$$\Delta w = \frac{1}{2}\sum_{j=1}^{s}\sum_{k=1}^{s}\frac{e_j e_k}{R_{jk}} + \frac{1}{2}\left(\frac{1}{D}-\frac{1}{D_i}\right)\sum_{n=0}^{\infty}\frac{(n+1)\Delta G_n}{[(n+1)+nD_i/D]b^{n2+1}} \quad (3)$$

$$\Delta G_n = \sum_{j=1}^{s}\sum_{k=1}^{s} e_j e_k r_j^n r_k^n P_n(\cos\vartheta_{jk})$$

The charge e_k is at a distance r_k from the center of the sphere, at a distance R_{jk} from the charge e_j, and the lines between e_k and the center, and e_j and the center meet at an angle of ϑ_{jk}. The P_n are the usual Legendre functions. b is the radius of the sphere, and $(r/b)^2 = x$.

In order to determine the effect of dipole–dipole interaction, it is necessary to evaluate ΔG_n. For the case in which dipoles are equidistant from the center on a diameter, ΔG_n becomes

$$\Delta G_n = 2a^{2n-2}(-1)^n M_1 M_2\left\{n^2\cos\zeta_1\cos\zeta_2 - \frac{n(n+1)}{2}\sin\zeta_1\sin\zeta_2\cos\varphi\right\} \quad (4)$$

Here ζ_1 is the angle between the first dipole and the diameter, ζ_2 the angle between the second dipole and the diameter, and φ the angle between the planes defined by the two dipoles and the diameter. Substituting in equation (3), expanding in a power series in D_i/D and retaining only the first term, equation (5) is obtained

$$\Delta w = \frac{M_1 M_2}{R^3}\left\{\cos\zeta_1\cos\zeta_2\left[\frac{2}{D_i}+4\sqrt{x}\left(\frac{1}{D}-\frac{1}{D_i}\right)S_1\right] - \sin\zeta_1\sin\zeta_2\left[\frac{1}{D_i}+4\sqrt{x}\left(\frac{1}{D}-\frac{1}{D_i}\right)\right]S_2\right\}$$

$$S_1 = \sum_{n=0}^{\infty}\left[n^2x^n(-1)^n - \frac{n_3}{n+1}\frac{D_i}{D}x^n(-1)^n\right] \quad (5)$$

$$S_2 = \sum_{n=0}^{\infty}\left[n(n+1)(-1)^n x^n - \frac{D_i}{D}n^2(-1)^n x^n\right]$$

The expression obtained after evaluating the indicated sums is rather complex. However, for the case in which the two dipoles are co-linear, actual numerical computations show that the expression may be replaced, as a first approximation, by equation (2).

The author wishes to thank Professor J. G. Kirkwood for his generous assistance in connection with the derivation given above.

Summary

The electrostatic equations derived by Kirkwood and Westheimer have been applied successfully to calculation of the effect of para substituents on the rate of saponification of the esters of phenylacetic, hydrocinnamic, benzoic and cinnamic esters, to the rate of alkaline hydrolysis of benzamides and the rate of reaction of benzyl chlorides with sodium iodide.

An approximate equation has been derived which applies to dipole–dipole interaction, and has been shown satisfactorily to account for the effect of substituents on the rate of alkylation of trimethylamine by substituted methyl benzoates.

CHICAGO, ILLINOIS RECEIVED MAY 16, 1940

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

Heats of Organic Reactions. IX. A New Calorimeter and the Denaturation of Methemoglobin by Alkali

BY JOHN B. CONN, G. B. KISTIAKOWSKY AND RICHARD M. ROBERTS

So many organic reactions cannot be studied calorimetrically in the vapor phase with convenience that we were compelled some time ago to construct a new calorimeter designed specifically to study liquid phase reactions. We have attempted to build a rather flexible unit suitable for work under greatly varying conditions, and thus, of course, had to sacrifice certain valuable characteristics. The calorimeter herein described is neither as sensitive as some nor as accurate as others, but if one is justified in evaluating a "utility coefficient," made up of sensitivity, accuracy, speed of operation, and a few other factors, the comparison will not be very unfavorable to the present instrument.

With this calorimeter we have measured the heats of hydrolysis of several acid anhydrides, the results of which work will be published shortly, and have now studied the irreversible denaturation of methemoglobin in solutions of high pH at 25°. We have chosen methemoglobin as the first object of study because of the ease of its preparation in the pure crystalline state, and because this protein is known to be soluble when denatured, except

in the neighborhood of the isoelectric point. The advantage of methemoglobin over hemoglobin is that evolution of gas on denaturation is avoided, and one is assured of the state of the protein. Since the oxidation involves only the valence of iron, the results presented here in all probability apply to hemoglobin as well. Alkaline denaturation was chosen for the first study because experiments revealed more favorable solubility–*p*H relationship and because the reaction is apparently not complicated by splitting of the hematin and by a partial reversal,[1] as is the acid denaturation.

The Calorimeter.—The calorimeter is of the twin-adiabatic-submarine design, but is equally suitable for use as a single unit. In the present work it has been used only in the latter capacity, and a description of the details of twin-adiabatic operation will be postponed until practical use has been made of it.

The two identical calorimeters, one of which is schematically shown in Fig. 1, are cylindrical gold cans (A) of 900 cc. volume, with tightly fitting thin covers (B), deproteinized rubber gaskets being used in the joint. The

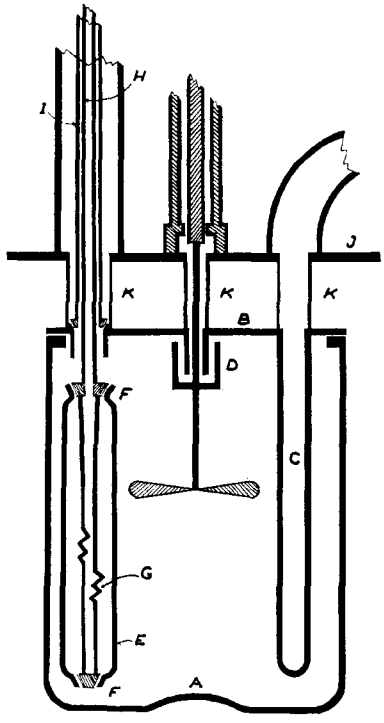

Fig. 1.—The calorimeter.

