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The Iron-Binding Protein of Swine Serum *

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In 1925 Fontès and Thivolle [1] showed that horse serum includes an iron-containing fraction. Subsequently Barkan [2] and Warburg [3] were able to confirm this observation. Barkan showed that at the pH of the blood the iron in the serum (»serum iron») was non-dialyzable. Barkan [4] further found that in native serum the serum iron is not ultrafiltrable. Starkenstein and Harvalik [5] enriched serum with iron; and on fractionation of such serum with ammonium sulphate (50 % saturation) the added iron was found in the globulin fraction. On salting of serum with ammonium sulphate Barkan & Schales [6] found that the protein-bound serum iron was precipitated at 50 % saturation. Vahlquist [7] investigated the protein-binding of the iron in serum through iron analyses on serum fractions that were separated by electrophoresis. He found that the γ-globulin fraction was poor in iron and that the albumin fraction contained less than one-half of the total serum iron. From these experiments he arrived at the conclusion that the main part of the iron was combined with the α-and β-globulin fractions.

Holmberg and Laurell [8] showed that serum contains a high-molecular component that is able to bind iron firmly. It was found that the amount of this component in the serum was decisive of its iron-binding capacity. These authors found the iron-binding capacity of serum from normal persons to be about 300 γ % Fe.

Schade and Caroline [9] were able by means of a microbiological method to show that normal serum contains an iron-binding component. They were further able to show that on fractionation of the serum after Cohn [10] this component is found in fraction IV : 2, which chiefly is made up of a »β-globulin fraction» poor in lipoid.

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* At the Sixth Meeting of Scandinavian Chemists in August 1947 Laurell gave an account of the studies described in the present paper.

In a survey of the protein components of human plasma Cohn [11] gives some numerical values for »β_1-metal-combining protein» (fraction IV : 7) taken from a paper by Oncley, Scatchard and Brown [12]. The molecular weight for fraction IV : 7, determined by osmotic measurements, was 93,000, and the partial specific volume was 0.725. In his paper Cohn gives an approximate iso-electric point = 5.6; and he also states that the metal-combining protein has been crystallized by Koechlin.

Laurell [13] has given an account of some methods for determination of the iron-binding component content of the serum and for variations of this protein fraction under physiological and pathological conditions. He also shows that the reaction

$$\text{iron} + \text{iron-binding protein} \rightleftharpoons \text{iron-containing protein}$$

is reversible.

Previously the isolation of the serum iron-protein complex has met with unsurmountable difficulties, partly because the available methods for fractionation were not sufficiently precise for this purpose, partly because an adequate knowledge about the binding of iron in the native serum iron-protein complex was wanting. In this respect, the conditions have now improved, partly through the alcohol precipitation method given by Cohn, partly because it now is practicable by means of the analytical methods given by Holmberg and Laurell [8], Schade and Caroline [9] and Laurell [13] to separate in the serum specifically bound iron (firmly combined with the iron-binding protein) from the less firmly bound iron (loosely combined with the serum proteins). (The serum proteins have a great tendency to combine with iron. This iron is not so firmly bound, however, as the iron in the iron-binding protein.) A circumstance that facilitates the fractionation is the phenomenon pointed out by Holmberg and Laurell [8] that when the iron-binding protein takes up iron, the mixture becomes reddish in color. Thus it is possible by measuring the intensity of this color to estimate the effectivity of the fractionation. This observation has also been confirmed by Schade and Caroline [9].

PURIFICATION OF THE IRON-BINDING PROTEIN

Swine serum was used in the experiments on fractionation. It offers two advantages above most other sera. In the first place, it possesses a relatively high iron-binding capacity (about 400 γ %). Furthermore, it is practically free from bilirubin, on which account it is easy through measuring the intensity

of color to follow the iron-containing protein in the course of the fractionation, as this is the entirely dominating colored constituent of swine serum if the withdrawn blood is fairly free from hemolysis.

The first step in the process of purification has consisted in salting-out as follows: A serum was diluted with an equal volume of water. Then as much ferrous salt was added as is required to saturate the iron-binding component with iron. Under thorough stirring, a saturated solution of ammonium sulphate, neutralized with ammonia, was added until the final concentration of the mixture became 60 % saturation. The ammonium sulphate solution was added through a fine glass tube (immersed in the serum). The rate at which the ammonium sulphate was added was regulated so that it took some hours to reach this concentration. The precipitation took place at room temperature, and also the ammonium sulphate solution had been saturated at the same temperature. The stirring was further continued for some hours after the addition of ammonium sulphate had been performed. After this, the mixture was left standing over night before the precipitate was separated. About 90% of the iron-binding component was found to be present in the solution. Some loss of this component is unavoidable as the precipitate is not washed — in order not to increase the volume of the solution unnecessarily.

Then the ammonium sulphate concentration was increased to 75 % saturation, and the mixture was left standing till the following day before the precipitate was separated by filtration. The precipitate consisted chiefly in albumin, the iron-containing protein, a relatively small amount of globulins and hemoglobin.

