CCCXI. THE TIME COURSE OF THE HEAT EFFECTS IN RAPID CHEMICAL CHANGES.

I. IMPROVED APPARATUS AND METHODS.

II. THE REACTIONS OF HAEMOGLOBIN WITH OXYGEN AND CARBON MONOXIDE.

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ROUGHTON [1930, 1, 2] has described a method of following the heat effects in rapid chemical changes and has applied it to the reactions of acids and alkalis with buffers, amino-acids and proteins. The principle of the method is as follows.

The two substances which are to react are contained in separate solutions R and L ; reaction between them is brought about by driving the two fluids into a special mixing chamber, wherein mixture occurs in $1/1000$ sec. or less. The mixed fluid emerging from the mixing chamber passes into an observation tube, at various points in which the temperature of the running fluid is taken by means of a thermocouple. The change in temperature due to the reaction between R and L is given by the difference between the reading with both fluids running and the average of the two readings when R and L are running separately. The corresponding time is simply equal to d/v , where d is the distance from the mixing chamber of the thermojunction and v is the rate of flow of the mixed fluid down the observation tube.

This method was developed to the extent of being able to measure, to within about 0.001° , the temperature change occurring in rapid reactions within 0.01 sec. or less from the beginning of such reactions. Roughton, however, pointed out that many, if not most, of the rapid chemical reactions which are of interest to the biochemist only involve changes, *in toto*, of 0.01 – 0.001° . Hence, if the course of these changes is to be followed accurately, the sensitivity of the method must be increased 5 or 10-fold, *i.e.* to within 0.0001 – 0.0002° . This objective has been realised in the papers presented below. Of these, Part I deals with the changes in apparatus and technique by which the greater sensitivity has been reached, whilst in Part II will be found some preliminary results of the method in regard to the reactions of haemoglobin with oxygen and carbon monoxide.

I. IMPROVED APPARATUS AND METHODS.

DESCRIPTION OF APPARATUS.

General arrangement of apparatus. The refinements outlined by Roughton [1930, 1] for a ten-fold increase in sensitivity, and those actually carried out in the present apparatus, consisted mainly in the improved thermal control of the entire apparatus, thus enabling a slower galvanometer of about 7 times greater sensitivity to be used. The 5 mm. observation tube of the previous paper was

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replaced by one of 2 mm. diameter: for a given rate of linear flow down the tube, the consumption of fluid is reduced to about one-sixth, thus giving more time for the taking of each individual galvanometer reading without undue sacrifice of fluid.

The general arrangement is shown in Fig. 1. The reagents are placed in stoneware bottles, *R* and *L*, of about 5 l. capacity, mounted symmetrically on a metal stand, and each connected in the manner shown in a vertical metal box *B*, containing the mixing chamber and observation tube and mounted on the same stand. The entire stand, with its bottles and metal box, is immersed in a 70-gallon tank containing water, which can be kept constant in temperature to $\pm 0.01^\circ$ (as checked by a Beckmann thermometer) by a small lamp turned on and off occasionally by hand and by vigorous stirring with compressed air. The apparatus is raised from (or lowered into) the tank, as required, by means of a block and tackle.

Fig. 1. Apparatus for determination of velocity of rapid reactions.
(For description see text.)

The reagent bottles, R, L. These were drilled at the top for the introduction of two or more glass inlet tubes, *i*, which are sealed in with tightly fitting rubber tubing, and the joints made rigid by means of metal collars filled with a cement of wax and resin. One of these inlet tubes passes to the bottom of the bottle and can be used to introduce the reagents or to withdraw samples of fluid during an experiment. The other is short and serves as an inlet for the compressed gas which is to force the reagent into the mixing chamber. This gas is brought to the same temperature as the reagents by being passed through 80 ft. of lead tubing lining the inside of the thermostat before it enters the bottle. Since in the present apparatus the gas only comes in contact with the upper layers of the reagent, and since the reagent is forced out at the bottom of the bottle less care need be taken than in the previous work as to the heat effects accompanying the sudden compression and/or the humidity of the gas.

Additional holes in the necks of the bottles can be used as points of entry for the various thermocouples used to indicate the difference in temperature between the reagents and the thermostat water, or between the contents of *R* and *L* respectively. Small adjustments of temperature, just before a run, can be made by adding warm water to the bath, or by heating one or other of the fluids in *R* and *L* by means of 3-watt electric bulbs *w*, suitably protected from water and sealed into the bottom of the bottles.

The reagents are stirred by stout glass rods, *g*, bent and flattened into vanes at their free ends, the rods being introduced through lanoline-lubricated ground

joints, *j*, fixed in rubber corks in the necks of the bottles. The glass rods are coupled at their top ends to the shaft of special electric motors by rubber tubing, and are rotated about 90 times a minute. This arrangement, which proved satisfactory in every way, enables the solutions to be stirred effectively when the bottles contain any gas other than air. This condition had to be met in our work on haemoglobin (in Part II).

The metal box B and its contents. The glass leads, *l*, which connect the lower parts of the bottles with the mixing chamber, are much shorter than the delivery tubes in the previous apparatus. They are air-jacketed by wide rubber tubing between the bottles and the metal box.

The mixing chamber and the observation tube are themselves situated symmetrically in the water-tight metal box *B*, the detachable front of which is fixed in place by wing-nuts. The flow of reagents into the mixing-chamber is controlled from the outside by brass rods, *r*, which pass through the roof of *B* and rest, at their lower ends, on short pieces of rubber tubing, *t*, inserted in the connections on either side of the mixing chamber and supported underneath by brass blocks.

The mixing chamber, *m*, is a simple three-way tap of suitable bore, its central limb being sealed as regularly as possible to the lower end of the observation tube.