covers have five holes, one in the center, and four on the circumference of a circle. To three of these are welded closed tubes containing, respectively, an electric heater, a 20-junction thermel projecting into the outer thermostat bath, and a 50-junction "main" thermel (C) reaching into the other calorimeter. The center opening, provided with a short open tube, carries a propeller-type stirrer with a mineral oil seal on the shaft (D); a gold-foil cylinder (not shown in the drawing) supported by the thermel tubes and not quite reaching to the top and bottom of the can, directs the liquid flow. Submerged in the liquid of the calorimeter and attached opposite the fifth opening in the calorimeter lid is the mixing device—a small (62 cc.) can (E) which can be washed, filled, and emptied into the calorimeter without taking the latter apart. The mixing can has conical holes (F) in its top and bottom. Into the bottom opening is ground a solid cone connected by spring supports (G) to the slightly larger cone fitting into the top opening of the can. The springs are adjusted so that when the upper cone is pressed tightly in position the springs also hold the bottom cone tightly against its seat. The use of a minute amount of vaseline–ceresin grease gives a completely tight seal, and no difficulties were ever experienced from leakage. The upper cone has a small central opening into which is welded a long stainless steel tube (H) (0.087″ I. D., 0.011″ wall) reaching outside the thermostat bath. By inserting into it a stainless steel tube of still smaller diameter, the can may be filled or washed out. On lifting the double-plug by means of the attached tube the contents of the mixing can are emptied into the calorimeter, the liquid flow in the latter being so directed that this operation takes but a minute or two. Tightly surrounding the tube attached to the cones is another thin-walled stainless steel tube (I) (0.110″ I. D., 0.005″ wall) with a ground cone of larger diameter attached to its lower end. This cone fits into the conically ground opening of the calorimeter lid and provides a tight seal against evaporation of liquid from the calorimeter. Both steel tubes are pressed down by springs mounted outside the thermostat bath and can be independently lifted, so that the calorimeter vessel can be filled without disturbing the contents of the mixing can. The chief advantage of this entire mixing device—arrived at after many other designs were tried—is that there is no need to dismount the calorimeter after each run, and experiments can follow each other in rapid succession. Three runs a day frequently have been made.

Each calorimeter is suspended inside a gold-plated copper can (J) which is wholly submerged in the thermostat-bath, the clearance between the cans being about 2.5 cm. Suspension is provided by five tubes (K) made by oxy-hydrogen welding from a 90% Pt–10% Rh foil 0.001″ thick; the tubes are soldered to the calorimeter cover opposite its five openings, and to the lid of the outer can, in which there are suitable openings. These tubes provide an all-metal and completely air-tight construction for the thermels and the heater; they ensure also that no vapors from the calorimeter reach the space between it and the insulating can. As an inspection of the heat conductivity data will show, the total heat conductance along the metal of these tubes is wholly negligible compared with the heat conductance of the air surrounding the calorimeter, or of the thermel wires. The thermels are constructed of carefully tested constantan and copper wires, B. and S. gages 34 and 40, respectively, except for the long middle section of the main thermel connecting the two calorimeters. Heavier wires are used here, the soldered joints

(1) Anson and Mirsky, *J. Phys. Chem.*, **35**, 185 (1931).

being located inside the thermostat bath. This rather complicated construction ensures small heat losses from the calorimeter but approximately doubles the sensitivity of the temperature measurements as compared with a thermel made wholly of thin wires. In the details of the thermel construction we followed the suggestions of White,[2] except that the soldered junctions were insulated by wrapping small strips of rice cigarette paper around them, followed by treatment with cellulose acetate solution; this gives a much neater and better heat-conducting construction than the silk-thread insulation suggested by him. Each junction was brought to the outside of the wire bundle and individually touched the wall of the containing tube, the end of the thermel resembling thus a miniature uncut bunch of bananas. The lag of the thermels is less than fifteen seconds, which is a sufficiently short interval, since the rate of heat exchange between the calorimeter and the surroundings amounts to approximately 1/600 of the thermal head per minute. The Newtonian law of cooling is obeyed at least up to two degrees of temperature difference; this is assisted by a thin (0.0005") aluminum shield surrounding the calorimeter on all sides midway between it and the outer can.

The stirrers (driven by a synchronous motor) are run on ball-bearings mounted on the lid of the outer can in effective thermal contact with the thermostat bath, and the shafts to the inside of the calorimeters are made of thin-walled stainless steel tubing. Neither of the short (20-junction) thermels nor the 50-junction "main" thermel projects outside the thermostat-bath.

The thermostat is provided with a very rapidly acting decalin-filled thermoregulator made of fifty feet of thin-walled Pyrex tubing of 8 mm. diameter. A metal needle valve connects a reservoir for excess liquid to the glass tubing of the regulator by a glass–Kovar seal, and makes changes of the setting easily possible. Together with a Thyratron-circuit and a "mechanical brain"-controlled low voltage heater described before,[3] this system keeps constant temperature (or follows temperature changes of the calorimeter) with an accuracy better than 0.001°. The thermostat is placed in a large air-bath roughly controlled at one or two degrees lower temperature, so that calorimetric runs can be made at temperatures well below or above room temperature.

The electrical equipment used in measuring temperature changes and in electrically calibrating the calorimeter already has been described.[3] The sensitivity is such that 0.00005° (0.1 microvolt) temperature difference between the twin calorimeters corresponds to 1 mm. displacement on the galvanometer scale; complete stability of the circuit suggests that a somewhat more sensitive galvanometer could be profitably used, but this was not deemed necessary.

In the runs here described one of the twin calorimeters was allowed to reach a stationary state in which the heat of stirring compensated for the heat losses to the outside, and was used as the constant reference temperature. Since the calorimeters are mounted entirely independently in the thermostat, temperature changes in one do not affect the other. The excess temperature of the comparison calorimeter over that of the thermostat is about 0.035°. The heat of stirring in the calorimeters filled with water amounts to about 0.05 cal. per minute.

Many preliminary experiments, the details of which we shall omit, have led to the following conclusions: (1) with the same liquid in the calorimeter, electrical calibrations of the energy equivalent (in joules per microvolt) check to somewhat better than 0.1%; (2) within this limit of error, the energy equivalent does not depend on the rate of electrical energy input when the latter is varied over nearly hundred-fold; (3) the rate of heat loss is constant, but the heat of stirring is rather sensitive to the level of the liquid in the calorimeter, and cannot be exactly reproduced from one filling to another; (4) keeping the mixing can closed or open does not alter the heat of stirring measurably; (5) raising the double-plug in the mixing can is equivalent to not more than 0.05 calorie heat change in a water-filled calorimeter; (6) thermal equilibrium between the main body of the calorimeter and the closed mixing can is established in less than fifteen minutes.