The precipitate was dissolved in water and dialyzed for two days against running tap-water in order to render the solution salt-free. The dialyzed solution was then mixed with so much sodium acetate-acetic acid buffer with pH 4.8 that the pH of the mixture became 5.2. The buffer was added under continous stirring during which the pH was watched by means of a glass electrode. The specific resistance of the solution was used for control of the salt concentration. The specific resistance of the solution should not be lower than 1000 ohms if a good separation is to be obtained in the following step of the procedure.

The temperature of the solution was then lowered to 0° C. Under continuous stirring, 60 vol. % ethyl alcohol, with a temperature of —5° C, was added slowly through a fine glass tube immersed in the solution. Throughout this part of the procedure it is essential to keep the temperature under +4° C. When the alcohol concentration in the mixture reached 20 vol.%, the addition of alcohol was stopped. A strong precipitation was obtained. The mixture was left standing over night at +4° C and then the precipitate was separated

by filtration at the same temperature in refrigerator. The solution thus obtained was brilliantly red in color.

Then the alcohol concentration in the filtrate was increased to 25 vol. %, and the resulting precipitate was removed by filtration after the mixture had been left standing for 24 hours at from —3° C to —5° C whereafter the precipitate now formed was removed by filtration (it was soluble in physiological salt solution, and it consisted partly in iron-containing protein, partly in impurities). The remaining protein in the mother-liquor consisted chiefly of the iron-containing protein. By lowering the temperature of the mother-liquor to about —15° C the iron-containing protein could be precipitated as a beautiful red sediment which again dissolved readily in neutral solutions. The solution of the fractionated iron-containing protein obtained in this way was freed from alcohol through dialysis against running water. In frozen condition, the solution of the iron-containing protein could be stored without any change taking place in its properties.

A reprecipitation of the iron-containing protein was performed after the schema outlined above in order further to rid it of impurities.

(The optimal fractionation is obtained when the specific resistance in the solution prior to the addition of alcohol is higher than 1000 ohms.)

PROPERTIES OF THE IRON-CONTAINING PROTEIN

The purified iron protein complex may be precipitated neither with 50 % saturated ammonium sulphate nor with saturated sodium sulphate solution. As is evident from the above, the complex is precipitated in mixture with the other serum proteins in the albumin fraction. Nor is the iron-containing protein precipitated on dialysis against distilled water till all the salt is removed. With a view to its aspects of solubility, there is no reason to designate the iron-containing protein as a globulin.

In order to test the purity of the iron-containing protein prepared in the way here described, experiments were carried out with electrophoresis, ultracentrifuging and diffusion.

The electrophoresis experiments were carried out with the apparatus given by Tiselius and optical reading *ad modum* Svensson [14]. For these experiments we employed a buffer containing primary and secondary sodium phosphate with a combined ionic strength of 0.1. Besides, the buffers were 0.1 molar in NaCl, so that the total ionic strength was 0.2. A preliminary experiment with a 1.2 % solution with pH 6.8 showed the substance to be homogeneous electrophoretically. Then a number of experiments were made at 0.0° C with 0.5 %

solutions with pH varying from 5.1 to 7.3. The iso-electric point for the iron-containing protein determined in these phosphate buffers was about 4.4 (extra polated value). Table 1 gives the mobility of the substance and the pH of the solution for the descending side. As will be noticed, the rate of movement was low. (With pH under 5 the iron partly splits off from the protein, on which account we have omitted some values obtained for the mobility in acetate buffers with pH lover than 5.)

The experiments with ultracentrifugation were performed in a Svedberg ultracentrifuge with solutions containing varying percentages of protein dissolved in a buffer made up of 0.025 *M* Na_2HPO_4, 0.025 *M* NaH_2PO_4 and 0.10 *M* NaCl. (60,000 r. p. m. Registration was made by Lamm's scale method.) Also the ultracentrifuge tests showed that the protein was practically pure.

Table 2 gives the values obtained for the sedimentation constant (in Svedberg units). (As to the performance of the ultracentrifuge experiments, the reader is referred to [15].)

From the values recorded in Table 2, the value for concentration 0 is calculated as $s_{20} = 5.8\ S$.

Table 1. Electrophoretical measurements.

pH	Mobility in cm^2/s-volt $\times 10^5$
7.35	2.5
6.85	2.2
6.75	2.4
5.95	1.3
5.45	1.5
5.10	0.8

Table 2. Ultracentrifuge experiments.

Protein concentration in %	Sedimentation constant in Svedberg units
0.80	5.60
0.60	5.86
0.30	5.78
0.26	5.69
0.20	5.68
0.15	5.91
0.08	5.83
0.00 (extrapolated)	5.80

Table 3. Diffusion experiments.

Protein concentration in %	Diffusion constant $D_A \times 10^7$
0.8	5.82
0.5	5.82

Table 4. Iron content of the iron-binding protein.