The observation tube, *o*, a glass capillary of internal diameter 2 mm. and length 12 cm. is widened at the top so as to join with a few cm. of 10 mm. diameter tubing, and then passes through the roof of the box to a funnel from which the effluent can either be sucked to waste or collected in an evacuated tonometer.

Construction of thermocouples. These were made from enamelled wire, no. 24 copper and no. 32 constantan, roughly as described in the previous paper, and then insulated with several layers of celluloid varnish. They were introduced into the observation tube from above *via* the open funnel. Two types of thermocouple were again used: (a) The "absolute" or "thermos" type in which one of the junctions is placed in the observation tube and the other, the "cold" one, is kept in a suitable enclosure, constant to $\pm 0.0002^\circ$ during the period of the experiment. In the first experiments, the cold junction was immersed in a cylinder consisting of alternate layers of copper and paraffin wax, in the metal box *B*, whilst in subsequent ones it was placed in a small vessel of mercury separated by an air gap from the walls of a large bottle immersed in the thermostat, the latter arrangement being more convenient. (b) The "differential" type, in which the junctions are only 3 cm. apart, and which therefore measures the difference of temperature between two points 3 cm. apart in the running fluid. As before, and as was indeed to be expected, this type proved much more satisfactory, both as regards the steadiness and the speed of response of the galvanometer reading.

Electrical connections, galvanometer etc. The copper leads from the thermocouples were soldered to rubber-covered copper cable leads, which were connected *via* appropriate "all-copper" switches and a manganin resistance box with the galvanometer.

For the present purpose sufficient sensitivity was given by a Kipp Zb moving coil instrument, shunted with 50 ohms and with minimum magnetic control. With the scale at 4 metres, the deflection per micro-volt with 0 ohms in series was about 120 mm., so that the accuracy attainable on the electrical side, if the deflections be read to 0.2 mm., is better than 0.00004° .

Calibration of thermocouples. Readings were usually taken either with 50 ohms or 0 ohms in series with the couple and the galvanometer. In the former case the

couples were calibrated by inserting the junctions in separate flasks, which were filled with well-stirred water at temperatures differing respectively from one another by 1 or 2°. The calibration with 0 ohms in series was not made directly, since it would have needed too small a temperature difference between the two flasks to be measured accurately with ordinary standard thermometers: instead it was calculated from the calibration with 50 ohms in series by the formula:

$$\theta_0 = \theta_{50} \times (50 + T + G)/(T + G),$$

where θ_0 , θ_{50} are the sensitivities of the galvanometer, with 0 and 50 ohms respectively in series; T is the resistance of the thermocouple, G is the resistance of the galvanometer and its leads, both of which were determined independently.

TYPICAL EXPERIMENT AND CONTROLS.

Control experiment with plain water in bottles R and L.

Roughton [1930, 1] pointed out that the method contained several physical sources of error, which might have to be allowed for by blank corrections. Of these the most important are fluid friction, heat conduction between the flowing fluids and their surroundings and local temperature effects in the neighbourhood of the thermojunctions. Theoretical calculation and experimental controls, however, both showed that the temperature effects due to these physical sources of error were individually and collectively within the range 0.0001–0.001°, and hence could be neglected in the previous work, since the accuracy there aimed at was only 0.001°. In the present work, with its hope of a five- or ten-fold increase in sensitivity, the effects had obviously to be investigated further: this was done, as before, by control experiments with plain water in each bottle. Since the procedure was the same as in ordinary experiments, in which positive thermochemical effects were to be looked for, it will be useful, at the outset, to describe it in detail.

At least 2 hours before the experiment was to begin, bottles *R* and *L* were each filled with 4 litres of water. The apparatus was lowered into the thermostat and kept there until the time of the experiment, the liquids being stirred periodically, and their temperatures brought to that of the bath water (to within $\pm 0.001^\circ$) by suitable short periods of electric heating. The pressure inside the bottles was increased to 20 cm. Hg above atmospheric pressure by connecting the gas leads of *R* and *L* to a cylinder of compressed oxygen or nitrogen *via* the 80 ft. length of tubing in the thermostat, the pressure being regulated automatically by a simple mercury blow-off valve. Stirring of the bath and reagent bottles was stopped, and, after the differential thermocouple had been placed in a suitable position in the observation tube the switch connecting it to the galvanometer was pressed down; this was done as early as possible before the beginning of the measurements, so as to allow time for the "parasitic" E.M.F.'s of the circuit to become stable. A series resistance of 10,000 ohms and a short-circuiting switch protected the galvanometer during the adjustment of the thermocouple. The series resistance was then reduced to zero (or in certain experiments to 50 ohms) and readings taken in the following sequence:

- (i) Fluid from *R* alone running for 20 sec. Galvanometer scale reading taken = $R_1 = 515.0$ mm.
- (ii) Fluid from both bottles running together for 20 sec. Reading = $B = 515.5$ mm.
- (iii) Fluid from *L* alone running for 20 sec. Reading = $L = 514.2$ mm.

In this example the two junctions of the thermo-couple were placed at 2 and 5 cm. respectively from the mixing chamber. Let x = relative delivery of R to L when both are running (*v. infra*). Then temperature effect, due to physical causes, is thus, in the case of the differential thermocouple

$$= B - \frac{xR + L}{x + 1} = 0.9 \text{ mm.} = 0.00022^\circ \text{ (warming).}$$

Actually in this example, and indeed in general, readings R and L are so close that $\frac{xR + L}{x + 1}$ can with sufficient accuracy be replaced by $\frac{1}{2}(R + L)$, provided, as is usually the case, that x lies between 0.4 and 0.6.

