THE EQUILIBRIA BETWEEN NATIVE AND DENATURED HEMOGLOBIN IN SALICYLATE SOLUTIONS AND THE THEORETICAL CONSEQUENCES OF THE EQUILIBRIUM BETWEEN NATIVE AND DENATURED PROTEIN

By M. L. ANSON and A. E. MIRSKY

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J., and the Hospital of The Rockefeller Institute for Medical Research, New York)

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The denaturation of hemoglobin by acid is partially reversible (Anson and Mirsky, 1931). If acidified hemoglobin is rapidly neutralized all the protein is precipitated. If the acidified hemoglobin is first made slightly alkaline and then after a few seconds brought to the neutral point only a third of the protein is precipitated. The soluble two-thirds has again the properties of native hemoglobin.

Equilibria in Salicylate Solutions.—It has already been shown (Anson and Mirsky, 1929 *b*) that concentrated sodium salicylate in neutral solution denatures hemoglobin and keeps denatured hemoglobin in solution. It will be shown in this paper that denaturation by salicylate is completely reversed when the salicylate is removed by dialysis under suitable conditions or when the salicylate solution is simply diluted with water. Salicylate not concentrated enough to denature hemoglobin completely produces an equilibrium mixture of native and denatured hemoglobin. The higher the salicylate concentration the higher is the percentage denaturation (see Fig. 1). At any given salicylate concentration the percentage denaturation is the same whether one starts with native or with denatured hemoglobin. Decreasing the hemoglobin concentration from 1 per cent to 0.5 per cent or raising the temperature from 25°C. to 35°C. has no detectable effect on the equilibrium in 0.25 M salicylate solution.

Differences between Native and Denatured Hemoglobin.—Hemoglobin denatured by salicylate has three properties characteristic of hemoglobin denatured by other means. It is insoluble under the same

conditions under which native hemoglobin is soluble; it is digested by trypsin which does not attack native hemoglobin; and it has the parahematin type of spectrum which is also given by a solution of hemin in pyridine. When the denaturation of hemoglobin by salicylate is reversed, the original properties of native hemoglobin are restored.

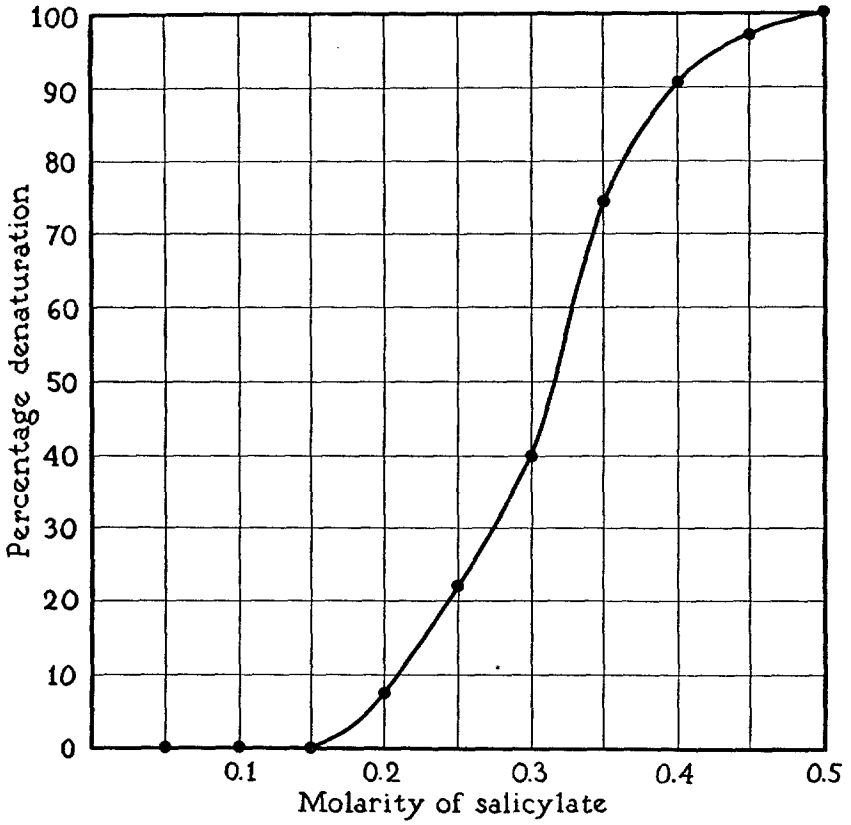

FIG. 1. The effect of salicylate concentration on the denaturation of hemoglobin

One can, if one so wishes, assume that salicylate converts hemoglobin not into denatured hemoglobin but into some other compound which also is insoluble, and digestible and has the parahematin spectrum. Before such an assumption need be considered seriously some difference between the hypothetical other compound and denatured hemoglobin must first be demonstrated.