Preparation of Methemoglobin.—Following standard procedure, horse plasma residues were diluted with three volumes of a solution containing 0.9% sodium chloride and 1.0% sodium citrate, and run at 37° through a Sharples super-centrifuge. The hemoglobin concentrate thus obtained was transferred to the cold room, shaken with ether, and then brought to *p*H 6.6 by careful and slow addition of 1 *N* hydrochloric acid with vigorous stirring. The thick suspension of oxyhemoglobin crystals which separated on standing was centrifuged, the centrifugate was washed twice with an equal volume of iced distilled water, and the oxyhemoglobin bottled as paste. These and the following operations were carried out with as much speed as possible, and the materials were never taken from the cold room until they were to be used in the calorimeter. It has been our experience that hemoglobin preparations which are more than three weeks old develop abnormal sensitivity to denaturation, although otherwise appearing perfectly good.

The conversion to methemoglobin was a modification of the procedure described by Levy.[4] Oxyhemoglobin paste was put into solution by dropwise addition of cold 1 *N* potassium hydroxide with violent stirring. The solution was centrifuged to remove stroma, and assayed by the oxygen capacity method, using the Van Slyke apparatus.[5] A calculated excess of 0.69 *M* potassium ferricyanide solution was added with stirring, and the product brought back to the isoelectric point by dropwise addition of 1 *N* hydrochloric acid.

The dark methemoglobin solution was filtered through cloth to remove a small amount of scummy insoluble matter and then brought to crystallization by mixing with an equal volume of saturated ammonium sulfate which had been adjusted to *p*H 6.6. (We have found that spontaneous crystallization of methemoglobin from the untreated solution is very sluggish and incomplete.) After three hours of stirring, the crystals were centrifuged down and washed three times with an equal volume of ice-cold 0.2 *N* potassium chloride solution (not all of the am-

(2) Walter P. White, "The Modern Calorimeter," Chemical Catalog Co., New York.

(3) Article I of this series, THIS JOURNAL, **57**, 65 (1935).

(4) Levy, *J. Biol. Chem.*, **89**, 173 (1930).

(5) Sendroy, *ibid.*, **91**, 307 (1931).

monium sulfate was removed by this procedure) and dissolved in the appropriate volume of 0.2 *N* potassium chloride for the stock solution. Before use, the stock solutions were centrifuged, a small amount of insoluble material being thus removed. In the preparation of the last (no. 5) stock solution the procedure was modified in that the methemoglobin solution was brought to *p*H 7.2 (to avoid partial crystallization) and was dialyzed under stirring until tests for potassium ferricyanide were negative. No ammonium salts were present in this preparation.

Experimental Details.—The general procedure in making a run was the following. The double-plug was fixed in position in the mixing can and the tightness of the joints was tested with a manometer. The two solutions were placed in the calorimeter; 62 cc. of one solution in the mixing can, and about 835 cc. of the other in the main body of the calorimeter. The calorimeter was then brought by electric heating to a temperature close to its stationary state, and after waiting a suitable time for thermal homogeneity to be reached the double-plug was raised, thus opening the mixing can. It was found that in general protein solutions increased in viscosity on addition of alkali and thus the heat of stirring was changed by as much as 20 to 30%. This made it necessary to adopt a special procedure for the calculation of the true temperature rise in the reaction. The calorimeter temperature was followed until the rate of change became constant, which was taken to mean that the chemical reaction was over and that only heat of stirring and heat losses to the outside remained. In the final experiments this was usually the case twenty to forty minutes after mixing. The calculation of the temperature rise due to reaction was made assuming that the viscosity and therefore the heat of stirring remained unchanged during the entire time after mixing the solutions. Since this whole correction of actual temperature readings amounted to only a very few per cent. of the total temperature change, the approximations introduced in such a calculation cannot significantly affect the results. Temperature rises were converted to heat content changes by using the same heat capacity for all runs; this was determined once for all by heating the calorimeter electrically after a run was completed and the rate of temperature change had become constant.

In all, five different stock solutions of methemoglobin have been used. The first two were for qualitative experiments, and no particular precautions were taken to duplicate conditions in different runs. In the other series of experiments we have attempted to add exactly the same quantity of alkali to the same quantity of methemoglobin by the time the final state in each calorimetric experiment was reached. However, the quantity of alkali added to the protein solution from the mixing can in the calorimeter varied from one experiment to another. Accordingly, different amounts of alkali were added to the methemoglobin solution before it was placed in the calorimeter. All solutions used in these experiments were carefully made 0.2 *N* in potassium chloride, hence the heats of mixing were very low. Thus, 0.023 mole of potassium hydroxide in 62 cc. of solution 0.2 *N* in potassium chloride gave 1.63 cal. heat evolution when mixed with 835 cc. of a 0.2 *N* potassium chloride solution. Mixing 62 cc. of a 0.2 *N* potassium chloride solution with 850 g. of the same methemoglobin solution at *p*H 12 as used in experiments 1 to 9 of Table I gave less than 0.1 cal. heat absorption. In the last series of experiments a constant quantity of methemoglobin stock solution was weighed out, to which was added a predetermined quantity of potassium hydroxide solution of known normality. When the resulting solution was to be one containing native protein, the alkali was first diluted with a large amount of 0.2 *N* potassium chloride solution (so as to be about 0.05 *N* in potassium hydroxide), and was added extremely slowly (during about thirty minutes) to a vigorously stirred (but not foaming) methemoglobin solution. The solution was then made up to 900 g. with a 0.2 *N* potassium chloride solution and 850 g. of it was used to fill the main body of the calorimeter. A predetermined amount of the same potassium hydroxide solution was measured out; to it was added enough 0.5 *N* potassium chloride solution to make it 0.2 *N* in potassium chloride. This solution was poured into the mixing can, and was washed down with enough 0.2 *N* potassium chloride solution to make 62 cc. of the mixture in each experiment.

In experiments on denatured methemoglobin a fixed amount of alkali solution was added more rapidly to the protein, bringing it to a *p*H of about 12, where denaturation was rapid and complete. After allowing this solution to stand for a short time, a predetermined quantity of 0.2 *N* hydrochloric acid was added very slowly while stirring the solution vigorously, and the whole was made up to 900 g. with 0.2 *N* potassium chloride solution; 850 g. was again used in the calorimeter. Into the mixing can was placed sufficient potassium hydroxide (in 0.2 *N* potassium chloride solution) so that after reaction the protein solution contained the same excess of alkali over hydrochloric acid as the total quantity of alkali added in experiments with native protein.

