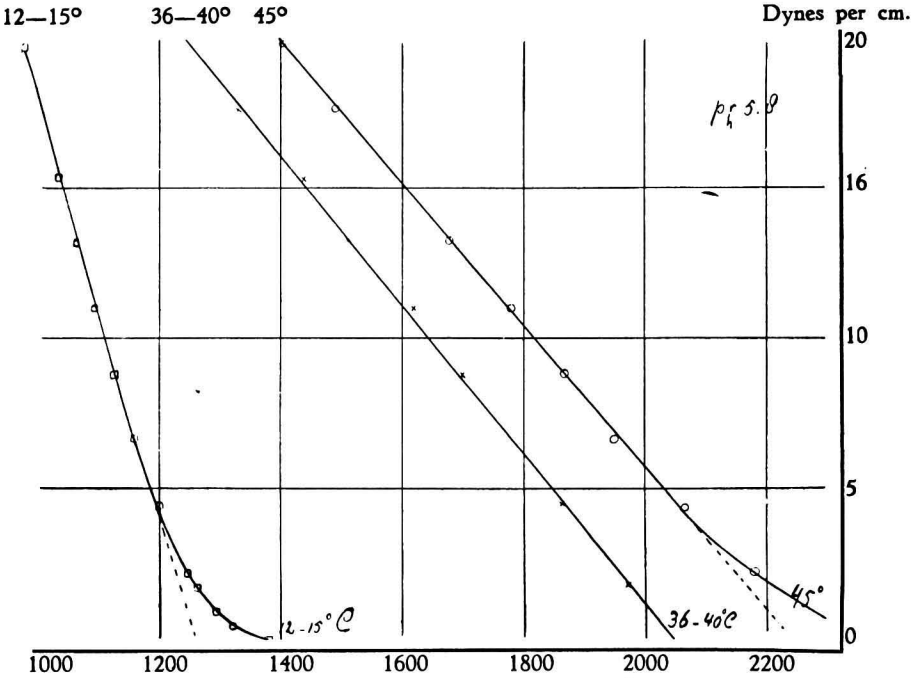
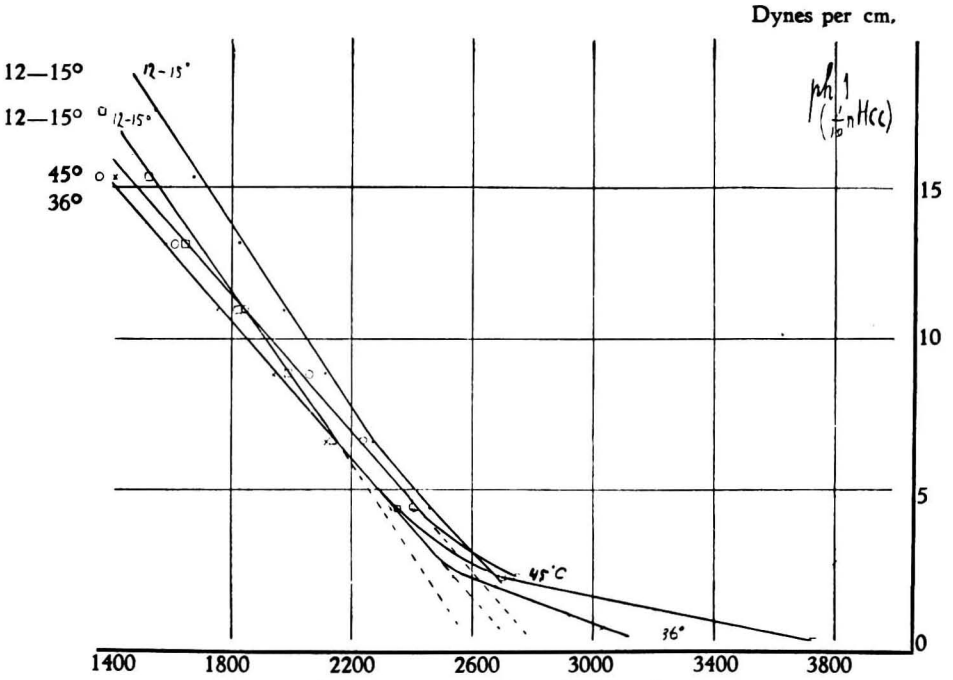


Fig. 9.

presence of a COOH or NH_2-group, the agreement is very good. The CO—NH-group can, accordingly, occupy no more than 20 A.U. in a spreading substance. There is, therefore, some reason to assume that the maximum velocity of spreading on the acid or heated water-surface of the oxy-hemoglobin is the real mono-molecular spreading (film a), depending on the circumstance that about all the CO—NH-groups have been drawn to the water-surface.