The same procedure was then repeated with the differential thermocouple in 2 or 3 other positions.

By that time sufficient fluid had passed through the leads and the mixing-chamber for them to reach a much more steady temperature state than at the start. The differential couple was then replaced by the "absolute" couple, the latter usually being adjusted in the middle of the capillary part of the observation tube, *i.e.* at 5 cm. from the mixing chamber. The absolute "couple" is of course affected by temperature drifts in the contents of the bottles during the experiment; to compensate for these a set of five readings was always made in the following order:

- (i) Fluid from R alone for 20 sec. Reading = $R_1 = 372.2$ mm.
- (ii) Fluid from both bottles for 20 sec. Reading = $B_1 = 367.2$ mm.
- (iii) Fluid from L alone for 20 sec. Reading = $L = 365.3$ mm.
- (iv) Fluid from both bottles for 20 sec. Reading = $B_2 = 364.9$ mm.
- (v) Fluid from R alone for 20 sec. Reading = $R_2 = 369.5$ mm.

If the solutions had not been adequately equilibrated as regards temperature, the 20-sec. running period had to be increased.

The temperature effect, due to physical causes, is for the "absolute" couple thus

$$= \frac{1}{2}(B_1 + B_2) - \frac{x(R_1 + R_2)/2 + L}{1 + x} = -1.8 \text{ mm.} = 0.0006^\circ \text{ (warming).}$$

In this case also, readings R and L were so close together, that the expression

$$\frac{x(R_1 + R_2)/2 + L}{1 + x}$$

could be replaced by

$$\frac{(R_1 + R_2)/2 + L}{2}.$$

As a rule the readings were less steady than the differential couple readings, especially if the absolute readings were, in contradistinction to the above procedure, taken before much fluid had been passed through the apparatus.

To obtain the rate of flow, the mixed fluid emerging into the funnel during 15 sec. run was collected in an evacuated tonometer and measured. The rate usually varied from 500 to 700 ml. per minute according to the position of the thermocouple in the observation tube. Allowance had to be made for this in calculating the respective time intervals.

In this control experiment it was not necessary to know the relative delivery of R to L with both fluids running together, *i.e.* x , since readings R and L were so close together. In "live" experiments, however, the relative delivery is always required whether R and L are close or not: in experiments with haemoglobin solution, it was calculated simply from the ratio of the respective Hb concentrations in the mixed fluid and in fluid L (assuming that the haemoglobin was placed in this bottle and not in R) as estimated colorimetrically: in all other

experiments, so far, one or both of the reagents has been capable of volumetric estimation (*e.g.* HCl, NaHCO₃ or NaOH). In these cases also, x can easily be calculated from the respective titres of the mixed fluid and of the original reagents R and L . If neither solution is naturally coloured or titratable, the simplest plan is to add a small amount of an indifferent coloured substance to L and to calculate the relative delivery by the same colorimetric method, as in the case of haemoglobin solutions.

Magnitude of blank errors.

These were measured by control experiments with plain water (or aerated blood solution) in bottles R and L .

(a) *With the differential thermocouple.* In size the blank effect shown in the control experiment above is of the order to be expected, namely 0.0002–0.0003°. The consistency is shown by the figures of Table I; from these it appears that the heat effects at specified points in the observation tube repeat themselves to within 0.1 mm. (*i.e.* 0.000025°), even when one of the bottles contains blood diluted 1 in 4, instead of water. Corresponding blank corrections have therefore been deducted from the differential couple readings in “live” experiments, and the residue, if any, attributed to temperature differences of thermochemical origin.

Up to a distance of 11 cm. from the observation tube the distal junction is seen to be 0.0002–0.0003° hotter than the one proximal to the mixing chamber: this is probably due, in the main, to the frictional resistance encountered by the fluid in travelling from one junction to the other and dissipated as heat. In the 11–14 position, however, the situation is reversed: this is probably to be explained by the fact that the observation tube widens out at a distance between 11 and 14 cm. from the mixing chamber, which means that at the 11 cm. point the fluid must be travelling much faster than at the 14 cm. point. The diminished “local turbulence” and drop of pressure in the neighbourhood of the latter probably account for its being apparently cooler by 0.00035° than the 11 cm. point.

Table I. *Test for blank corrections with differential thermocouple.*

Series resistance in circuit = 0 ohm.				
Driving pressure applied to R and L = 20 cm. Hg air pressure.				
Distance of differential junctions from mixing chamber (in cm.)	2–5	5–8	8–11	11–14
Heat effect in mm. galv. deflection:				
(a) Water + water	+0.8	+1.4	+1.7	–1.5
(b) Water + blood solution (1 in 4)	+0.9	+1.4	$\begin{cases} +1.4 \\ +1.4 \end{cases}$	$\begin{cases} -1.5 \\ -1.25 \\ -1.4 \end{cases}$

1 mm. = 0.00025°.

Here + means that junction distal to mixing chamber is warmer.

Here – means that junction distal to mixing chamber is cooler.

(b) *With the absolute thermocouple.* The blank corrections for the absolute thermocouple in various positions in the observation tube are shown by the figures given in Table II. Consistent results were obtained in two separate experiments. The corrections turn out, not unexpectedly, to be distinctly greater than those for the differential thermocouple but are, from the physical point of view, quite reasonable in size. A specially extended series was taken with the thermocouple at 5 cm., since this was the staple position used in most of our experiments. The mean effect at this position = 2.1 mm. galv. deflection = 0.00067°.