The Change in Spectrum on Denaturation.—The change in spectrum resulting from the denaturation of hemoglobin is the basis of the method used to estimate the percentage denaturation. Hemoglobin consists of a native protein, globin, joined to an iron porphyrin complex, heme. The spectrum of heme is changed by combination of heme with native globin and the spectrum of the heme-globin compound is changed when the globin is denatured. The denaturation of hemoglobin can, therefore, be followed spectroscopically, heme acting as an indicator of the change in the protein with which it is combined. This was first pointed out for the compounds of reduced (ferrous) heme. The compound of reduced heme and native globin has the spectrum of reduced hemoglobin while the compound of reduced heme with denatured globin has the spectrum of hemochromogen (Anson and Mirsky, 1925; 1928). Analogously the compound of oxidized heme and native globin has the spectrum of methemoglobin while the compound of oxidized heme and denatured globin has the spectrum of parahematin (Keilin, 1926). Parahematin has no distinct absorption in either the yellow or the red, whereas alkaline methemoglobin and hematin have a band in the yellow and acid methemoglobin and hematin have a band in the red. The spectrum of parahematin is thus qualitatively different from the spectra of the other hemoglobin derivatives.

Because of technical difficulties the spectroscopic study of the denaturation of hemoglobin and its reversal has not been satisfactory. The difficulties are these. In neutral solution both globin hemochromogen and globin parahematin are insoluble. In alkaline solution, globin hemochromogen prepared from hemoglobin can combine with extra reduced heme and globin parahematin dissociates to a greater or lesser extent into denatured globin and oxidized heme. It is doubtful whether the pure spectrum of globin parahematin has hitherto been observed. In the neutral salicylate solutions used in the present experiments globin parahematin is neither precipitated nor dissociated and so the difficulties which have been mentioned are avoided.

The Change in Absorption of Green Light on Denaturation.—Viewed in the monochromatic green light easily obtained by the use of color filters from the mercury arc lamp, all pigments appear to be of the same color. Different pigments, however, differ in the extents to

which they absorb the green light. Denatured methemoglobin prepared by the addition of salicylate to a neutral phosphate solution of native methemoglobin absorbs the green light about twice as strongly as does native methemoglobin. Hence one can measure the extent of denaturation of methemoglobin by measuring the extent of absorption of green light with a colorimeter. The greater the percentage denaturation, the greater the absorption of green light.

Aggregation.—The solubility of denatured hemoglobin in dilute neutral salicylate solution is limited. If the protein concentration is too high there results, first, association or aggregation of the protein molecules, and then visible precipitation. Aggregation of the molecules of denatured hemoglobin increases the absorption of green light by an equilibrium mixture of native and denatured hemoglobin in two different ways. First, the aggregated pigment has a greater absorbing power than the unaggregated. Secondly, if as a result of aggregation denatured hemoglobin is removed from the equilibrium mixture

$$\text{unaggregated native hemoglobin} \leftrightarrows \text{unaggregated denatured hemoglobin}$$

then more denatured hemoglobin is formed to maintain the equilibrium and there is an increase in the total amount of denatured protein and hence in the light absorption. This complicating effect of aggregation on the study of an equilibrium has already been discussed in connection with the equilibrium

$$\text{reduced heme} + \text{pyridine} \leftrightarrows \text{pyridine hemochromogen}$$

(Anson and Mirsky, 1929*a*; 1930). The same formation of denatured from native hemoglobin which takes place when the denatured hemoglobin is aggregated also takes place when the denatured hemoglobin is digested.

