DIFFERENTIATION OF OXYHEMOGLOBINS BY MEANS OF MUTUAL SOLUBILITY TESTS.

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(Received for publication, July 6, 1923.)

In recent papers[1] the authors were able to show by serological tests that hemoglobin exhibits species specificity to a high degree. It seemed of advantage, however, to demonstrate these differences, undoubtedly chemical in character, by some independent method.

The solubility of an individual protein itself might be taken as a criterion of chemical entity, as has been suggested recently by Cohn.[2] As, however, this method requires the attainment of absolute purity, aside from the possibility that several substances might have the same solubility within the limits of error, another method was sought.

Of the possibilities which suggested themselves, a promising one seemed the use of the well known fact that the solubilities of substances which do not react with each other are additive. As a consequence it would be expected that if two samples of oxyhemoglobin were different each would dissolve in a saturated solution of the other as in pure water itself. On the other hand, if two preparations were identical, a saturated solution of one would obviously be saturated to both.

As the method used in the present work was a comparative one, extreme accuracy was not aimed at. Hence the solubility determinations were carried out simply at room temperature. 1 cc. samples of the solutions were used, and the content in hemoglobin was determined by drying to constant weight *in vacuo* at 40–50°C.

[1] Landsteiner, K., *Verslog Kon. Acad. van Wetensch. te Amsterdam*, 1921, xxix, 1029. Heidelberger, M., and Landsteiner, K., *J. Exp. Med.*, 1923, xxxviii, 561.

[2] Cohn, E. J., *J. Gen. Physiol.*, 1921–22, iv, 697.

The preparations of oxyhemoglobin were made according to the method recently published by one of us.[3] In each case freedom from salts was controlled by conductivity measurements, and in several instances electrometric pH determinations were run on the solutions in order to make certain that the observed solubility effects were not due to differences in the pH of the solutions.

As an example of the manipulation used the following experiment (No. 2) is described.

Recrystallized horse and dog oxyhemoglobin were used. The conductivities of the aqueous solutions were respectively 4.3×10^{-5} and 2.7×10^{-5}. Each oxyhemoglobin in the form of a moist crystalline paste was mixed with water in a tube and shaken mechanically for $\frac{3}{4}$ of an hour. That equilibrium was approximately attained within this period was shown by a determination of the hemoglobin in solution, and a comparison of the amount found with the hemoglobin content after the next shaking, the latter value being the one given at the head of each column in the tables. Usually a slight increase was noted. At the end of the period of shaking the tubes were centrifuged and the supernatant liquid from each was poured in equal parts into two tubes. To one of these was added more of the homologous protein, while to the other the oxyhemoglobin of the other species was added. The four tubes were now shaken again for $\frac{1}{2}$ hour and centrifuged. In every case before taking the analytical samples the solutions were filtered through small analytical filters in order to hold back any crystal fragments. 1 cc. portions of each were now dried and the residue was weighed.

The results are given in the following tabulations, in which the figures represent the weight of the residue from 1 cc. in gm. In each case the value after "difference" should approximate that at the head of the opposite column.

Experiment 1.

Dog + dog	0.0275	Horse + horse	0.0080
Dog + horse	0.0384	Horse + dog	0.0302
Difference	0.0109	Difference	0.0222

[3] Heidelberger, M., *J. Biol. Chem.*, 1922, liii, 31.

Experiment 2.

Conductivities: horse HbO_2 4.3×10^{-5}; dog HbO_2 2.7×10^{-5}.

Dog + dog	0.0238	Horse + horse	0.0158
Dog + horse	0.0372	Horse + dog	0.0417
Difference	0.0134	Difference	0.0259

Experiment 3.

Dog + dog	0.0260	Horse + horse	0.0151
Dog + horse	0.0395	Horse + dog	0.0400
Difference	0.0135	Difference	0.0249

Experiment 4.