Table II. *Test for blank corrections with absolute thermocouple.*

Fluid R = fluid L = tap water.			
Series resistance in circuit = 0 ohm.			
Driving pressure = 20 cm. Hg.			
Relative deliveries of two fluids equal.			
Distance of absolute junction from mixing chamber in cm.	2	5	8
Heat effect in mm. galv. deflection:			
(a) Water + water	$\begin{cases} +2.3 \\ +2.3 \end{cases}$	+1.9	$\begin{cases} +1.6 \\ +1.8 \end{cases}$
(b) Water + water	—	$\begin{cases} +1.8 \\ +2.3 \\ +2.0 \\ +2.4 \end{cases}$	+2.5

1 mm. = 0.00032°.

Here + means that reading B is warmer than the mean of readings R and L .*Accuracy of the method and résumé of procedure.*

With total temperature changes of 0.05° or more in the observation tube, the measurements are believed to be accurate to within about 1 % of the total change, as was the case in Roughton's earlier work. In the case of the smaller changes, of the order of 0.005–0.01°, which it was the special object of this work to measure, a higher "absolute" accuracy was reached, especially with the differential thermocouple. This is shown by

The degree of agreement between duplicates. Table III shows a series of duplicate determinations made both with the differential and the absolute thermocouples. In the case of the differential couple, duplicates usually agreed with one another to within the limit to which the galvanometer deflections could be read by eye, *i.e.* to 0.12 mm. = 0.00003°. In the case of the absolute couple the divergence was naturally much greater, but did not on the average exceed 0.00026°. It is probable that a single set of observations with the absolute thermocouple is as a rule correct to 0.0002°, when the total temperature change is less than 0.01°; with the differential couple the accuracy seems to be about five times better, *i.e.* to within 0.00004°.

Table III. *Duplicate readings of difference between temperature of mixed fluid and average temperature of separate fluids R and L .*

Readings expressed in mm. galv. deflection. Series resistance in circuit = 0 ohm.

Differential thermocouple		Absolute thermocouple	
Reading 1	Reading 2	Reading 1	Reading 2
1.40	1.40	10.8	10.7
1.40	1.40	14.6	15.2
1.50	1.25	12.1	12.5
9.40	9.30	13.3	12.2
6.20	6.66	13.3	14.2
0.45	0.50	11.8	10.0

Average discrepancy between duplicates.

0.14 mm. = 0.00003°

0.80 mm. = 0.00026°.

That these claims are not exaggerated has been checked in two ways:

(i) *Comparison of the rate of combination of carbon monoxide with haemoglobin by the thermal and optical methods.* In these experiments, which are described

more fully in Part II, it was found that the rate of combination of CO with haemoglobin by the present method agreed to within 2% with the rate as measured independently by the optical method of Millikan [1933]. This degree of agreement could hardly have been attained if the thermal methods were appreciably less accurate than has just been claimed.

(ii) *Measurement by the absolute couple of the rapid heat of combination of HCl with NaOH.* Two experiments of this kind were made to test the accuracy and reliability of the absolute couple.

(a) Fluid $R=0.0054\text{ }N\text{ HCl}$ (containing trace of CO_2), fluid $L=0.0214\text{ }N\text{ NaOH}$.

Relative delivery of R to $L=1/1.21$.

Expected temperature rise $=0.0343 \pm 0.0004^\circ$.

Observed temperature rise $=0.0340 \pm 0.0004^\circ$ (mean of two observations).

In this experiment, the total temperature change was too large for the maximum absolute accuracy to be expected.

(b) Fluid $R=0.002\text{ }N\text{ NaOH}$, Fluid $L=0.00013\text{ }N\text{ HCl}$ (containing trace of CO_2).

Relative deliveries of R and L equal.

Expected temperature rise $=0.0008 \pm 0.0002^\circ$.

Observed temperature rise $=0.0009 \pm 0.0002^\circ$ (mean of 4 observations).

The uncertainties of 0.0004 and 0.0002° in the respective "expected temperature rises" in the two cases are due to the difficulty of estimating the concentration of such weak HCl solutions accurately. In both cases a small correction was inserted for the heat of neutralisation of the traces of dissolved CO_2 in the HCl solution.

The agreement, on the whole, is quite as good as can be expected and we have hence felt justified in applying the method to the problems described in Part II, and to others since then.

Résumé of procedure.

For the convenience of the reader, the procedure in a typical "live" experiment may now be briefly recapitulated. The two reagents are placed in bottles R and L in the thermostat, and by a 2-hour period of appropriate stirring and electric heating are brought to within 0.005° of each other and the bath. This preliminary period may with advantage be prolonged to 4 hours or more. The solutions are then driven by compressed gas (pressure used $=20\text{ cm. Hg}$) at the correct temperature into the mixing chamber in the sequence R , both, L , for periods of 20 sec. each, and the corresponding readings taken with the differential thermocouple at 2 or 3 different places in the observation tube. A set of readings with the absolute thermocouple, at say 5 cm. from the mixing-chamber, is then taken in the sequence R , both, L , both, R for periods of 20 sec. each again. Further similar sets of readings with either of the thermocouples are then taken as desired.

The rate of flow is then measured, and the relative deliveries of R and L are calculated from the colour or titre of the mixed fluid, as compared with that of R and/or L . If neither R nor L possesses naturally a colour or titre, a small amount of an indifferent coloured substance may be added to one of them.

The above observations are then used to calculate the corresponding temperature differences, from which are subtracted blank corrections according to Tables I and II.

From these data it is easy to plot the thermochemical temperature change *versus* time from the beginning of the reaction. An example is shown in Fig. 2.

The interpretation of such temperature-time curves varies with the reaction under study, and no general rules can be laid down here. In computing the magnitude of the corresponding heat changes in calories, it has to be remembered that (i) the heat of dilution of the solutions on mixture may be significant, and in this case, if not obtainable from standard Tables, must be measured by special experiments, (ii) the specific heat of the mixed solution may differ from unity.

