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ON THE OCCURRENCE OF TWO KINDS OF HÆMOGLOBIN IN NORMAL HUMAN BLOOD.

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In the fifth chapter of Barcroft's book on hæmoglobin he discusses its specificity, and suggests the possibility of more than one hæmoglobin in the same blood, the differences being located in the globin part of the molecule. The curious change in "span" (*i.e.* the distance between the bands for oxy- and CO-hæmoglobin) in the blood of the same rabbit makes Barcroft put the question, "Why and how should the globin of a rabbit's hæmoglobin alter on hæmorrhage?"

In limiting the problem to the unity and variability of hæmoglobin in one kind of blood, especially in human blood, we find that most methods of research on the specificity of hæmoglobin are not sensitive enough to detect the presence of, say, 10 p.c. of a modified hæmoglobin in 100 p.c. total. Only the estimation of the so-called alkali resistance, which gives enormous differences in blood of different species, might be sensitive enough. This paper describes the photo-electric measurement of the rate of denaturation of human hæmoglobin at *p*H about 12, as a means of differentiation between more than one kind of pigment in the same blood.

Method.

Von Krüger [1888, 1925, 1927], who first studied the rate of hæmoglobin denaturation in alkaline solutions ("Zersetzungszeit"), added 1/5 vol. *N*/4 NaOH to the solution of hæmoglobin and measured the time from this moment to the visual disappearance of the typical absorption bands. In this way he found the very large differences in "Zersetzungszeit" in the hæmoglobin of mammalian species, for instance for human blood 1 min., for rabbit's blood 30 min., for horse blood 80 min., for ox blood 24 hours. A more exact study was made by Haurowitz

[1929], who measured the rate of extinction in the region of the β bands (538–544) and also in the α region (572–580) by means of the spectrophotometer.

For production of the alkaline reaction he used the same process as von Krüger, viz. the addition of 1/5 vol. $N/4$ NaOH.

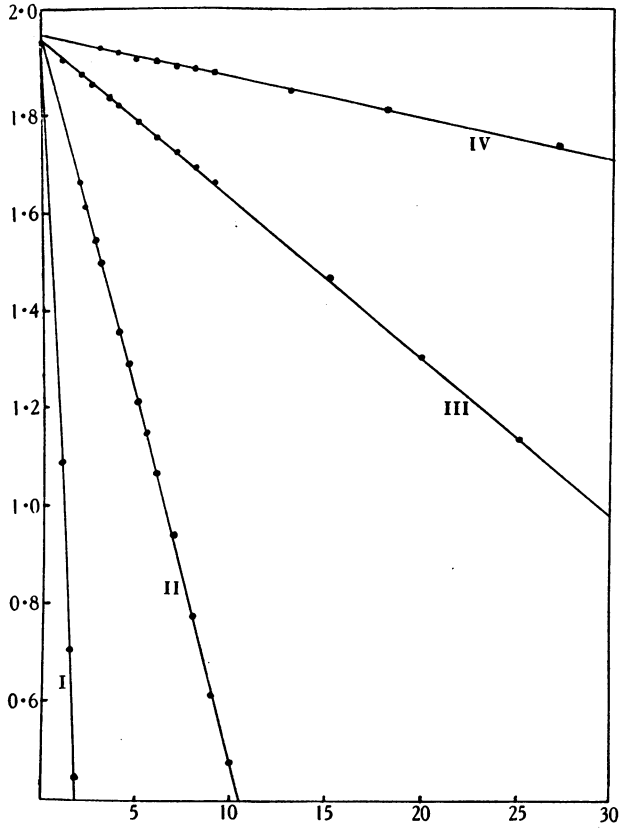

Fig. 1. Effect of *p*H on rate of alkali denaturation at 19·6° C. Vertical axis: log percentage unchanged hæmoglobin in total mixture; horizontal axis: time in minutes. (I) *p*H 12·12; (II) *p*H 11·89; (III) *p*H 11·66; (IV) 11·55.

The sensitivity of this method is very much dependent on the investigator's experience, and on the time which may be taken for each determination. In our opinion it will be difficult to estimate a decrease in extinction, smaller than that corresponding to a 2 p.c. change of oxyhæmoglobin to globin hæmochromogen. For our purpose we have tried to combine a good sensitivity with the exclusion of the subjective

factor, by using a photo-electric arrangement, and by keeping the conditions which govern the speed of reaction sufficiently constant.

The two factors on which the rate of alkali denaturation of a certain type of hæmoglobin is dependent are (*a*) the concentration of OH ions, and (*b*) the temperature.

Fig. 1 shows the influence of *p*H on the rate of denaturation at constant temperature. The hæmoglobin was obtained from a human blood, which did not contain more than 5 p.c. of the resistant form of hæmoglobin (to be described later). The ratio's log conc. HbO_2 : time are straight lines for about 80 p.c. completion of the reaction.