Conductivities: horse HbO_2 3.2×10^{-5}; dog HbO_2 3.5×10^{-5}; guinea pig HbO_2 2.9×10^{-5}.

pH electrometric;* respectively: 6.69, 6.82, 6.76.

Dog + dog	0.0256	Horse + horse	0.0107
Dog + horse	0.0356	Horse + dog	0.0293
Difference	0.0100	Difference	0.0186
Dog + dog	0.0256	Guinea pig + guinea pig	0.0046
Dog + guinea pig	0.0274	Guinea pig + dog	0.0318
Difference	0.0018†	Difference	0.0272
Horse + horse	0.0107	Guinea pig + guinea pig	0.0046
Horse + guinea pig	0.0140	Guinea pig + horse	0.0159
Difference	0.0033	Difference	0.0113

*The writers are indebted to Dr. A. B. Hastings for these determinations.

†That this value is too low is probably due to the fact that, as mentioned above, saturation was not always complete at the end of the first shaking. This would have little influence except in a case such as this, in which the solubility of the second type of hemoglobin is much less than that of the first.

Experiment 5.

Conductivities: guinea pig 2.8×10^{-5}; rat 3.8×10^{-5}.

pH electrometric, respectively: 6.69, 6.95.

Guinea pig + guinea pig	0.0040	Rat + rat	0.0039
Guinea pig + rat	0.0071	Rat + guinea pig	0.0086
Difference	0.0031	Difference	0.0047

It will be seen from the above experiments that in four of the species tested, differences between the oxyhemoglobins were indicated by the increase in the dissolved hemoglobin. This increase was in most cases not far from the solubility of the added type of oxyhemoglobin in water alone. It thus appears that the method is capable of bringing to light chemical differences between the oxyhemoglobins of not too closely related species. The trial of this procedure in the study of other proteins would therefore seem desirable.

As a control on the method Experiment 6 was performed. In this experiment two different lots of horse oxyhemoglobin were compared as before. As was to be expected the result was negative.

Experiment 6.

Comparison of two different preparations of horse oxyhemoglobin (I and II). Conductivities: I 2.2×10^{-5}; II 3.3×10^{-5}.

I + I	0.0120	II + II	0.0107
I + II	0.0118	II + I	0.0107

Experiment 7.

Conductivities: horse HbO_2 3.3×10^{-5}; donkey 1.5×10^{-5}.

Horse + horse	0.0166	Donkey + donkey	0.0086
Horse + donkey	0.0139	Donkey + horse	0.0128
Difference	−0.0027	Difference	0.0042

In Experiment 7 oxyhemoglobins of two closely related species, namely the horse and the donkey, were investigated. This case was of special interest in that our serological tests indicated so slight a dissimilarity as to render difficult a serological differentiation. In the present series of experiments, also, this case differed from the others, as no addition of solubilities took place. While it would seem that the final values were between the solubilities of the two preparations, too much stress cannot be laid upon this point, as the absolute value of the solubility of horse oxyhemoglobin varied in the different experiments, and the cause of this variation has still to be determined.

Since, from general considerations, identity of the two proteins is improbable, it would appear that the two oxyhemoglobins behave

as isomorphous compounds. It is interesting that such a relationship should be found in a case in which the proteins appear compatible *in vivo*, as is shown by the ability to cross these animals. In this connection we must mention the comprehensive work of Reichert and Brown[4] on the crystallography of hemoglobin with respect to species differences. Its possible bearing upon the point just mentioned has been stated by Loeb.[5]

CONCLUSIONS.

1. The rule of addition of solubilities is applicable to the differentiation of the oxyhemoglobins of not too closely related species.

2. The oxyhemoglobins of the horse, dog, rat, and guinea pig show differences when tested by this method. The oxyhemoglobins of the donkey and horse show a similarity which is best explained by the assumption of isomorphism.

[4] Reichert, E. T., and Brown, A. P., *Carnegie Inst. Washington, Pub. 116*, 1909.
[5] Loeb, J., *Science*, 1917, xlv, 191.