The apparatus of the present research can deal with time intervals, from mixture, of 0.005–0.5 sec. This range could, if desired, be extended both in an upward and downward direction.

Forecast of further improvements.

The limits of error of the temperature measurements are in the present apparatus much smaller than the size of the blank corrections for non-chemical sources of heat which there seems no way of avoiding. It therefore looks doubtful whether the temperature sensitivity can be developed much further, nor at the moment is there any call, since the apparatus with its existing accuracy has already a wide possible field of work before it. What, however, is badly needed is a radical economy in the quantity of the fluids required for an experiment, 4 litres being an outrageous, if not impossible, demand in many cases of biochemical interest. We hope, in due course, to address ourselves to this problem, but the solution of it would probably involve some rather radical changes in design; it may, therefore, not be attempted until the potentialities of the present technique shall have been more fully worked out.

II. THE REACTIONS OF HAEMOGLOBIN WITH OXYGEN AND CARBON MONOXIDE.

Elsewhere Roughton [1935] describes measurements, by the usual slow calorimetric method, of the heat of combination of haemoglobin with oxygen and carbon monoxide. Reduced blood or haemoglobin solution was shaken with O₂ or CO in thermos flasks, and the temperature of the contents was registered thermo-electrically at suitable intervals. Under these conditions 15 min., at least, were required for the temperature conditions to become steady again. This delay must have been in part due to the slowness of diffusion of O₂ (or CO) from the gas phase to the haemoglobin in the liquid phase, and in part due to the slowness with which the heat, liberated by the reaction, was distributed throughout the contents and walls of the flasks. As to the actual rate of heat liberation itself these measurements gave no guide—the time so occupied might have been anything from 0.01 sec. to 5 min.

The measurement of the true speed at which the heat of these reactions is liberated is of interest in two connections.

(a) From the theoretical standpoint it is important to know whether the rate of heat liberation runs parallel with the rate of combination of O₂ (and/or CO) as followed in other ways, such as by the spectroscopic method of Hartridge and Roughton [1923, 1, 2] and the photo-electric method of Millikan [1933]. If the heat effects were found to lag behind or to be divisible into more than one phase in time it would mean that the primary process of combination of O₂ (and/

or CO) with haemoglobin, as revealed by the optical change, must be followed by secondary tautomeric changes after the O_2 (and/or CO) molecule is combined, such as have indeed been found by Roughton [1934] in special cases. Such secondary tautomerisations would be a new feature in the haemoglobin reactions, and would certainly entail a re-formulation of their physico-chemical mechanism.

(b) Failing any discrepancy between the speed of the reaction as followed thermally on the one hand, and optically on the other, the thermal method would provide a valuable alternative means of measuring the rate of the reactions, especially in haemoglobin solutions of high concentration and in suspensions of intact red cells. In the latter, especially, the optical method may be rather inaccurate, on account of the uncertainty due to the scattering of light by the walls of the red cells and of the irregular paths which the light must take through the biconcave shaped cells.

In the present paper comparisons are given of (i) the heat of combination of 1 mol. O_2 with haemoglobin by the usual slow method [Roughton, 1935] and by the rapid thermal method of Part I (ii) the rate of combination of CO with haemoglobin by the optical method and by the rapid thermal method. Preliminary observations are also given of the rate of uptake of O_2 and of CO by suspensions of intact red cells, and in conclusion the further scope of the method, particularly in regard to the kinetics of the reactions of CO_2 in whole blood is pointed out.

METHODS.

Preparation of solutions.

Reduced haemoglobin solutions. Prepared from ox blood containing 1 % boric acid and stored at $+0.5^\circ$. The blood was reduced by shaking in an evacuated vessel for about 30 min. at about 40° with intervals during which small quantities of purified nitrogen were introduced and the vessel again evacuated. It was then laked by dilution with eight times its volume of air-free water.

Reduced corpuscles. Ox corpuscles were washed once with 1 % NaCl and then diluted with NaCl solution to the volume of blood originally used. The suspension was reduced and diluted with air-free 1 % NaCl to the same extent as in the preparation of reduced Hb solution.

Oxygen, nitrogen and carbon monoxide. Cylinder oxygen was used throughout; cylinder nitrogen needed purification when used in the preparation of reduced haemoglobin, and for this purpose it was shaken with alkaline $Na_2S_2O_4$ containing Na anthraquinone- β -sulphonate and stored at increased pressure in a stoneware bottle. Carbon monoxide was prepared from sodium formate and commercial sulphuric acid and stored over alkaline sodium hydrosulphite under pressure. Special precautions—thorough washing out of all leads immediately after the preparation—were needed in order to remove all traces of formic acid from the gas.

Determination of rapid heat and time course of its production.

The general procedure has already been described in some detail in Part I, but certain points peculiar to experiments with haemoglobin require special mention.

4 litres of the reduced haemoglobin solution or corpuscle suspension, stored under nitrogen at a slight positive pressure, were transferred to the evacuated bottle *L*, after all leads had been duly washed out with nitrogen. Similarly 4 litres of water equilibrated with O_2 or CO at atmospheric pressure were transferred to bottle *R* and kept under a slightly increased pressure of O_2 or CO.

After all temperature adjustments had been made, the experiment could proceed exactly as described in Part I, measurements with the differential thermocouple usually preceding those with the absolute couple, in cases where a gradient was expected; in this way the absolute couple could be used under the most favourable conditions.

Samples of effluent were taken in an evacuated tonometer while *L* and *R* were both delivering liquid, and the relative delivery of *L* and *R* was determined colorimetrically, by comparing this effluent with an exactly twice diluted sample from *L*.

