[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

Heats of Organic Reactions. XII. The Reaction between Methemoglobin and Salicylate

BY RICHARD M. ROBERTS

The effect of sodium salicylate upon hemoglobin and methemoglobin has frequently been cited as an example of completely reversible denaturation of a protein.[1,2,3,4] The properties of hemoglobin treated with sodium salicylate are strikingly different from those of the native protein, yet the protein regains its original properties upon removal of the salicylate by dialysis. Anson and Mirsky, in a study of the reaction between bovine methemoglobin and sodium salicylate, took as their measure of the extent of denaturation the fractional increase in absorption of green light upon addition of salicylate. Increase in concentration of salicylate beyond 0.5 molar had no further effect on the absorption; Anson and Mirsky therefore assumed that denaturation was complete at this concentration.

In order to test the validity of this conclusion from colorimetric experiments, and to obtain more information on the nature of the reaction, the heat of reaction of sodium salicylate with methemoglobin and carboxyhemoglobin of the horse was investigated. Since several coördination compounds of iron and salicylate are known,[5] it is necessary to consider the possibility of combination between salicylate and the iron of the prosthetic group of hemoglobin as well as with the protein itself. A study of the saturation effect described by Anson and Mirsky was consequently supplemented by an attempt to determine whether there exists a significant difference in heat of reaction of sodium salicylate with methemoglobin, in which the iron is able to form additional bonds, and with carboxyhemoglobin, in which the bond-forming power of the iron is exhausted.

Experimental Procedure

Crystalline carboxyhemoglobin prepared from horse blood was recrystallized once. The crystalline paste was dissolved in water, thoroughly dialyzed in the cold to remove residual salts, and centrifuged. The supernatant solution was used as the stock solution of carboxyhemoglobin for calorimetric experiments.

To prepare a stock solution of methemoglobin, the crystalline paste of carboxyhemoglobin was suspended in water in a tonometer, and as much carbon monoxide as possible was removed by evacuating, shaking, and admitting air repeatedly. The suspension was then dissolved in phosphate buffer of *p*H 7, a calculated excess of potassium ferricyanide was added, and the solution was allowed to stand overnight in the cold room. The resulting methemoglobin solution was then thoroughly dialyzed, and centrifuged.

Kjeldahl analyses were performed on all stock solutions of protein used. Salts of ordinary C. P. grade were used without further purification to prepare stock solutions for the calorimetric work.

Before proceeding with the thermal measurements, the colorimetric experiments of Anson and Mirsky with sodium salicylate were repeated, using horse methemoglobin instead of bovine methemoglobin. A plot of our results was substantially identical with that of Anson and Mirsky.[1]

The calorimetric technique of the present experiments has been previously described in detail.[6] To eliminate heat evolution due to small changes of *p*H, all solutions (including water used in dilution runs) were made 0.139 molar in Na_2HPO_4–KH_2PO_4 buffer of *p*H 7.3.

In order to obtain the heat of reaction of protein and salt at a given dilution to form a mixture at the same dilution, three measurements were necessary. The three thermochemical equations may be represented as

$$P(\mathrm{A}) + xS(\mathrm{B}) = P,xS(\mathrm{C}) + \Delta H_1 \quad (1)$$

$$xS(\mathrm{B}) = xS(\mathrm{C}) + \Delta H_2 \quad (2)$$

$$P(\mathrm{A}) = P(\mathrm{C}) + \Delta H_3 \quad (3)$$

Subtracting Eqs. 2 and 3 from Eq. 1 one obtains

$$P(\mathrm{C}) + xS(\mathrm{C}) = P,xS(\mathrm{C}) + \Delta H_1 - \Delta H_2 - \Delta H_3 \quad (4)$$

In these equations P and xS are protein and x moles of salt; A, B and C are the respective dilutions. In the experiments reported here, the protein was always placed in the inner can (62 cc.)

(1) Anson and Mirsky, *J. Gen. Physiol.*, **13**, 129 (1929).
(2) Anson and Mirsky, *ibid.*, **17**, 399 (1934).
(3) Roche and Combette, *Compt. rend. soc. biol.*, **126**, 950 (1937).
(4) Holden, *Australian J. Exptl. Biol. Med.*, **15**, 43 (1937).
(5) Weinland and Herz, *Ann.*, **400**, 219 (1913).
(6) Conn, Kistiakowsky and Roberts, THIS JOURNAL, **62**, 1895 (1940).

and the salt solution in the outer can (840 cc.), and brought to thermal equilibrium. The double plug was then raised, allowing the two solutions to mix, and observations of the temperature were made (usually for about thirty minutes) until the reaction was complete. To obtain ΔH_2 and ΔH_3, runs were subsequently made omitting the protein (Eq. 2), and omitting the salt (Eq. 3).