Preliminary experiments established that methemoglobin solutions brought to *p*H 11.9 were wholly denatured, as evidenced by an almost colorless supernatant liquid over the precipitate formed on bringing the solution to the isoelectric point of methemoglobin (*p*H 6.8). Only at *p*H lower than 10.9, after standing for some fifteen minutes, was considerable color left in the solution after bringing it to the isoelectric point. In the main series of experiments all solutions were brought to approximately *p*H 11.9 on opening the mixing can.

The *p*H at which denaturation begins, as evidenced by partial precipitation at the isoelectric point, could not be determined exactly because it depends on the manner in which alkali is added to the protein solution. However, observing much care in this addition, we were able to obtain solutions at *p*H 9.3 which gave no precipitate in the centrifuge when returned to the isoelectric point. On adding acid to denatured protein at a high *p*H, precipitation occurs well before the isoelectric point is reached. Again, the exact *p*H of coagulation depends on the mode of addition of the acid. We have been able to obtain solutions at *p*H 9.9 which gave no precipitate on centrifuging. These observations limited the ranges in which the quantity of alkali contained in the mixing can could be varied.

The 50 g. of methemoglobin solution remaining after removing 850 g. for use in the calorimeter was used for *p*H and precipitation tests. In experiments with native

protein this solution was titrated with dilute acid to the isoelectric point at the same time that the mixing can was opened in the calorimeter, and the precipitate, if any, was collected by centrifuging. This precipitate was dissolved in alkali, the supernatant liquid was then brought to the same *p*H as the dissolved material (high enough to cause complete denaturation) and the relative concentrations were determined colorimetrically. This procedure is subject to some criticism but no native solutions in which denatured protein was detected by the precipitation test were used for quantitative calculation. The sole requirement for denatured proteins was the absence of a precipitate at the *p*H at which they went into the calorimeter.

The *p*H measurements were made on a Hellige-type glass electrode meter, and the values of *p*H given in this article refer to the Hellige scale. The meter was adjusted against a buffer at frequent intervals; the absence of sodium salts from all solutions used probably made deviations from true *p*H small even in the most alkaline range, but no greater accuracy than 0.1 *p*H unit on the Hellige scale can be claimed.

Results

Before using a new stock solution of methemoglobin in the calorimeter, its titration curve was determined, and the data thus obtained were used in computing the necessary amounts of alkali. As our protein stock solutions nos. 3 and 4 contained some ammonium sulfate, the titration curves are devoid of absolute meaning; they are similar to those given by Wyman.[6] A comparison of the titration curves of stock no. 3 with stock no. 5 (free from ammonium salts) suggests about 0.01 to 0.02 *N* concentration of ammonium salts in the former.

Before discussing the final measurements we wish to consider various preliminary runs which are represented graphically in Fig. 2. The heat changes for different runs are plotted there on shifted scales; to convert them to temperature changes it may be noted that one microvolt on the main thermel, our unit of temperature change (approximately 0.0005°C.),i s equivalent to 0.466 calorie. In all runs in Fig. 2 corrections have been applied for the heat of stirring and for the heat losses to the outside.

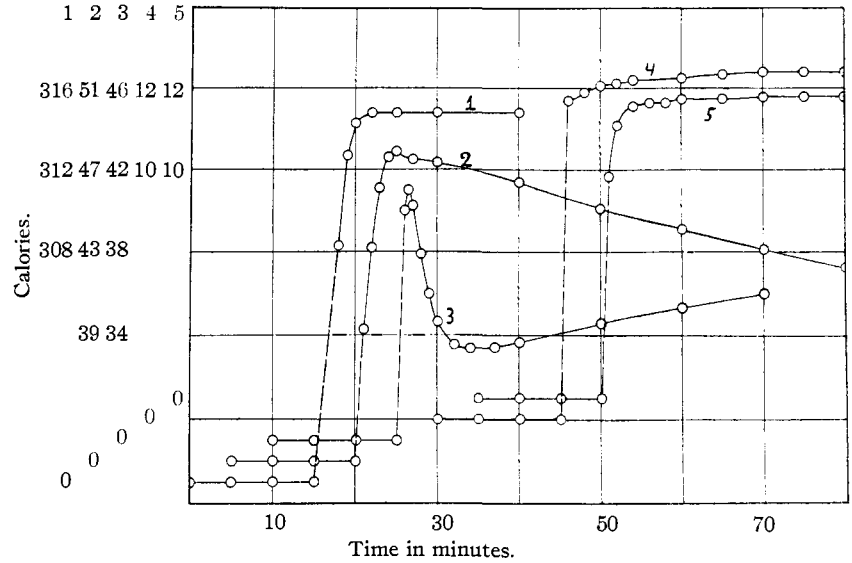

Fig. 2.—Thermal changes in methemoglobin solutions at different *p*H.

Curve 1 shows the heat of partial neutralization by hydrochloric acid of approximately the same amount of potassium hydroxide as was used in the main series of experiments, the final *p*H being 10.7. It will be noted that the heat evolution is more than 99% complete only four minutes after raising the double-plug, although the alkali used was in excess and thus the acid displacing the alkali in the mixing can had in turn to be washed out by alkali. This demonstrates that the mixing is extremely rapid and does not introduce important lags in the observed temperature of the calorimeter.

Curve 2 shows that when native methemoglobin at its isoelectric point is mixed in the calorimeter with enough alkali to bring its *p*H to 10.1, the initial rapid heat evolution is followed by a slow absorption of heat, not wholly completed even one hour after the instant of mixing.

Curve 3 gives the results of a similar experiment with a greater amount of alkali, the final *p*H in this case being 11.2. Here the initial heat evolution is followed by a considerable heat absorption, which, in turn, is succeeded by a still slower heat evolution. The latter eventually subsides also but its exact course is difficult to follow on account of the uncertainties in the stirring heat correction.

Curve 4 shows that at a still higher *p*H, 11.8, (the starting solution in this case was already at *p*H 9.25 and therefore the total heat evolution on mixing was small) the heat absorbing reaction has become so fast that it is masked by the initial heat

(6) Wyman, *J. Biol. Chem.*, **127**, 1 (1939).