The O_2 or CO content of fluid *R*, and the haemoglobin content of fluid *L* were both determined with the Van Slyke-Neill apparatus at the end of the experiment.

The rise of temperature due to the reaction was usually about 0.005° , and in good experiments, could be determined to within $\pm 0.0002^\circ$. This corresponds to an experimental error of about 250 calories in the heat of combination of 1 mol O_2 with Hb.

Determination of slow heat.

The method was in most respects similar to that described by Roughton [1935]: the heat insulation of the thermos flasks was, however, improved by enclosing the flasks completely in a copper jacket through which thermostat water was circulated by means of a rotary pump. With this arrangement the change in temperature of the water during circulation, when the contents of the flasks had sufficiently equilibrated, was less than 0.01° . Temperature adjustment of the thermos flasks to that of the circulating water was made by short periods of electric heating, the internal circulation described by Roughton being dispensed with. The difference in temperature between the contents of the flasks and the circulating water was determined by a second thermocouple.

An additional improvement consisted of the use of ordinary ground-glass syringes for gas reservoirs, a change which made it possible to introduce the O_2 or CO into the thermos flasks without stopping the shaker; one syringe was filled with O_2 (or CO) and the other with N_2 , and at the appropriate instant the contents of both syringes were delivered simultaneously into the respective flasks. In this way, any extraneous heat effects due to compression of gas, water vapour *etc.* are exactly balanced. The entire process of introducing the gas only took about 10 sec.

In good experiments, the possible error in the heat determination by this method is of the order 500–1000 calories per mol O_2 combining with Hb.

EXPERIMENTAL RESULTS.

(1) *Slow and rapid heats of reaction of O_2 with haemoglobin.*

Our first concern was to ascertain whether a temperature gradient could be detected in the observation tube, or whether, as we anticipated from previous work with the optical methods, the reaction would prove to be too rapid for such a gradient to be observed. We found as expected, that at least 98 % of the total temperature rise in the observation tube was complete by the time fluid had travelled 2 cm. from the mixing chamber, *i.e.* in 0.008 sec. The process is thus too rapid for its time course to be followed by the present apparatus, nor would it be of any use to reduce the rate by lowering the $[O_2]$ in fluid *R*, for this would also make the resultant temperature rise too small to be accurately measured.

We next proceeded to see whether the observed temperature rise, which remained constant during the time required for the liquid to traverse the remainder

of the capillary part of the observation tube, would also remain constant for a further relatively long period, or whether, as seemed possible for the reasons already indicated, there would occur slow secondary processes accompanied by an absorption or evolution of heat. For this purpose the thermojunction was placed in the wide part of the observation tube and deflections were read in the following order: fluid from bottle *R* was run for 20 sec., suddenly turned off, and the galvanometer readings taken at 10 sec. intervals. Then similar drift readings were taken after *R* and *L* had been running together for 20 sec., and again after fluid from *L* alone had been running. In this way drift readings were obtained for successive 10 sec. periods after the completion of the reaction, and any secondary heat production or absorption would have shown itself by a difference between the mean rate of drift of *R* and *L* running separately and that of *B* (*R* and *L* running together). Table IV makes this procedure clearer, and Table V summarises the results of all experiments of this kind (5 in number) which were made, the figures given representing the mean apparent "delayed" temperature change (in mm. galvanometer deflection) for successive 10 sec. periods after mixing. It is clear that although the figures indicate without exception a slight absorption of heat during the first 40 sec. and a slight extra heat production during the subsequent 40 sec., the individual changes are well within experimental error and can be safely disregarded.

Table IV. *Temperature drift in stationary fluids in observation tube.*Exp. of February 5th, 1935. Hb + O₂.

Procedure before readings taken as described in text.

Time sec.	<i>R</i>		<i>B</i>		<i>L</i>		Mean drift <i>L</i> and <i>R</i> mm.	Drift of <i>B</i> - mean drift of (<i>L</i> + <i>R</i>) mm.
	Galvano- meter readings mm.	Drift in 10 sec.	Galvano- meter readings mm.	Drift in 10 sec.	Galvano- meter readings mm.	Drift in 10 sec.		
10	341.5		362.0		379.5		-0.55	
20	341.0	-0.5	361.0	-1.0	378.9	-0.6	+0.35	-0.45
30	341.5	+0.5	361.5	+0.5	379.1	+0.2	+0.35	+0.15
40	342.0	+0.5	362.0	+0.5	379.3	+0.2	+0.35	+0.15
50	342.2	+0.2	362.0	0	379.1	-0.2	0	0
60	342.8	+0.6	362.2	+0.2	379.2	+0.1	+0.35	+0.15
70	343.0	+0.2	362.2	0	379.0	-0.2	0	0
80	344.0	+1.0	363.0	0	379.3	+0.3	+0.65	+0.15
90	345.2	+0.6	364.0	+1.0	374.9	+0.6	+0.90	+0.10

N.B. Negative sign in last column indicates delayed heat absorption.

Table V. *Apparent slow temperature changes subsequent to reaction of Hb + O₂.*

Mean values from 5 experiments.

Period after mixing (sec.)	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90
Mean extra heat production, in mm. galvanometer deflection	+0.12	-0.09	-0.25	-0.42	+0.08	+0.07	+0.18	+0.12
As % total rapid heat	+3	-2.2	-6.3	-10.5	+2	+1.7	+4.5	+3

(Approx.)

Mean = -0.6% of total heat = -0.00003°.

