Medicine. — *The spreading of* WARBURG's *yellow enzyme.* By E. GORTER and J. J. HERMANS.

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As an example of a protein with prosthetic group we have spread WARBURG's yellow enzyme prepared from fresh yeast. This substance has been examined by WARBURG and CHRISTIAN [1,2]), THEORELL [3]), WEYGAND [4,5]) and others. For convenience sake let us briefly summarize the results obtained by these authors as far as these results are of interest to the present investigation.

The yellow ferment consists of a protein bound to an active group: lactoflavin phosphoric acid. Its molecular weight is $(71 \pm 3) \cdot 10^3$. It contains 15.9 % nitrogen and 0.043 % phosphorus [3]). The protein itself is free from phosphorus. Consequently, since it is very likely that 1 molecule of the active group is combined with 1 molecule of the protein [3]), the molecular concentration of the yellow ferment can be calculated from the molecular phosphorus content.

The yellow ferment owes its colour to the lactoflavin: $C_{17}H_{20}N_4O_6$. As will be described below, we have made use of its absorption of light in the Stufenphotometer, applying the blue filter S 47. We have used KOSCHARA's [6]) value for the extinction: $\varepsilon_1 = 2.80$. This applies to a concentration of 100 γ lactoflavin per cc and a layer of 1 cm. At the time of our experiments we did not know of the value given by KUHN [7]) ($\varepsilon_1 = 3.23$). It was shown, however, by ROTTIER [8]) that KUHN's result must be considered as too high, the actual value being 2.67 ± 0.05. The difference between this latter value and the one used by us is immaterial in view of our imperfect knowledge of the molecular weight and the uncertainties in the determination of nitrogen and phosphorus..

We have prepared the yellow enzyme in the way described by WARBURG and CHRISTIAN [1]).

> 30 kg fresh yeast from the brewery was washed several times with water. After sedimentation of the yeast, the adhering water was pressed off and the yeast dried at a temperature of 0—5° C. The dry substance was ground till it quantitatively passed a B 20 sieve; we obtained 2 kg of dry powder. This powder was mixed with 7 liter of water, stirred 16 hours at a temperature of about 0° and finally 2 hours at 37°. The yeast was then centrifuged off, and to the liquid obtained (3 l.), 800 cc of lead acetate (liquor plumbi subacetici DAB [6]) was added while stirring. After one night at low temperature, the white precipitate was filtered off, the filtrate mixed with 150 cc 1 molar phosphate of p = 7.5, and then filtered again. To the 2.5 l. thus obtained, 1.3 l. of acetone was added at 0°, and the mixture kept at a temperature below zero.
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> The precipitate was again filtered off and the liquid (3.5 l.) saturated with carbondioxyde and then mixed with 2 l. acetone, always keeping the temperature below zero. An oily precipitate was formed, which was separated from the liquid and then solved in distilled water at 0°.

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1) O. WARBURG and W. CHRISTIAN, Biochem. Z. **254**, 438 (1932); **266**, 377 (1933).
2) O. WARBURG and W. CHRISTIAN, Biochem. Z. **298**, 367 (1938).
3) H. THEORELL, Biochem. Z. **290**, 293 (1937); **278**, 263 (1935).
4) F. WEYGAND and H. STOCKER, Z. physiol. Chem. **247**, 167 (1937).
5) F. WEYGAND and L. BIRKHOFER, Z. physiol. Chem. **261**, 172 (1939).
6) E. KOSCHARA, Z. physiol. Chem. **232**, 101 (1935).
7) R. KUHN, Ber. deutsch. chem. Ges. **68**, 1765 (1935).
8) P. B. ROTTIER, thesis Delft 1942.

The raw material thus obtained was purified by adsorption and eluation in several stages as described by WEYGAND [4]). It is claimed by WEYGAND and BIRKHOFER [5]) that this process may be directly applied to the aqueous extract of the yeast. Our attempts, however, to obtain the yellow ferment in this way were unsuccessful. We even found it impossible to isolate a pure product if we did not first purify the raw material, prepared in WARBURG's manner, by repeated precipitation with acetone. If this pre-purification was omitted, the process of adsorption and eluation led to a degree of purity of 70 % at most. The adsorption was brought about at $p_H = 5.2$ by a suspension of aluminium-hydroxyde, for the preparation of which we refer to WILLSTÄTTER and KRAUT [9]). Eluation followed in a solution of ammonium sulphate and ammonia at $p_H = 8.2$. The yellow ferment was then precipitated at $p_H = 6$ by ammonium sulphate 50 % at 0° and centrifuged off at 10.000 revolutions per minute, since it was found that a velocity of 4000 revolutions was ineffective. For further particulars the reader is referred to WEYGAND and BIRKHOFER [5]). We may add that both the adsorption and the eluation appeared to be greatly furthered if sufficient time was allowed to elapse in the process (usually 1 or 2 hours).