TABLE I

HEAT CHANGES ON MIXING METHEMOGLOBIN AND POTASSIUM HYDROXIDE SOLUTIONS AT 25°

No.	Protein nitrogen in the calorimeter, g.	Millimoles added in advance: KOH	Millimoles added in advance: HCl	KOH in mixing can of the calorimeter	pH of solution: Before mixing	pH of solution: After mixing	Heat evolution ($-\Delta H_{298}$) on mixing, in gram calories[a] (total charge)	Comments
				Stock No. 3				
1	0.978	8.11	0	28.32	8.85	11.80	17.75	No denaturation in the original (8) soln.
2	.978	9.90	0	26.57	9.05	11.85	15.15	No denaturation
3	.978	11.65	0	24.79	9.25	11.80	12.40	No denaturation
4	.978	13.38	0	23.05	9.60	11.90	10.72	6.5% denaturation at the time of mixing
5	.978	13.38	0	23.05	9.35	11.90	13.65	More denaturation than in run No. 4; not determined
6	.978	36.45	16.50	16.50	9.95	11.85	11.80	Solution clear before mixing; denatured protein
7	.978	36.45	14.94	14.95	10.10	11.80	9.46	Solution clear; denatured protein
8	.978	36.45	13.36	13.36	10.30	11.85	7.08	Solution clear; denatured protein
9	.978	36.45	11.79	11.78	10.65	11.85	5.31	Solution clear; denatured protein
				Stock No. 4				
10	4.34	26.8	0	101.3	8.75	12.2	83.8	No denaturation before mixing
11	4.34	34.2	0	93.8	8.95	11.9	71.8	No denaturation before mixing
12	4.34	41.0	0	87.0	9.25	12.2	63.5	3% denaturation before mixing
13	4.34	128.1	49.6	49.6	10.0	11.9	37.6	Denatured protein; solution clear
14	4.34	128.1	44.2	44.2	10.3	12.1	29.7	Denatured protein; solution clear
15	4.34	128.1 + 10.5	55.0	44.5	10.3	12.2	30.0	On adding HCl precipitate formed; redissolved on adding additional alkali
16	1.20	18.05	0	17.52	10.05	11.90	5.92	2.7% denaturation; part of the same solution as in the next experiment, but fresh
17	1.20	18.05	0	17.52	9.75	11.75	16.09	9.7% denaturation; solution left overnight in the cold room before being placed in the calorimeter
				Stock No. 5				
18	1.48	14.2	7.95	7.95	11.0	11.8	3.24	Solution brought to pH 12 before adding acid
19	1.48	6.25	0	7.95	10.7	11.6	2.70	Solution brought initially to pH 11.1 and allowed to stand at 25° for 3 hours.

[a] Gram calorie used here is defined as equal to 4.1833 international joules.

evolution and only the "tail-end" of the final heat evolution is noticeable. In a few experiments a slight cooling lasting for less than five minutes followed the initial heat evolution even at *p*H 11.8.

Curve 5, finally, shows an experiment with denatured protein brought to *p*H 10.0 from 11.9 and raised again to *p*H 11.9 on mixing in the calorimeter. This curve is very similar to that describing the neutralization of alkali by free acid and shows that no secondary reactions accompany the initial heat evolution.

Curves 2 and 3 of Fig. 2 are extremely interesting from a qualitative point of view in that they show the existence of an endothermic reaction, the rate of which is considerably slower than that of the ionic reactions immediately following the opening of the can. The endothermic reaction is greatly accelerated by increasing *p*H and undoubtedly is connected with the denaturation of the protein. From the quantitative point of view, however, these curves are poorly suited for evaluation of the heat absorption in the process of denaturation, because of overlapping of several processes.

It was therefore decided to adopt a different procedure, by which no uncertainties due to a faulty kinetic interpretation of the data would be attached to the final result. Consider the following schemes

$$\text{(Native protein} + n\ \text{KOH)} + m\ \text{KOH} \longrightarrow \text{(Denatured protein} + (n+m)\ \text{KOH)} + \Delta H_1$$

$$-$$

$$\text{(Denatured protein} + n\ \text{KOH)} + m\ \text{KOH} \longrightarrow \text{(Denatured protein} + (n+m)\ \text{KOH)} + \Delta H_2$$

$$\text{(N. P.} + n\ \text{KOH)} \longrightarrow \text{(D. P.} + n\ \text{KOH)} + \Delta H_1 - \Delta H_2$$

or

$$\text{(Native protein at } p\text{H } X) + M\ \text{KOH} \longrightarrow \text{(Denatured protein at } p\text{H } Y) + \Delta H_3$$

$$-$$

$$\text{(Denatured protein at } p\text{H } X) + N\ \text{KOH} \longrightarrow \text{(Denatured protein at } p\text{H } Y) + \Delta H_4$$

$$\text{(N. P. at } p\text{H } X) \longrightarrow \text{(D. P. at } p\text{H } X + (N - M)\ \text{KOH}) + \Delta H_3 - \Delta H_4.$$

The first two steps of each scheme can be measured in the calorimeter (or at least obtained by extrapolation, as will appear below), and hence the third step is given as their difference. The first cycle gives the reaction at a variable *p*H but a constant quantity of alkali ions, the second gives the reaction at a constant *p*H, but with addition of $N - M$ moles of potassium hydroxide. In some respects the second cycle has thermodynamic advantages, but unfortunately the *p*H of unbuffered alkaline solutions of methemoglobin, at least

as measured by a glass electrode, is not very steady; consequently we have chosen to investigate primarily the first cycle, and to obtain the second only incidentally.

Table I gives the results of the experiments performed; the various column headings are self-explanatory and need no further comment.

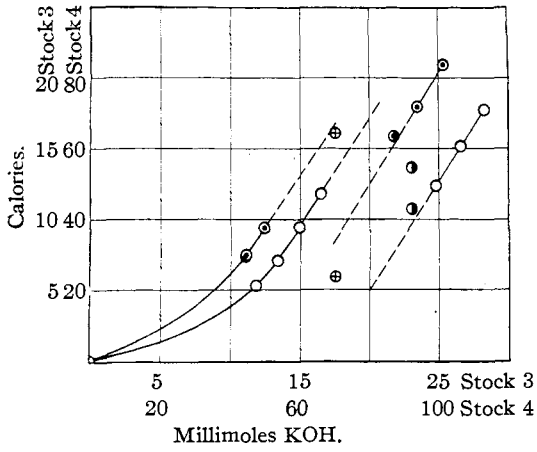

Fig. 3.—Heat changes plotted against moles of KOH in the mixing can: empty circles, stock 3; dotted circles, stock 4; shaded circles, partially denatured solutions; crossed circles, experiments nos. 16 and 17.