To avoid aggregation in experiments on the effect of salicylate on the equilibrium between native and denatured hemoglobin, the hemoglobin concentration is kept as low as is consistent with accurate colorimetric measurements. Bovine hemoglobin is used because compared with hemoglobin from other common animals it is relatively soluble and requires a relatively high concentration of salicylate for denaturation. The solutions cannot be made more alkaline to avoid

aggregation because the optical properties of the pigments change and become much more alike than they are in neutral solution.

If salicylate is added to native methemoglobin the absorption of green light at first increases rapidly with time, then remains constant, and finally increases again very slowly. If the same final conditions are obtained by the addition of water to denatured methemoglobin in

TABLE I

Effect of Salicylate Concentration and Time on the Absorption of Green Light by Hemoglobin Solutions

Molarity of salicylate	Time, *min.*							
	0.5	5	10	15	30	40	60	120
0	20				20			
0.05 (*a*)				20.2		20.1		
0.10 (*a*)				20.1		20.1		
0.15 (*a*)		20.0		20.0				
(*b*)		20.0		20.0				
0.20 (*a*)	20.0	19.7	19.2	19.1	18.7	18.7	18.0	17.5
(*b*)	18.0	18.7	18.7	18.7	18.7	18.3	17.4	17.4
0.25 (*a*)	18.0	17.3	16.9	16.8	16.7	16.7	16.0	16.0
(*b*)	15.7	16.7	16.7	16.7	16.7	16.0	16.0	16.0
0.30 (*a*)	17.0	15.1	14.7	14.7	14.6	14.5	14.0	13.8
(*b*)	12.3	14.2	14.7	14.7		14.6	13.9	13.9
0.35 (*a*)	13.5	12.3	11.8	12.0	12.0	12.0	11.8	11.8
(*b*)	11.3	12.0	12.0	12.0	11.9	12.0	11.8	11.8
0.40 (*a*)	13.0	11.2	11.0	11.0				
(*b*)	10.8	11.0		11.0				
0.45 (*a*)	13.0	11.0	10.7	10.7				
0.50 (*a*)		10.5			10.5			

The figures represent colorimeter readings. (*a*) means that the equilibrium was reached by the formation of denatured hemoglobin, (*b*) by the formation of native hemoglobin. (See experimental part of text.)

concentrated salicylate solution then the light absorption first decreases rapidly, then remains constant, and finally increases very slowly. The value of the light absorption which is constant for a while is the same whether the experiment is started with native or with denatured hemoglobin. These results, which are given in Table I, suggest that a true equilibrium is measured before slow aggregation or other change takes place. Aggregation, however, is not definitely

excluded at any stage of the reaction. The state of dispersion of the protein can be decided conclusively only by direct molecular weight determinations.

That there is an equilibrium between a red form and a brown form of hemoglobin is an observed fact whose validity does not depend on the existence or non-existence of aggregation. If aggregation does take place before the equilibrium can be reached and measured then one cannot tell from the total effects of salicylate concentration and temperature on the percentage denaturation to what extent the salicylate concentration and the temperature influence the degree of aggregation and to what extent they influence the equilibrium between unaggregated native and denatured hemoglobins. If, on the other hand, the equilibrium is not being disturbed by aggregation then any theory of denaturation must be in harmony with the facts first, that the curve relating percentage denaturation to salicylate concentration is S-shaped (see Fig. 1) and, secondly, that temperature has little effect on the equilibrium between native and denatured hemoglobin although it has a great effect on the equilibrium between native and denatured trypsin (Anson and Mirsky, 1934). The theory of denaturation we shall now present which is in harmony with the facts which have just been stated is simply a restatement in other words of the existence of an equilibrium between the native form of a protein N and its denatured form, D.