Results and Discussion

The results of a number of measurements with carboxyhemoglobin and methemoglobin and various salts are collected in Table I. Accurate measurements of the heat of reaction of carboxyhemoglobin and sodium salicylate proved impossible. It was found that the temperature rise, although initially rapid, became very slow, and the reaction was incomplete even after an hour. Values of the heat of reaction given in the table for two concentrations are rough estimates. No further experiments were made with carboxyhemoglobin. It is possible that the slow heat evolution observed in the case of carboxyhemoglobin is due to a gradual replacement of carbon monoxide by salicylate. Methemoglobin did not show this behavior.

TABLE I

HEATS OF REACTION

Dilution of HbOH: Protein N, g., 0.294; ΔH_3, cal., 0.14

Conc. of salt after mixing, moles/l.	Protein N reacting, g.	ΔH_1, cal.	ΔH_2, cal.	$-\Delta H = -(\Delta H_1 - \Delta H_2 - \Delta H_3)$, cal.	$-\Delta H$, cal./g. N
HbOH and Sodium Salicylate					
0.100	0.294	−0.46			
	.000		0.27	0.87	2.96
.184	0.294	−0.05			
	.000		1.00	1.19	4.05
.300	.294	2.23			
	.000		2.46	0.37	1.26
.425	.294	3.05			
	.000		4.37	1.46	4.97
.497	.294	3.46			
	.000		5.82	2.50	8.50
.596	.294	4.51			
	.000		7.96	3.59	12.21
.695	.294	6.42			
	.000		10.37	4.09	13.92
.794	.294	8.33			
	.000		12.79	4.60	15.65
HbCO and Sodium Salicylate					
.497	.178	4.28			
	.000		5.82	1.68	9.44
.596	.178	5.69			
	.000		7.96	2.41	13.54
HbOH and Sodium Benzoate					
.500	.204	−3.78			
	.000		−3.64	0.28	1.37
.800	.204	−9.24			
	.000		−9.24	0.14	0.69
HbOH and Potassium Chloride					
.500	.204	2.00			
	.000		2.50	0.64	3.14
.800	.204	5.14			
	.000		5.96	0.96	4.71
HbOH and Sodium Chloride					
.820	.201	5.01			
	.000		6.01	1.14	5.67

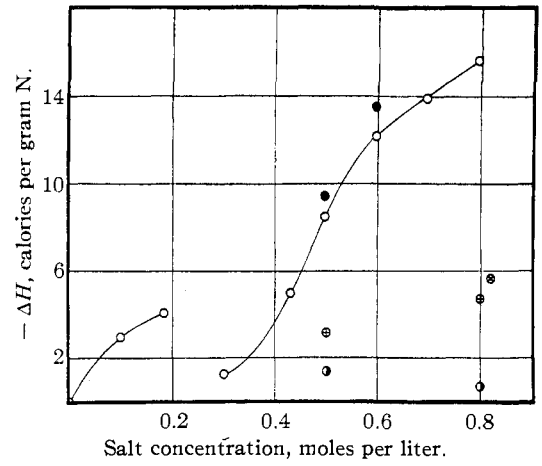


Fig. 1.—○, Methemoglobin + sodium salicylate; ◑, methemoglobin + sodium benzoate; ⊕, methemoglobin + potassium chloride; ⊗, methemoglobin + sodium chloride; ●, carboxyhemoglobin + sodium salicylate.

In Fig. 1 the heat of reaction $-\Delta H = -(\Delta H_1 - \Delta H_2 - \Delta H_3)$, corresponding to Eq. 4 has been plotted in calories per gram of nitrogen against the salt concentration in the final mixture. The two full circles are for carboxyhemoglobin; all other points are for methemoglobin.

The two heats estimated for carboxyhemoglobin and sodium salicylate lie very close to those for methemoglobin. The state of the iron thus seems to have very little influence on the heat of reaction of the protein with salicylate, indicating that most of the reaction occurs elsewhere on the protein molecule.