In Fig. 3 some of these data are plotted so as to show heat evolution as a function of the alkali added in the mixing can. Except for those experiments in which partial denaturation occurred, the points for stock no. 3 fit two nearly parallel and nearly straight lines over the limited range of the experiments. Further out the lines do bend, but this is immaterial for the following. The vertical distance between the two lines gives the heat change occurring on denaturation when a constant quantity of alkali is present in the solution; within experimental error this heat change is independent of the amount of alkali actually in the solution; in other words, it is independent of the *p*H of the solution, at least over the range 9 to 10. In this method of evaluating the heat of denaturation of methemoglobin such quantities as the heats of dilution or of reaction with the ammonium ion, which depend upon the amount of alkali in the mixing can, cancel out; the same is not quite true of the heat of denaturation at constant *p*H discussed below. The substantially straight-line relationship between the quantity of alkali added and the heat evolution shows that over the range of *p*H 8.8 to 10.3 the heat of titration of methemoglobin is constant. From the data of Table I this heat is readily evaluated and is found equal to 1.5 kcal. per mole of potassium hydroxide. If the reaction consuming hydroxyl ions is $XH^+ + OH^- \longrightarrow X + H_2O$, then, since the heat of neutralization of a strong acid by a strong alkali is *ca.* 13.7 kcal., it follows that the heat of ionization of the basic groups titrated in this range is about 12.2 kcal. (ΔH = 12.2 kcal.), a value in close agreement with the figure given by Wyman,[6] 11.5 kcal. However, part of the basic groups titrated in this range are the ammonium ions and hence the measured value is a composite of several processes.

In the case of the concentrated methemoglobin solutions (stock no. 4) the two lines in Fig. 3 are very much less accurately fixed because of a smaller number of experiments and of the longer extrapolation due to partial denaturation at lower *p*H and precipitation at higher *p*H. Also, the two lines are not quite parallel, which may be due to experimental errors, but also to secondary heat effects produced by the high concentrations of the protein and the alkali.

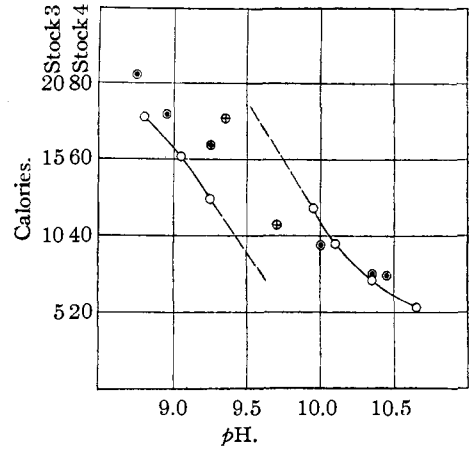

Fig. 4.—Heat changes plotted against initial *p*H of methemoglobin solutions: empty circles, stock 3; dotted circles, stock 4; crossed circles, experiments nos. 16 and 17.

In Fig. 4 we have plotted heat changes as a function of the *p*H of the solution before mixing. The plot has no such accuracy as that given in Fig. 3, since despite the fact that the same total quantity of alkali was always added, the final *p*H varied over a range of two-tenths of a unit and hence the initial *p*H is also in doubt by about ± 0.1 unit. Nevertheless it is seen that two approximately straight lines are again obtained on the plot and the vertical distance between them gives

the heat of denaturation at constant pH. This value is considerably smaller than that at constant quantity of alkali, which is in accord with our observation that pH is decreased in the process of denaturation. The best demonstration of this appears in runs 3 and 4 or 16 and 17 of Table I; for the latter experiments parts of the same alkali-protein solution were used. In experiment 17 the solution was allowed to denature extensively on long standing and the pH was lowered by 0.3 unit. About the same decrease was found on comparing two titration curves, one made as rapidly as possible, the other by allowing the protein solution to stand for about an hour after each change of one unit in pH. Closer inspection of all of these experiments suggests that the change of pH accompanies the first stage of denaturation, preceding that in which formation of a material insoluble at the isoelectric point occurs.

The reaction at constant pH can be written in the following manner:

(Heat of denaturation at constant quantity of alkali) + (Heat of addition of enough alkali to bring the solution to the initial pH) = (Heat of denaturation at constant pH).

The first term we take from Fig. 3 as 12.3 cal.; the second can be taken from the mean slope of the straight lines on Fig. 4, and is found to be -4.3 cal. for an increase in pH of 0.3 unit. The sum is $+8.0$ cal., which is sufficiently close to the direct result of Fig. 4, 9.8 cal., to show the self-consistency of the data obtained with the stock no. 3 solution of methemoglobin. The true value for the heat of denaturation at constant pH is nearer to 8.0 cal. than 9.8 cal. because the considerable uncertainty in the slope of the lines drawn in Fig. 4 affects the former value less than it does the latter.

No definite conclusions can be drawn from the experiments on the concentrated solution, no. 4, also plotted in Fig. 4. When one allows for the minimum uncertainty of 0.1 pH unit for each point on the graph, almost any value for the heat of denaturation may be obtained. This is due, however, to the greatly increased errors of extrapolation and does not disprove in any way the validity of the other results. The errors involved in the determinations of the heat of denaturation of methemoglobin cannot be evaluated exactly but a fairly accurate estimate can be given for stock no. 3. The accidental calorimetric errors in the individual determinations are about 2% for the smallest and about 1% for the larger heat changes. The extrapolation resorted to in Fig. 3 increases these errors to about 6% in the heat of denaturation. To this must be added an error arising from the assumed straight line character of extrapolation but since the last three points on each curve fall on straight lines and because these latter are parallel, we take such error to be only 3% of the heat of denaturation. Systematic errors of calorimetric nature are wholly insignificant and the chemical errors, mostly of accidental character, are about 0.5 to 1% in each experiment, so that in extrapolation another error of about 3% is added to the heat of denaturation. The total error for stock no. 3 in Fig. 3 is found therefore to be $(6^2 + 3^2 + 3^2)^{1/2} = 7.5\%$. The case of the stock no. 4 is much less favorable and one can only make a guess that the resultant error, due mainly to long extrapolations, is two to three times as large as above.

In converting the observed heat change to a molal quantity the problem arises of the determination of the methemoglobin in the calorimeter. Three determinations were made on stock no. 3: two independent semi-micro-Kjeldahl determinations were made (a) on a sample which was dialyzed for forty-eight hours under efficient stirring and (b) on a sample which was denatured at pH 12, precipitated at pH 6.8 and redissolved. They gave 2.066 and 2.070 mg. of nitrogen per cc. of stock, respectively. Oxygen absorption was determined by the method of Conant[7] and gave 0.0167 ($\pm$0.0002) cc. at N. T. P. per cc. of stock. This gives, if the percentage nitrogen is taken as 16.8 and the equivalent weight of methemoglobin as 16,700, 2.09 mg. nitrogen per cc. of stock. The oxygen determination was not wholly satisfactory because the reduced hemoglobin gradually deteriorated, as was shown by a progressive decrease of oxygen absorption in successive samples from the same reduced batch. However, this determination cannot give too high a value and hence the three determinations are in excellent agreement.