Let us suppose that there is added to the equilibrium mixture $N \rightleftarrows D$, a substance, X, which can combine reversibly with both N and D or modify N and D in any reversible way. There then results the double equilibrium

$$\begin{array}{ccc} N + X & \rightleftarrows & NX \\ \downarrow\uparrow & & \uparrow\downarrow \\ D + X & \rightleftarrows & DX \end{array}$$

The total native protein is $N + NX$, the total denatured protein $D + DX$. There are two ways in which the equilibria can be influenced reversibly. First, the equilibrium constants can be changed by a change in the solvent, in the temperature, or in the rate at which any form of energy is being absorbed. Secondly, the amounts of N and D combined with X can be increased by an increase in the

concentration of X. If X has the same affinity for D as for N, then the fraction of D converted into DX by the addition of X is the same as the fraction of N converted into NX and there is no change in the total percentage denaturation. If X has a greater affinity for D than for N, relatively more D is converted into DX than N into NX and the percentage denaturation is increased. The necessary relation between the denaturation equilibria and the X combination equilibria is given by the identity:

$$\frac{N/D}{NX/DX} \equiv \frac{N/NX}{D/DX}$$

Given the mere fact that the addition of X causes denaturation one cannot decide whether the cause of the denaturation is a change in the equilibrium constants of the individual equilibria or a greater combination of X with D than with N, or whether X acts in both ways. The decision must be made on the basis of measurements of the individual equilibria or on the basis of chemical probabilities. It is likely, for instance, that a change in percentage denaturation brought about by acid is due to a difference in the affinities of acid for the native and denatured forms of the protein.

In practice when X is added to a protein there usually results a whole series of X compounds and hence the complicated equilibrium.

$$\begin{array}{ccccccc} N & \rightleftarrows & NX_1 & \rightleftarrows & NX_2 & \cdots\cdots & NX_n \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ D & \rightleftarrows & DX_1 & \rightleftarrows & DX_2 & \cdots\cdots & DX_n \end{array}$$

The shape of the curve relating the concentration of X to the percentage denaturation depends on the values of all the equilibrium constants. By substituting a suitable set of values into the involved equation representing the complicated equilibrium one can obtain the S-shaped curve which relates the concentration of salicylate to the percentage denaturation of hemoglobin by salicylate. Such curve fitting is of little theoretical significance so long as the numerous equilibrium constants are chosen arbitrarily. What are needed are X combination curves to go with X denaturation curves; for instance, acid titration curves to go with measurements of the effect of pH on denaturation. The presentation of the detailed mathematical formu-

lation of the theory of equilibria had best be postponed until data for testing the equations are available.

The heat of the denaturation $N \rightarrow DX$ caused by the addition of X to N is equal to the heat of the reaction $N \rightarrow NX$ plus the heat of the reaction $NX \rightarrow DX$. Since the reaction $N \rightarrow NX$ may be either endothermic or exothermic depending on the nature of X, the heat of the denaturation $N \rightarrow DX$ will vary with the nature of the denaturing agent.

EXPERIMENTAL

Equilibria.—The stock solutions are a freshly filtered 1 per cent solution of dialyzed bovine methemoglobin prepared according to Anson and Mirsky (1931) in a buffer made up of equal parts 0.1 M K_2PO_4 and 0.1 M KH_2PO_4 and a filtered 1 M solution of sodium salicylate which is stored in the cold. Only such a sample of salicylate should be used which on filtration yields a water-clear solution. Monochromatic green light for the colorimetric measurements is obtained from the mercury vapor lamp by means of the two color filters supplied by the Corning Glass Company for the isolation of the green line. The standard which is set at 20 is made up by adding 9 parts of water to 1 part of the hemoglobin solution. All the experiments described in the experimental part are carried out at 25°C.

To reach the equilibrium corresponding to X tenth molar salicylate solution by formation of denatured hemoglobin, 10 ml. of solution are made by adding X ml. of the 1 M salicylate to a mixture of 1 ml. hemoglobin and $10 - (X + 1)$ ml. water. To reach equilibrium from the other side by the formation of native hemoglobin, 10 ml. of solution are made by adding X ml. of salicylate to a mixture of 1 ml. hemoglobin and $7 - (X + 1)$ ml. water and then after 3 minutes adding 3 ml. more of water. The colorimetric readings at various times are given in Table I.

Since denatured methemoglobin absorbs green light $\frac{20}{10.5}$ or 1.9 times as strongly as native methemoglobin, the relation between the colorimetric reading, R, and the fraction, D, of the protein which is denatured is given by

$$(1 - D) + 1.9D = \frac{20}{R}$$

or

$$D = \frac{20 - R}{0.9R}$$

The greater the percentage denaturation, the less accurate is the estimation of the percentage denaturation by this colorimetric method. Fig. 1 shows the relation between the salicylate concentration and the percentage denaturation.