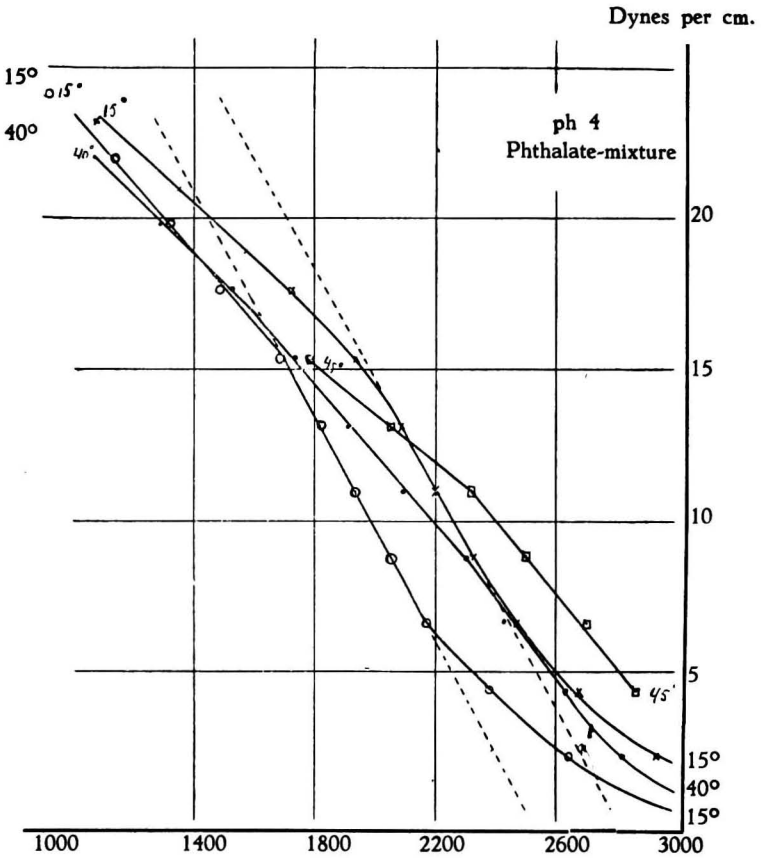
The values being half so great on neutral water (film b) it must be

[1]) From the values for the separate amino acid groups, as given by PLIMMER, Chemical constitution of the proteins, Part I Analysis, 1917, 132.



Surface per molecule in 10^{-16} cm^2.

Fig. 10.



Surface per molecule in 10^{-16} cm^2.

Fig. 11.

assumed that only half of the polar groups participate in the spreading, or that in this case always two molecules lie associated, the layer being bi-molecular under these circumstances.

The latter supposition seems more probable, because acid and rise of temperature both as a rule counteract association, and because from the low values found for the initial spreading a strong association of the oxy-hemoglobin may be inferred.

Besides in this connection we wish once more to point out that the maximum spreading (to ± 3200 A.U. per molecule) obtained on a buffer liquid of ph 3, can no more be increased by heating than inversely the maximum spreading obtained by heating, by acidification.

On the other hand the bi-molecular spreading (± 1600 A.U.) can be doubled both by heating and by acidification of the water.

Assuming that the spreading to 3200 A.U. per molecule is the mono-molecular spreading, we find that the thickness of the layer must be ± 6 A.U. For the molecular volume is 20000, when the Sp. Gr. is assumed to be 1.275. Though the smallest spreading (film c) can never be measured accurately, we wish yet to state that it repeatedly amounted to about $^1/_{50}$ of the maximum spreading, and that the size of the hematine, which very easily spreads from ether, is of the same order of magnitude, viz. ± 70×10^{-16} cm^2. Perhaps this gives some insight into the association of the molecules in the oxy-hemoglobin solution used.