Measurements were made with sodium benzoate, sodium chloride and potassium chloride, and in no case was a heat of reaction comparable to that of salicylate observed. Sodium benzoate, which is most nearly like sodium salicylate, showed a negligible heat of reaction with methemoglobin, nearly independent of concentration. The heats of reaction of potassium chloride and sodium

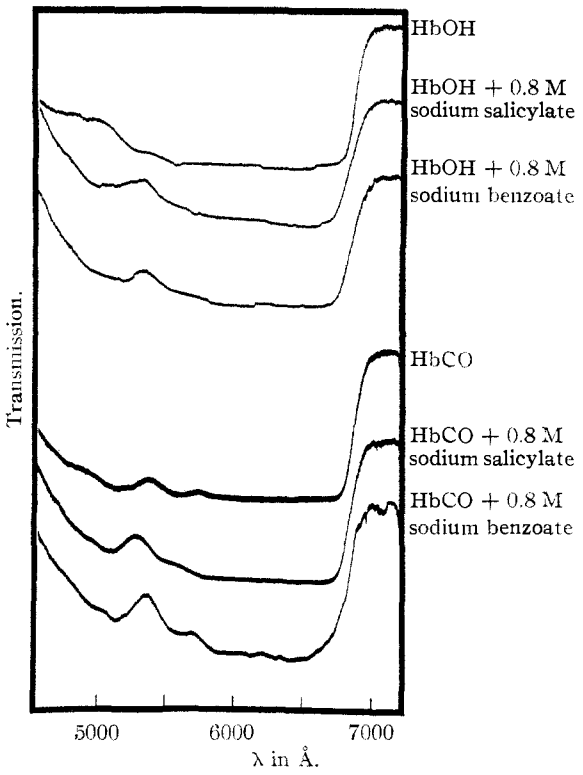


Fig. 2.

chloride were much greater than that of sodium benzoate (see Table I and Fig. 1). The highest point, for sodium chloride at 0.8 molar, is only about one-third of the value for salicylate. The reaction between salicylate and methemoglobin therefore appears to be specific.

A plot of the heat of reaction against salicylate concentration in the case of methemoglobin increases until a region is reached, between about 0.20 and 0.28 *M*, in which the protein is insoluble.[7] At higher concentrations the products of reaction are again soluble, but the ΔH curve shows a sharp discontinuity over this region. The curve then rises steadily as far as 0.8 *M*, the limit of the present experiments. It might be possible to explain the discontinuity by assuming, as did Anson and Mirsky,[1] that the reaction occurs in several stages, each stage having a different heat of reaction; however, the failure of the heat of reaction to become independent of salicylate concentration, even when the molar excess of salicylate is 3000-fold, suggests that the reaction is not stoichiometric in character. Furthermore, the ease with which the salicylate is removed by dialysis, with restoration of the original properties of the protein, argues for a weak interaction rather than a profound disruption of the protein molecule. The reaction may in part consist of the formation of hydrogen bonds between salicylate and some amino acid residues of the protein.

The nature of the visible absorption spectrum of hemoglobin depends chiefly upon the state of oxidation of the iron, and upon the formation of coördination complexes with the iron. Microphotometer tracings of the absorption spectra of methemoglobin and carboxyhemoglobin alone and in the presence of salicylate and benzoate are shown in Fig. 2. It was found that although the spectra of methemoglobin and carboxyhemoglobin are very different, the spectrum of methemoglobin in salicylate is strikingly similar to that of carboxyhemoglobin alone, very probably indicating the formation of a coördination complex betweeen salicylate and the iron of methemoglobin. It was found that salicylate had very little effect on the spectrum of carboxyhemoglobin, since the iron is here already fully saturated. Despite this fact, the heat of reaction of carboxyhemoglobin and salicylate was about the same as that of methemoglobin, in which a marked change in spectrum occurs. On the other hand, sodium benzoate alters the spectrum of methemoglobin in much the same way as salicylate at similar concentration, yet the heat of reaction of sodium benzoate with the protein is nearly zero. These observations, together with the fact that the ΔH curve shows no indication of saturation of methemoglobin with salicylate in the concentration range investigated, point to the conclusion that the colorimetric method of estimating the extent of reaction of salicylate with the methemoglobin molecule as a whole is unreliable.

The author wishes to thank Professor G. B. Kistiakowsky for many helpful suggestions, and the Rockefeller Foundation for its support of the project of which this work is a part.