Preparation no	No of atoms of iron per protein molecule
1	1.5
2	1.3
3	1.3
4	1.4

Also diffusion experiments were carried out with the iron-containing protein dissolved in the same buffer as in the ultracentrifuge tests. In these experiments the concentration varied between 0.5 and 0.8 %. Registration was made by Lamm's scale method. No dependence upon the concentration could be established. The values of the diffusion constant D_A, calculated by the »method of areas» have been summarized in Table 3. The mean value obtained was a diffusion constant $D_A = 5.8 \times 10^{-7}$.

No determination of the partial specific volume has been made. We have reckoned with $V = 0.725$ as given in the articles by Cohn [11] and by Oncley, Scatchard and Brown [12].

By means of these values for the sedimentation constant, diffusion constant and partial specific volume, on employment of Svedberg's formula [15], we thus obtained the molecular weight M = 88,000 for the iron-containing protein here prepared. The »frictional ratio» f/f_0 was calculated to be 1.25. When the molecule is assumed to have the form of a revolution ellipsoid, the ratio between the length and the width of the molecule is 4.9, and the length of the molecule is 170 Å units, while its width is 35 Å units.

The amount of specifically bound iron was determined in four quite different preparations (new serum for each preparation) of the iron containing protein. The results are shown in Table 4. As is seen from the table the iron content varies between 1 and 2 atoms of iron per molecule (mol.wt. = 88,000). It suggests that most probably the iron binding protein can specifically combine with a maximum number of two atoms of iron. The fact that we have not obtained a whole number of iron atoms per molecule can perhaps be explained in different ways. Probably it depends on the facts that a part of the iron has been split off during the preparation and that the ability to combine with iron is easily reduced. (During the preparation the solution has been maintained at pH 5.2.)

SUMMARY

A description is given of the isolation of the iron-binding protein of swine serum. The properties of the thus isolated iron-containing protein are investigated. The molecular weight is estimated as being 88,000, and the iso-electric point as 4.4.

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REFERENCES

1. Fontès, A., and Thivolle, L. *Compt rend. soc. biol.* **93** (1925) 687.
2. Barkan, G. *Z. physiol. Chem.* **171** (1927) 205.
3. Warburg, O. *Biochem. Z.* **187** (1927) 255.
4. Barkan, G. *Z. physiol. Chem.* **216** (1933) 1, 217; **221** (1933), 241.
5. Starkenstein, E., and Harvalik, Z. *Arch. exp. Path. Pharmakol.* **172** (1933) 74.
6. Barkan, G., and Schales, O. *Z. physiol. Chem.* **248** (1937) 96.
7. Vahlquist, B. *Acta Paediat.* **28** (1941) suppl. 5.
8. Holmberg, C. G., and Laurell, C.-B. *Acta Physiol. Scand.* **10** (1945) 307.
9. Schade, A. L., and Caroline, L. *Science* **104** (1946) 340.
10. Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J., Melin, M., and Taylor, H. S. *J. Am. Chem. Soc.* **68** (1946) 459.
11. Cohn, E. *Experientia* **3** (1947) 125.
12. Oncley, J. L., Scatchard, G., and Brown, A. *J. Phys. and Coll. Chem.* **51** (1947) **184.**
13. Laurell, C.-B. *Acta Physiol. Scand.* **14** (1947) suppl. 46.
14. Svensson, H. *Arkiv Kemi, Mineral., Geol.* **22 A** (1946) no. 10.
15. Svedberg, T., and Pedersen, K. O. *The Ultracentrifuge* Oxford (1940).

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Preliminary Communications

Isolation of a Phosphorus-rich Substance of High Molecular Weight from *Aspergillus niger*

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While investigating phosphorus metabolism in moulds, Mann made the very interesting observation that extracts from *Aspergillus niger* contained not only pyrophosphate but also metaphosphate [1]. However, he made no determinations of the molecular weight of this metaphosphate. During investigations of the enzymatic breakdown of synthetic polymetaphosphate of very high molecular weight we made preliminary experiments which showed that a fraction of the naturally occurring metaphosphate (from *A. niger* is not dialyzable and therefore may possess a relatively high molecular weight[2].

In 1936 MacFarlane isolated from yeast a nucleic acid preparation which contained 16—18 % P[3]. Later Wiame also isolated from yeast such preparations which contained 17 % P and 8 % N [4, 5].

In our continued experiments we have now shown that it is possible to isolate from *A. niger* a high molecular, non-dialyzable substance with a very high phosphorus-content. This substance does not contain nucleic acid.

For the experiments we have used a culture of *A. niger* v. Tiegh. (no. 594 from the National Collection of Type Cultures maintained in Britain by the Medical Research Council). The mould was cultivated at 28° C on a medium of the following composition: 10 g glucose, 0.2 g K_2HPO_4, 0.4 g $NaNO_3$, 0.05 g $MgSO_4$, $7H_2O$ and 100 ml water. After 8 days' growth the mould was ground in a »Waring