As a final test of the correctness of these results, and a general test of the reliability of the method for absolute thermochemical measurement, the heat of reaction of dissolved O₂ and Hb deduced from the steady temperature rise found in the observation capillary was compared, in a series of experiments, with the heat of reaction of similar Hb solutions, prepared from the same sample of blood

and subjected to the same experimental treatment, with gaseous oxygen, the heat being measured by the usual slow calorimetric method. If the foregoing results were reliable, and if the methods were quantitatively satisfactory, the two methods of measurement should have given identical results. Table VI includes all the tests of this kind, which have so far been carried out.

Three of the experiments were, on technical grounds, known to be imperfect, and are therefore bracketed in Table VI. The remaining five, however, show agreement within experimental error, both at alkaline and at neutral reaction and in presence or absence of 1% boric acid.

Table VI. *Summary of slow and rapid heats of reactions of 1 mol O₂ with Hb.*

Date	p _H and condition of blood	Slow	Rapid
13 March } 22 Feb. }	1% boric blood of 20 Feb. stored at 0°	6500	7300
14 March } 15 Feb. }	1% boric blood of 18 Feb. stored at 0°	(7100	7700)
14 March } 20 Feb. }	1% boric blood of 18 Feb. stored at 0°	(10100	6300)
20 March } 22 March }	1% boric blood of 13 March stored at 0°	(7500	6300)
25 March } 26 March }	Blood of 19 March without H ₃ BO ₃ . Stored— 10°	7700	6300
27 March } 28 March }	1% H ₃ BO ₃ blood of 26 March. Stored at 0°	6000	6300
2 April	1% H ₃ BO ₃ blood of 1 April. Alkaline (p _H ca. 9.5)	7100	6800
3 April	1% H ₃ BO ₃ blood of 1 April. Neutral	4700	5100

In the rapid experiments the thermo-couple was at 5 cm. from the mixing chamber throughout. The heat values in both columns refer to the reaction of O₂ in solution with haemoglobin in solution.

The values shown in Table VI are apparently much lower than those given by Roughton [1935]. Most of the difference is due to the fact that Roughton's results by the slow method are given for the reaction O₂ (in gas phase) + Hb → O₂Hb, whilst those in Table VI are for O₂ (in solution) + Hb → O₂Hb and must therefore be lower than the former by an amount equal to the heat of solution of O₂ at 17°, viz. 2800 calories. It is possible, in addition, that the eight-fold dilution of the blood used in the present paper causes a slight lowering in the heat of reaction.

(2) *Rate of reaction of CO and haemoglobin by thermal and optical methods.*

The first experiment with CO water showed that, in agreement with the optical results of Roughton [1934] the CO + Hb → COHb reaction occurs much more slowly than the O₂ + Hb → O₂Hb reaction; at such a rate, indeed, that the detailed course of heat production could be very easily followed by observations with the differential thermocouple placed in various positions along the observation tube. The curves so obtained at first showed signs of a small absorption of heat in the upper part of the tube, but further investigation with pure water in each bottle showed that this effect was due to the blank correction already referred to in Part I (p. 2627). The curves could then be converted into % COHb-time curves by:

(i) Application of the blank corrections (*v.* Tables I and II).

(ii) Determination of the absolute temperature rise at any given point in the observation tube by means of the absolute thermocouple.

(iii) The necessary small corrections for differences in rate of flow of liquid where the position of the differential couple was varied or where the absolute couple was in the tube.

(iv) A knowledge of the total CO capacity of the haemoglobin solution.

These final curves were of the usual type, approaching asymptotically an end-point and having a half reaction time, τ , of about 0.01 sec.; the results of a typical experiment are given in Fig. 2. Six experiments in all were performed, with such consistent results that only one need be shown. The end-point in each case corresponded to a heat of reaction of about 8000 cal.

Fig. 2. Rate of $\text{CO} + \text{Hb} \rightarrow \text{COHb}$ by 2 separate methods: $L = 1.4 \text{ mM Hb}$ (borated blood 1 in 4). $R = 1.0 \text{ mM CO}$; temperature $= 20^\circ$.

Fig. 3. Rate of uptake of O_2 and CO by red blood corpuscle suspensions.

As a final check on these curves, they were compared with those obtained on the same sample of blood by the optical method, for which we have to thank Dr G. A. Millikan. Fig. 2, depicting the results of a typical thermal experiment, likewise gives the points obtained in the same material by the optical method. The agreement is excellent, so that the conclusions drawn in the case of the

O₂-Hb reaction also apply here: there is no evidence for the existence of secondary heat processes, and no reason to suppose that the heat production does not proceed precisely in step with the optical change.

(3) *Comparison of rate of reaction of O₂ and CO with (a) Hb in solution and (b) Hb in intact corpuscles.*

Whereas in the case of the reaction of O₂ with Hb in solution no temperature gradient could be detected in the observation tube, it was easy to show that with corpuscle suspensions containing the same average concentration of haemoglobin, a gradient could be consistently observed, so that the effective half-reaction time was increased from < 0.002 sec. to about 0.007 sec. In the case of the slower reaction with CO, the diffusion effect was naturally less significant but was nevertheless unmistakable, the time of half reaction being increased from 0.010 to 0.016 sec. at 20.5°. Some preliminary experiments are shown in Fig. 3.

DISCUSSION.

It is clear that the slow and rapid heats of these haemoglobin reactions are identical within the limits of experimental error. This is evidence against the existence of any secondary reactions, at any rate of marked heat content.