After 4 adsorptions and eluations the product was practically pure, provided the raw material had first been precipitated 3 times by acetone. The final solution was dialysed against distilled water till it was free from ammonium ions. We obtained 45 cc of a solution containing 4.26 mg pure substance per cc. The concentration was determined and the purity controlled as follows.

a. The nitrogen content of the solution was determined by a micro-Kjeldahl to 0.680 ± 0.003 mg/cc. (Two determinations gave 0.677 and 0.684 respectively). Assuming that no impurities are present, this means that the solutions contained 4.28 ± 0.04 mg yellow enzyme per cc.

b. Phosphorus was determined after a method based on the reaction between phosphate, molybdate and hydroquinone. This method was made applicable to very small quantities of phosphorus by the introduction of the Stufenphotometer.

> 4 cc of the solution were destructed by 1 cc concentrated sulphuric acid in a KJELDAHL destruction flask. Hydrogen peroxide 30 % was added to complete the destruction; finally this hydrogen peroxide was removed by the addition of water, and heating. The mixture was then neutralised with NaOH, using phenolphtalein as indicator. After the addition of 2 cc trichloro-acetic-acid 20 %, 0.8 cc ammonium-molybdate 5 % in dilute sulphuric acid and 0.4 cc hydroquinone 0.5 % in dilute $NaHSO_3$, the volume was made up to 10 cc with water, and the mixture kept in the dark for half an hour. The intensity of the blue colour was measured in the Stufenphotometer applying the yellow filter S 57. To find the concentration of phosphate, a reference curve must be made, using different dilutions of a standard phosphate solution. When constructing this curve, care should be taken to add some sodium sulphate to the mixtures, since it appeared that the sodium sulphate formed in the neutralisation of the sulphuric acid to some extent affects the blue colour of the reaction product. We found the method to be quite satisfactory, giving the phosphorus content within a few per cent.

Our final result for the solution of the yellow enzyme was 1.80 ± 0.03 γ phosphorus per cc, i.e., 4.21 ± 0.07 mg yellow ferment per cc. The agreement with the determination of nitrogen is obviously quite satisfactory; we may safely take the concentration to be about 4.26 mg/cc.

c. After diluting the solution ten times, the extinction in the Stufenphotometer with filter S 47 proved to be $\varepsilon_5 = 0.298$ (layer of 5 cm thickness). Or, after reducing to a 1 cm layer: $\varepsilon_1 = 0.0596$. With KOSCHARA's value 2.80 for 100 γ lactoflavin per cc, we

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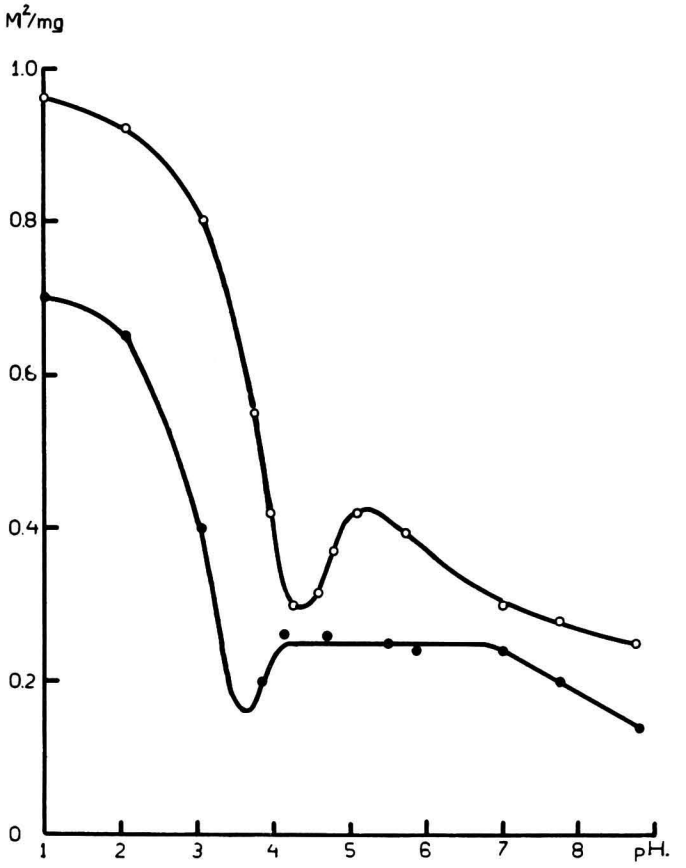
[9]) R. WILLSTÄTTER and H. KRAUT, Ber. deutsch. chem. Ges. **56**, 1117 (1923), **57**, 1082 (1924).