Also in a somewhat different way the values found for the spreading of the protein might be accounted for: viz. by assuming that the protein molecule is a rectangular prism of the following dimensions 6, 12 and 280 A.U., in which polar groups are present in all three planes. The smallest plane $6 \times 12 = 72$ A.U^2. is then the initial spreading and the size of the hematine molecule; the largest plane 12×280 A.U^2. explains the final spreading, the bi-molecular spreading being 6×280 A.U^2. On the other assumption it must also be assumed that the thickness of the molecule is 6 A.U., but then the shape of small flat blocks with a surface of 3200 A.U^2. may be assigned to the molecule. If this plane is a square, 56.5 A.U. is found for length and breadth (fig. 12). When groups of 48 molecules associated are considered, this difference disappears, as appears clearly from figure 12.

Summary:

On a water-surface there exist three forms a, b and c of a hemoglobin film, which differ from each other in many respects. One film (a) is the maximum spreading (± 3200 A.U. per molecule), is more easily compressible, and is formed either by increase of the hydrogen ion concentration or by rise of temperature. The other film (b) has half the extension and is found at a hydrogen ion concentration between 5.5 and 8 and at lower temperature. There occurs a third film (a), which is very unstable, often undergoes little change only on a hydrochloric acid solution

of $^1/_{1000}$ normal, and exists a very short time at the beginning of the experiment also on neutral liquids.

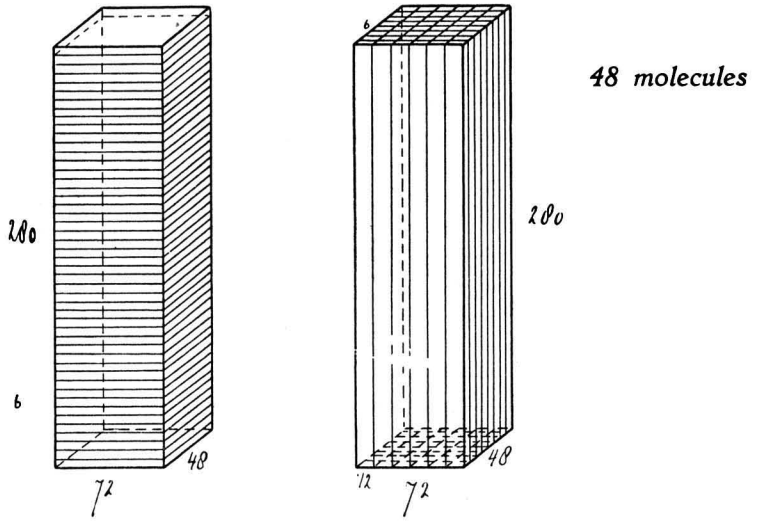


Fig. 12.

Application.

The supposition that the hemoglobin molecules are long needles, at the extremity of which the hematine particle is found with the active iron atom in the centre, is rather attractive. Thus all the iron atoms would be placed in one plane. If it is calculated how many of these groups of 48 molecules can find room in one red blood corpuscle in such a way that the iron atoms lie free on a surface, it appears that such a grouping may be reached by constructing spheres, at the surface of which the hemoglobin molecules lie with the iron towards the outside. In this way exactly half of all the hemoglobin may be got rid of. Hence it is possible to dispose of all the hemoglobin either by giving every sphere a double layer, or by imagining that on an average a double layer of these spheres has been placed along the surface. We mention the result of this calculation only because it is in accordance with the law of BÜRKER, viz. that the proportion of the quantity of hemoglobin and the surface of the chromocytes of different animal species is constant. In this connection we will also point out that it has been established by us that the lipoids of the whole chromocyte occupy a bi-molecular layer [1]) at the surface.

In conclusion it may be added that the doubling of the spreading of the hemoglobin under influence of acid, also appeared to be valid for other proteins, that the film of these proteins must have the same order of thickness, and that we consider it possible that a theory of muscular contraction should reckon with this influence of acid on the size of the protein molecule.

[1]) GORTER and GRENDEL, Journal of exp. medicine Apr. 1 1925 vol. XLI p. 439.