The hypothesis of secondary changes, following the primary combination of O₂ or CO with haemoglobin, is not supported by the results of a direct search for secondary heat effects following the primary evolution of heat: in the case of the rapid O₂ + Hb → O₂Hb reaction it is shown that the total heat is evolved within 0.008 sec. or less of mixing, and that the mixed fluids show no further temperature change over a period of 80 sec. In the case of the CO + Hb → COHb reaction, the evolution of heat followed the same course as the optical change, and thus again the search for secondary changes gave negative results. The excellent agreement between the thermal and optical data, however, suggests that results, by the thermal method, of experiments, in which optical data are either unobtainable or suspected to be inaccurate, can be accepted with confidence.

An example is provided by the preliminary experiments on suspensions of intact red blood corpuscle suspensions, where reliable optical data are difficult to obtain, on account of the optical heterogeneity of the medium and the consequent scattering of light. The experiments demonstrate quantitatively the importance of diffusion, a factor which had on theoretical grounds been shown by Roughton [1932] to be of importance in regulating the rate of uptake of dissolved gases by the red blood corpuscles. They suggest that further experiments on the same lines might provide the quantitative data necessary for a fuller knowledge of the diffusion coefficients of O₂ and CO in the contents of the corpuscles and in the membrane surrounding them. In principle it might be objected that the time relations in thermal records of reactions proceeding within the red blood corpuscles are distorted by the slowness of thermal conduction from the corpuscle to its surroundings: fortunately it is easy to show that in the times at present under consideration the error thus introduced is negligible. Assuming the corpuscle to be a plane sheet of thickness 1.4 μ and thermal conductivity = that of water = 1.4 × 10⁻³, it is found (by calculations similar to those of Roughton [1932] on diffusion of O₂ and CO within the corpuscle) that the transfer of heat from the inside of the corpuscles to the surroundings would be 98 % complete within a period of 0.0003 sec., whereas the half-reaction times in these experiments are of the order of 0.01 sec., i.e. 30 times greater.

Further scope of the method in blood reactions. The method is now being applied to the kinetics of the CO_2 reactions, which differ from those of O_2 in blood in not being accompanied by colour changes, which can conveniently be followed by optical methods. Already it has been possible to measure the heat of the separate reactions $\text{H}^+ + \text{HCO}_3^- \rightarrow \text{H}_2\text{CO}_3$, and $\text{H}_2\text{CO}_3 \rightarrow \text{H}_2\text{O} + \text{CO}_2$, the usual methods of calorimetry having hitherto only been able to give the sum of the heats of these two reactions, without any indication as to their individual values. Having obtained this basal information it is hoped now to proceed to the thermal study of the kinetics of the $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$ reaction in the red blood corpuscle, which contains large amounts of the catalyst for this reaction—carbonic anhydrase. The thermal method also appears to be the most promising one for determining the rate of carbamino-combination of CO_2 with haemoglobin—a process which has recently leapt into importance in the physiological transport of CO_2 by the blood. There is thus a large field awaiting the application of the method in blood kinetics and possibly in other biochemical problems also.

SUMMARY.

1. In Roughton's method for recording the time course of the heat effects in rapid chemical changes, the two reagents are driven by separate leads into a mixing chamber and thence into an observation tube: the temperature of the running fluid at various points of the latter is measured thermoelectrically to an accuracy of $\pm 0.001^\circ$. The present method was required, and was designed for, the study of reactions in which the total temperature change does not exceed 0.005 – 0.01° and hence necessitates, as regards the temperature measurement, a 5- to 10-fold greater precision than its predecessor. This has been obtained in part by special attention to the dimensions and lay-out of the apparatus, but in the main by immersing the whole apparatus in a water thermostat, the leads, mixing chamber and observation tube being, however, separated from the water by air jackets. These improvements have made it feasible to use a galvanometer of 7 times greater sensitivity per mm. deflection than heretofore.

2. Tests show that blank effects and/or errors of non thermochemical origin are of the order of 0.0004 – 0.0008° , and that by following exactly the technique described in the text:

(a) The "absolute" temperature difference between one junction in the running fluid and the other (the "cold" one) at a standard temperature outside can be measured to an accuracy of $\pm 0.0002^\circ$ on the average.

(b) The difference in temperature between two points in the observation tube, through which the mixed fluid is running, can be measured to an accuracy of $\pm 0.00004^\circ$ on the average. The method is thus competent to follow, to within 1–2 %, the time course of heat effects, over a period of 0.0 – 0.01 sec., in which the total temperature change does not exceed 0.01° .

3. The method has been applied to the study of the rapid thermochemistry of the reactions of haemoglobin with oxygen and carbon monoxide. It is found that:

(a) In the case of the $\text{O}_2 + \text{Hb} \rightarrow \text{O}_2\text{Hb}$ reaction, the heat liberated within 0.008 sec., does not differ within the limits of error from the total heat liberated in 10–15 min., as measured by the usual slow methods of calorimetry; nor is there any evidence of delayed heat effects during the intermediate period between these two extremes.

(b) The rate of the $\text{CO} + \text{Hb} \rightarrow \text{COHb}$ reaction, as measured by the thermal method, agrees very closely with the rate given by Millikan's photo-electric

method. These results not only confirm the validity of the rapid thermal method, but also show that the primary combination of O_2 and/or CO with haemoglobin is not followed by any secondary reactions, at any rate of appreciable heat content, and that the heat changes do keep pace exactly with the optical changes in the reaction. The thermal method can thus be used with confidence for rapid reactions in which optical methods are inapplicable or less suitable, such as the uptake of O_2 and CO by haemoglobin in intact red cell suspensions, of which some preliminary instances are given.

4. The further scope of the method, particularly in the study of the kinetics of the CO_2 reactions in whole blood, is pointed out. On the technical side the main desideratum is no longer an increase in accuracy, but rather a diminution in the volume of reagents required for each experiment, since this at present amounts to about 4 litres.

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