Taking the heat change in the calorimeter at constant quantity of alkali as 12.3 cal., one readily calculates that the heat of denaturation under these conditions is $\Delta H = 138$ kcal. ($\pm$14) per mole. At constant pH the value is approximately 100 kcal.

For the stock no. 4 the two Kjeldahl determinations, made as above, gave 9.44 and 9.29 mg. of nitrogen per cc., differing a little more than de-

(7) Conant, Scott and Douglas, *J. Biol. Chem.*, **76**, 223 (1928).

sirable. Color comparisons of the two stock solutions were then made under the following conditions: (a) fresh stock solutions at *p*H 6.8; (b) after denaturation by alkali; (c) after addition of some potassium ferricyanide to ensure complete absence of oxyhemoglobin; and (d) after denaturation of these last solutions. All comparisons were made on solutions so diluted that the actual color depths were almost identical and all gave substantially the same ratio, 1:3.0 (±10%). This ratio differs very much from that following from the Kjeldahl determinations, 1:4.5, and we are at a complete loss to account for the discrepancy. Unfortunately the situation cannot be remedied because calorimetric work had to be done immediately after preparation of these solutions and they were exhausted after the tests here described were completed. The only possibility which occurs to us is that the stock no. 4, for the preparation of which very large quantities of hemoglobin had to be handled, contained some other protein.

There is, thus, not much point in calculating the results for stock no. 4 and, depending on whether the Kjeldahl or the color determinations are taken for correct, values from 100 to 150 kcal. per mole, at constant alkali, are obtained, which are uncertain by a further 20% because of various calorimetric errors.

The figure of 138 kcal. obtained for stock no. 3 is fortunately not affected by these imperfections but its thermodynamic meaning has not been as yet properly defined. In this connection it must be pointed out first of all that the denatured state of the protein is well established: as a comparison of experiments 14 and 15 will show, a precipitation and resolution has no effect on the thermal state of the protein. The length of standing at *p*H 12 also has no effect on the thermal properties of the protein. Having found that at this *p*H thermal changes in the calorimeter cease after very few minutes, no particular precautions were taken to time the interval between bringing a solution to *p*H 12 and changing its *p*H to a lower value. These intervals varied quite considerably in experiments with denatured protein reported in Table I and yet the resultant heat changes were quite constant.

There remains the question as to whether the denatured state of the protein in these experiments is the same as that obtained on "complete" denaturation at some *p*H in the range from 9 to 10 or is characteristic of *p*H 12. This question is extremely difficult to answer experimentally and runs 18 and 19 of Table I give only partial information. This tends to show that even after standing for such a long time at *p*H 11 that all thermal changes should have ceased (*cf.* Fig. 2), the protein has not reached quite the same state as that material which has been at *p*H 12 for a short time, the difference in heat content being about 4 kcal. per mole.

Further light on this question is thrown by spectroscopic studies, the results of which are represented in Fig. 5.

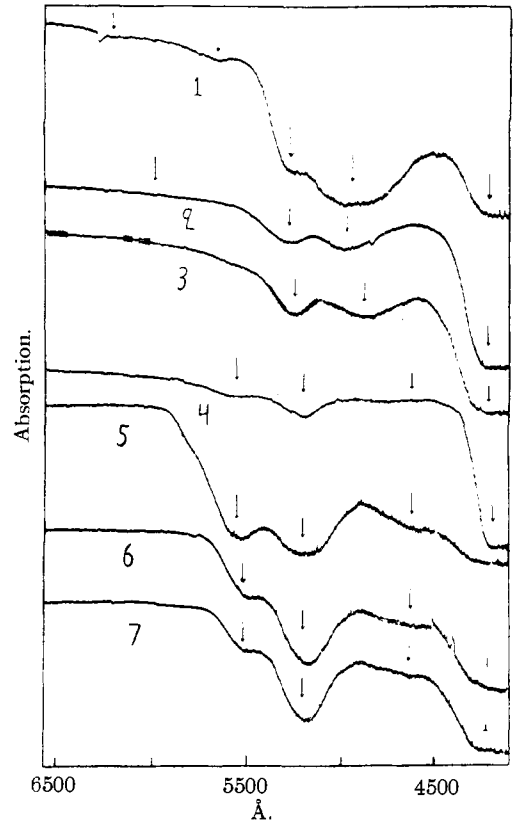

Fig. 5.—Absorption spectra of methemoglobin denatured under various conditions: (1) native methemoglobin at *p*H 6.8; (2) methemoglobin denatured by acid; (3) methemoglobin denatured by acid and taken to *p*H 12; (4) methemoglobin denatured by alkali at *p*H 12; (5) methemoglobin immediately after bringing to *p*H 11.2; (6) the same sample twenty minutes later; (7) methemoglobin denatured at *p*H 12 and then brought to *p*H 11.2. The arrows indicate visually observed bands.

The comparison of microphotometer curves 1, 2, 3 and 4 shows that the spectrum observed for acid-denatured methemoglobin and belonging presumably to at least partially free hematin is

retained on bringing the *p*H to 12, but is significantly different from that produced by alkaline denaturation at this *p*H. The most plausible interpretation of these curves is that no splitting of the hematin occurs on basic denaturation. The following three curves show that the spectrum produced on bringing the *p*H of native methemoglobin to 11.2 suffers gradual changes during some twenty minutes (about the time that a cooling is observed in the calorimeter, *cf.* Fig. 2) but reaches then a state which is indistinguishable from that of a solution which had been brought to *p*H 12.0 and then to *p*H 11.2. It may be noted that further standing at *p*H 11.2 was accompanied by no observable changes in the position or intensity of the absorption bands.

This spectroscopic evidence may be interpreted as favoring the supposition that the final state of methemoglobin is independent of the *p*H at which it was denatured but the thermochemical evidence just discussed seems to us more weighty and we are inclined to believe that even after a very protracted standing at some lower *p*H the protein would not reach quite the same thermodynamic state which it does at *p*H 12. The heat change accompanying such long standing would be somewhat lower than the 138 kcal. determined previously, although, if experiments 18 and 19 are indicative of the general trend,[8] it would be of the same order of magnitude.

On the other hand, if the heat absorbing reaction, shown in Fig. 2, is taken alone as representative of the process of "pure" denaturation, the heat change accompanying it is considerably larger and may be crudely estimated as of the order of 200 to 300 kcal. heat absorption per mole of methemoglobin.