Summary

The heats of reaction of methemoglobin and carboxyhemoglobin with sodium salicylate at *p*H 7.3 were measured. It was found that the state of oxidation of the hemoglobin iron was without effect on the heat of reaction at a given salicylate concentration.

The heat of reaction of methemoglobin and sodium salicylate increased with salicylate con-

(7) At the same protein concentration at which methemoglobin is precipitated by 0.20–0.28 *M* sodium salicylate, carboxyhemoglobin remains soluble, indicating that interaction between salicylate and the prosthetic group markedly lowers the solubility of the protein.

centration up to 0.8 *M*, the limit of the present experiments. It is therefore improbable that the reaction is stoichiometric. It is suggested that hydrogen bond formation between salicylate and side chains of the protein molecule may account for a part of the heat of reaction.

The heats of reaction are compared with changes in the absorption spectra of carboxyhemoglobin and methemoglobin in salicylate and benzoate. It was found that the two effects are unrelated. Colorimetric estimation of the extent of reaction of salicylate with methemoglobin is therefore unreliable.

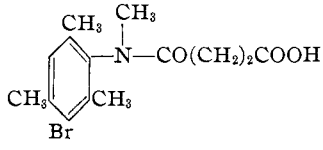
CAMBRIDGE, MASS. RECEIVED MARCH 10, 1942

[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

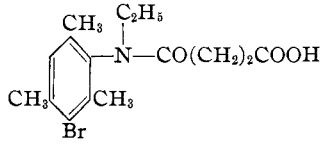
Restricted Rotation in Aryl Amines. III. Preparation and Resolution of N-Succinyl-1-methylamino-2-methylnaphthalene and N-Succinyl-1-methylamino-4-chloro-2-methylnaphthalene[1]

BY ROGER ADAMS AND A. A. ALBERT[2]

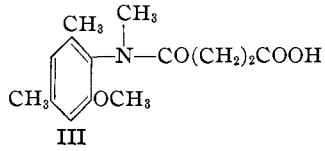
Restricted rotation in simple aromatic amines has been demonstrated by preparation and resolution of three compounds shown in I, II, and III.[1a,1b]



I

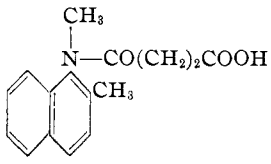


II

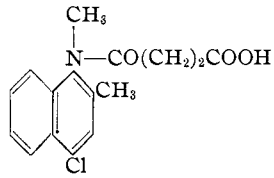


III

Two analogs in the naphthalene series have now been prepared and are shown in IV and V.



IV



V

The half-life periods of these five compounds determined in boiling *n*-butanol are shown in the table.

HALF-LIFE PERIODS OF RESTRICTED ARYL AMINES IN BOILING *n*-BUTANOL

Compound	Half-life in hours
I	9.0
II	28.0
III[a]	2.7
IV	5.7
V	4.1

[a] Taken in boiling methyl acetate, b. p. 56°.

A comparison of the values of IV and V with I (compare also with II) indicates that the interference of a —CH= group of the aromatic nucleus is less than that of a CH_3— group. This agrees with the observations in parallel compounds with restricted rotation in the substituted aryl olefin series.[3] It is also of interest to note that the 4-chloro substituent in compound V reduces the half-life of the product to 20% less than that of the corresponding unchlorinated derivative (IV).

Compound IV was synthesized by the general procedure used in the benzene series. 1-Amino-2-methylnaphthalene was N-methylated and then succinylated. Compound V was formed from 1-nitro-2-methylnaphthalene by reduction with stannous chloride in ethanolic hydrochloric acid to 1-amino-2-methyl-4-chloronaphthalene followed by N-methylation and succinylation.

The 1-ethylamino-2-methylnaphthalene was also synthesized and succinylated. The product, however, did not form salts suitable in properties for resolution.

Experimental

1-Nitro-2-methylnaphthalene.—This was prepared by the nitration of 2-methylnaphthalene in glacial acetic

(1) For previous papers see (a) Adams and Stewart, THIS JOURNAL, **63**, 2859 (1941); (b) Adams and Dankert, *ibid.*, **62**, 2191 (1940).

(2) An abstract of a thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Chemistry, Eastman Kodak Fellow, 1941–42.

(3) Adams and Miller, THIS JOURNAL, **62**, 53 (1940); Adams, Anderson and Miller, *ibid.*, **63**, 1589 (1941); Adams and Binder, *ibid.*, **63**, 2773 (1941).