find a concentration of 2.13 γ lactoflavin per cc. Since the molecular weight of lactoflavin is 376, and that of the yellow ferment is $(71 \pm 3)10^3$, this concentration corresponds to 0.402 ± 0.016 mg yellow ferment per cc. That is, in the original solution, 4.02 ± 0.16 mg/cc. Accordingly, the purity of the product amounts to some 94 %. If we had used ROTTIER's



○ Yellow enzyme, isoelectric point 5.22 [3]).
● Protein separated from the yellow enzyme, isoelectric point 5.78 [3]).

value for the extinction, we would have found 4.21 ± 0.17 mg ferment per cc, or 99 %. We have also measured the extinction of the lactoflavin after separation from the protein (see below). This led to the same value for the concentration within experimental error.

The spreading was performed in the usual way [10]). The solution was slowly blown out of a pipette on to the surface. After 1 minute [11]) the film was compressed and the pressure-area curve determined. They were all of the type common to proteins. Extrapolating the straight part of the curve, we obtained the area of the film at zero pressure

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

[11]) In the immediate neighbourhood of the iso-electric point reproducible results could be obtained only if two minutes or more elapsed before compression. Outside the iso-electric region 1 minute suffices; in the iso-electric point, however, spreading appears to be slightly more complete after 2 minutes, without increasing further if this time is increased to 3 minutes or more. For the influence of time on the spreading compare also: ref. 10.

for different p_H-values. These areas are recorded in the graph. Below $p_H = 3.5$ the liquid in the tray was dilute HCl. With p_H between 2.5 and 3.5 sodium chloride $\pm$ 0.002 n was added to attain an ionic concentration comparable to that of the higher p_H-values. For p_H larger than 3.5 we used acetate-veronal buffers 0.0033 molar. All p_H-values were measured by the potentiometric method (hydrogen electrode against calomel electrode) and are accurate to 0.01 units.

The graph further gives the corresponding areas for the protein alone. This protein was obtained in the way described by WARBURG and CHRISTIAN[12]: 10 cc of the solution containing 4.26 mg yellow ferment per cc were mixed with 10 cc saturated ammonium sulphate at 0°. No precipitate was formed. Through the further addition of 4 cc HCl 0.1 n, the protein was separated from the active group, and precipitated by the ammonium sulphate present. After centrifuging at 12.000 revolutions per minute, the protein was washed with half-saturated ammonium sulphate and finally solved in 6 cc 0.03 molar phosphate buffer of $p_H = 7.5$. This solution was dialysed against the said phosphate buffer till it was free from ammonium ions. The nitrogen content was determined by a micro-Kjeldahl and multiplied by a factor 6.25 to give the protein content. The spreading was brought about as usual. Here again it appeared that the spreading time is slightly larger in the iso-electric point (compare note 11). Finally, we may add that the prosthetic group when separated from the protein did not spread at all.

It is seen in the graph that spreading is more complete if the prosthetic group is present in the molecule. It is worth noting that this also applies to p_H-values below 3. In the bulk phase the protein is separated from the prosthetic group if $p_H < 3$. Apparently this reaction does not, or only incompletely, take place in the film, since otherwise no difference should be observed below $p_H = 3$ in the spreading of the protein alone and the protein bound to the active group.

The fact that the prosthetic group itself does not spread is interesting from a biological point of view. One of the functions of the protein in the yellow ferment thus appears to be its ability to spread, thereby greatly furthering the reactivity of the active group in all kinds of biological systems.

It would seem to us that no very detailed conclusions can as yet be drawn regarding the nature of the bonds between protein and prosthetic group, as could be done in the case of several other complex proteins[12]. From the fact that the minimum on the acid side is shifted to larger p_H-values if the prosthetic group is present, it might be inferred that the prosthetic group enters into relation with some of the NH_2-groups or other basic groups of the protein. The depth of this minimum, however, is not altered to a very pronounced extent and some caution is therefore desirable as regards the precise meaning of this shift to larger p_H.

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[12]) E. GORTER and L. MAASKANT, Proc. Kon. Akad. v. Wetensch., Amsterdam, **40**, 71 (1937); E. GORTER, Trans. Faraday Soc. **33**, 1125 (1937); E. GORTER, H. v. ORMONDT and TH. M. MEYER, Biochem. J. **29**, 38 (1935).