All in all, it must be conceded that while thermodynamically the initial and the final states of the protein here studied have been satisfactorily defined, the precise chemical nature of the reactions occurring remains quite unexplored. It may well be that formation of sulfhydryl groups, splitting of ammonia or something else accompanies the process of "pure" denaturation in the reaction studied in the calorimeter. The lack of definition, however, is not a fault of the calorimetric technique but of the extraordinarily incomplete chemical knowledge of the process of "pure" denaturation; and so long as chemical experiments have not provided a more precise meaning for this concept, thermochemical data must remain correspondingly formal.

In concluding we should like to comment on statements made in the literature[9] that denaturation is an "all or nothing process," and that denaturation is measured by insolubility at the isoelectric point. The first of our stock solutions was allowed to stand for a rather long time in the cold room, and it developed gradually a much greater sensitivity to denaturation than the fresh methemoglobin solutions used in the later work. Perfectly clear solutions were obtained on adding to the clear isoelectric solutions variable amounts of alkali but on reducing the *p*H by as little as one unit the entire protein came down. That is, a solution denatured at *p*H 9.5 gave precipitate at *p*H 8.5, but a solution denatured at 8.5 remained clear at this hydrogen ion concentration, while giving a precipitate nearer the isoelectric point.

On bringing methemoglobin to *p*H 11.2 and reducing the *p*H almost immediately thereafter to the isoelectric point, practically the entire protein is precipitated. Nevertheless, heat changes persist for a considerable time at this *p*H as shown by Curve 3 of Fig. 2.[10] Very illuminating also are experiments 16 and 17 of Table I.[11] According to colorimetric tests on precipitated protein, standing overnight in the cold room (the solution was at 25° when put into the cold room) increased denaturation from 2.7 to 9.7% or by 7.5×10^{-6} mole of methemoglobin. The heat change on adding an identical quantity of alkali in the calorimeter was altered by 10.2 cal., however, which means, if precipitation is a true measure of denaturation, that the heat of denaturation is 1400 kcal. per mole. This improbable figure suggests very clearly that insolubility at the isoelectric point is indicative of only one step of denaturation of methemoglobin and that denaturing processes occur before this stage is reached, while others follow it. This view of the denaturation process may seem to invalidate the method developed

(8) Similar experiments were made at still lower *p*H but here, after even four hours of standing, the calorimeter indicated incomplete denaturation, manifested by a slight cooling after the initial heating. Differences of the heat changes in experiments paired like 18 and 19 were somewhat larger, but of the same order of magnitude.

(9) *Cf.* Schmidt, "Chemistry of Amino Acids and Proteins," Charles C. Thomas, Springfield, Ill., 1938, p. 422.

(10) It is to be noted that the particular experiment shown in Fig. 2 was made with the sensitized protein of the first stock solution; similar curves were obtained later with the fresh methemoglobin solutions; this shows that the sensitization of the protein does not eliminate heat effects found with fresh solutions.

(11) Absolute values of the heat changes in these experiments should not be compared directly with those in the main series because of different alkali–protein ratios.

earlier for the calculation of the heat of denaturation of methemoglobin: it may be argued that the solutions which were denoted as native in Table I, because no precipitate formed on bringing them to the isoelectric point, were actually partially denatured. However, the slopes of the lines drawn through the experimental points in Fig. 3 are identical for the native and denatured solutions. If in the "native" solutions partial denaturation occurred, it would certainly depend on the *p*H of these solutions and hence the slope would be entirely different from that for the denatured solutions.

The magnitude of the heat of denaturation here reported is about twice as large as that reported by Anson and Mirsky[12] for trypsin and it is interesting to note that the molecular weights of the two proteins stand also in the 2:1 ratio. However, the thermal data on this type of protein reaction are still too meager to attempt their correlation with the structure of proteins.

Dr. Thomas S. Chambers has made preliminary experiments on the denaturation of methemoglobin and participated in the preparation of the stock solutions, for which our thanks are due him. We wish also to thank Dr. W. F. Ross of Harvard and Radcliffe for many valuable suggestions and discussions of the problem. To the Rockefeller Foundation our thanks are due for the financial assistance without which this work would not have been possible.

(12) Anson and Mirsky, *J. Gen. Physiol.*, **17**, 393 (1934).

Summary

1. A calorimeter is described which is suitable for observations on slow reactions with small heat changes.

2. It is shown that denaturation of methemoglobin at *p*H 10 to 12 is a measurably fast reaction accompanied by heat absorption in the initial stages and by heat evolution toward its end. The rate of these processes increases rapidly with *p*H.

3. By an indirect calorimetric procedure the heat of denaturation is determined to be: ΔH = 138 (±14) kcal. per mole of methemoglobin, at constant quantity of alkali in the solution. At constant *p*H the heat of denaturation is about 100 kcal.

4. The process of denaturation is shown to be very complex and the precipitability of methemoglobin at the isoelectric point is found to be not a good measure of denaturation.

CAMBRIDGE, MASS. RECEIVED MAY 10, 1940

[CONTRIBUTION FROM THE BELL TELEPHONE LABORATORIES]

X-Ray Examination of Polyisobutylene[1]

BY C. S. FULLER, C. J. FROSCH AND N. R. PAPE

Polyisobutylene was first studied from the X-ray standpoint by Brill and Halle.[2] These investigators pointed out that when this material was stretched to the maximum the amorphous X-ray diagram was replaced by a fiber pattern, analogous to the case of natural rubber. They found the identity period in the fiber direction to be 18.5 Å. and concluded that the molecules in the stretched state are not planar zigzag but because of the methyl side-groups are probably helical. The authors have re-examined this polymeric substance and in agreement with the work of Brill and Halle find that the reflections appear instantaneously and without orientation phenomena just as in the case of natural rubber. The identity period reported by these authors has been verified and good evidence has been found that the chain molecules in the crystalline regions of the stretched substance assume a coiled form and possess a 1:3 disposition of the methyl groups. Although the present work cannot claim to have arrived at a definite structure for crystalline polyisobutylene, it has been shown that the orthorhombic cell, a = 6.94 Å., b = 11.96 Å., c = 18.63 Å., is in agreement with the observed data.

Material and Procedure Employed.—Polyisobutylene[3] consists of long molecules which in the stretched material may be regarded as essentially linear in form. In the case of the lower molecular weight products there is insufficient interaction between molecules to enable the substance to be held in the stretched condition without relaxation. In the case of the very high molecular weight products, however, the behavior is decidedly rubber-like

(1) Presented before the Division of Rubber Chemistry of The American Chemical Society at Cincinnati, Ohio, April 12, 1940.

(2) Brill and Halle, *Naturwissenschaften*, **26**, 12 (1938).

(3) Thomas, Sparks, Frolich, Otto and Mueller-Cunradi, THIS JOURNAL, **62**, 276 (1940).