The Digestion Test.—Hemoglobin in salicylate solution is not digested by trypsin unless the salicylate concentration is high enough to cause some denaturation as shown by the optical test. If a 1 per cent solution of hemoglobin in 0.3 M salicylate, which by the colorimetric test is about 40 per cent denatured and 60 per cent native, is diluted with equal volume of water and placed in boiling water the protein precipitates. If 1.7×10^{-3} hemoglobin units (Anson and Mirsky, 1933) of trypsin are added to each ml. of the solution before it is diluted and heated, then digestion of the denatured hemoglobin takes place, more denatured hemoglobin is formed to maintain the equilibrium and so on until after 20 minutes no precipitate is formed if the solution is diluted and heated. A 1 per cent solution of hemoglobin in 0.1 M salicylate which by the colorimetric test is all native even after 20 minutes treatment with trypsin yields the same sort of precipitate on dilution and heating as does hemoglobin not treated with trypsin. The dilution is made with 0.2 M salicylate instead of with water so that the final salicylate concentrations of the solutions which are heated are 0.15 M in the two cases. Native hemoglobin prepared from denatured hemoglobin behaves like native hemoglobin which was never denatured. To denature the hemoglobin 1 ml. of salicylate is added to 1 ml. of hemoglobin. Brown native hemoglobin is obtained again from the red denatured hemoglobin by the gradual addition of 7.5 ml. water. Finally 0.5 ml. trypsin solution is added. Since trypsin is probably destroyed under the conditions under which hemoglobin is inactivated there is probably more active trypsin in the solution of native hemoglobin which is not digested than in the solution of denatured hemoglobin which is digested.

The Solubility Test.—Denatured methemoglobin is insoluble in 0.3 saturated ammonium sulfate. 1 ml. salicylate is added to 1 ml. hemoglobin and the protein is then precipitated by the addition of a mixture of 12 ml. water and 6 ml. saturated ammonium sulfate. Native hemoglobin is soluble under the same final conditions. No

precipitate results from the addition of 6 ml. saturated ammonium sulfate to a solution prepared by adding 1 ml. hemoglobin to a mixture of 12 ml. water and 1 ml. salicylate. "Reversed" hemoglobin behaves like native hemoglobin. 1 ml. salicylate is added to 1 ml. hemoglobin to bring about denaturation; 12 ml. are then added gradually to bring about the reversal of denaturation. When finally 6 ml. of saturated ammonium sulfate are added only a slight haze is obtained. The clear brown filtrate from this solution stays clear.

SUMMARY

The denaturation of hemoglobin by salicylate in neutral solution is completely reversible.

There is a mobile equilibrium between native and denatured hemoglobin in neutral salicylate solution. The higher the salicylate concentration the greater is the percentage denaturation.

When there is a mobile equilibrium between the native and denatured forms of a protein, denaturation is caused by the addition of any substance which has a greater affinity for the denatured than for the native form.

Theoretically the heat of denaturation must vary with the denaturing agent and must depend on the heat of combination of the denaturing agent with the protein.

REFERENCES

Anson, M. L., and Mirsky, A. E., 1925, *J. Physiol.*, **60,** 50.
Anson, M. L., and Mirsky, A. E., 1928, *J. Gen. Physiol.*, **12,** 273.
Anson, M. L., and Mirsky, A. E., 1929 *a*, *J. Gen. Physiol.*, **12,** 581.
Anson, M. L., and Mirsky, A. E., 1929 *b*, *J. Gen. Physiol.*, **13,** 121.
Anson, M. L., and Mirsky, A. E., 1930, *J. Gen. Physiol.*, **14,** 43.
Anson, M. L., and Mirsky, A. E., 1931, *J. Phys. Chem.*, **35,** 185.
Anson, M. L., and Mirsky, A. E., 1933, *J. Gen. Physiol.*, **17,** 151.
Anson, M. L., and Mirsky, A. E., 1934, *J. Gen. Physiol.*, **17,** 393.
Keilin, D., 1926, *Proc. Roy. Soc. London, Series B*, **100,** 129.