The solution of oxyhæmoglobin was made, by diluting oxalate blood with 0·15*N* Na_2HPO_4 solution, containing 1 p.c. of saponin, the final concentration of hæmoglobin being 1·36 p.c. This solution was centrifuged till it was quite clear and put into the absorption vessel of 1 cm. transverse diameter. The alkali denaturation was started by injection of 1 c.c. NaOH of various concentrations, by means of a precision syringe.

A special set of experiments showed that the described procedure enabled us to place the oxyhæmoglobin quickly in a medium of known and sufficiently constant *p*H; *p*H in each experiment was determined by the bubbling hydrogen electrode. Great care was taken to keep change of temperature in the reaction vessel within 0·1° C.

The temperature coefficient of protein denaturation being in many cases very large [Pauli, 1933], it is not surprising that a slight change in temperature has a definite effect on the rate of formation of globin hæmochromogen. In our experiments the temperature did not change over more than 0·1° C. in each measurement, although differences of temperature between separate experiments may be larger.

THE COLORIMETRIC ESTIMATION OF THE RATE OF CHANGE OF OXY-HÆMOGLOBIN TO ALKALINE GLOBIN HÆMOCHROMOGEN.

We used two methods, a bicolorimetric estimation without standard solutions and a photo-electric determination with calibration by means of known mixtures of HbO_2 and globin hæmochromogen.

The second method being the more exact one, it was solely used later on, although the bicolorimetric procedure too can give the rate of denaturation with sufficient sensitivity and may be briefly described for the use of those who wish to make analogous measurements.

Human, hæmolysed blood, containing 2·78 mg. Hb per c.c. was saturated with pure CO, made very clear by centrifugalization and divided in 3 × 5 c.c. in test-tubes *A*, *B* and *C*. Carboxyhæmoglobin is used, because its change on alkaline denaturation gives the most pronounced change in colour; the same observation has been made for the bicolorimetric determination of methæmoglobin [Clark, 1933]. To *A* is given 5 c.c. buffer solution so that the reaction is fixed at *p*H 12·00 at 18° C.; the tube is then placed at 37° C. for 15 min.,

the oxyhæmoglobin is thereby completely converted into the darker denaturation product. After addition of 5 c.c. distilled water to *B*, 5 c.c. of *A* and of *B* are filled in the lower and upper right cups of the colorimeter. *C* is placed in a 25° C. bath, together with a tube, containing 5 c.c. of the alkaline buffer solution. The contents of these two tubes are then quickly mixed and poured into the left colorimeter cup, fixed at 4 cm. distance and having a constant temperature circulating water jacket. From this moment the stopwatch is started, and colours are matched by making suitable optical mixtures of *A* and *B*, the total depths of liquid at both sides remaining 4 cm.

The best light for sufficient sensitivity was obtained by illuminating a piece of paper, brilliantly painted in the complementary colour of carboxyhæmoglobin, by a powerful Liesegang globoscope and sending the reflected light directly through the colorimeter cup; the light was turned off between measurements. The bicolorimeter readings are directly proportional to the amount of oxyhæmoglobin in total pigment; the method will indicate a change of about 3 p.c. of conversion. The results obtained were similar to those given by the photo-electric method which will now be described.

A method, suitable for our purpose, must be capable of distinguishing quantitatively between, say, a mixture of 95 p.c. globin hæmochromogen and 5 p.c. oxyhæmoglobin and a mixture of 94·5 and 5·5 p.c. respectively. We were not able to obtain a suitable monochromatic light of sufficient strength, but use a $CuSO_4$ filter, which cuts off the extreme red, which is not absorbed by globin hæmochromogen and to which the selenium cell is rather sensitive.

A 1000 c.p. Pointolite lamp is connected in series with an extra resistance and galvanometer; during the measurement an assistant regulates the current continuously at exactly 3 amp., which ensures sufficient steadiness of illumination and discards with the necessity of a differential method. An 8·0 lens concentrates the light on the surface of a selenium-oxyde "Sperrschicht" photo-electric cell, after it has passed through a $CuSO_4$ filter and the hæmoglobin solution (20 mm. of a 1·2 p.c. $CuSO_4$ solution). The selenium cell is connected in series with an E.M.F. of 300 mv. and a 10,000 ohm fixed resistance; the drop in potential over this resistance is measured by a Cambridge electrostatic valve potentiometer.

The sensitivity of this arrangement is seen in Fig. 2; for our purpose the most important change is that from 90 p.c. globin hæmochromogen and 10 p.c. oxyhæmoglobin to 100 p.c. of globin hæmochromogen. This gives for a 1·0 p.c. solution of human oxyhæmoglobin a drop of 28 mv., with an accuracy of at least 1 mv.; for the change from 80 to 100 p.c. globin hæmochromogen the drop is 61 mv.

If the concentration of hæmoglobin is doubled, the drop for the last 10 p.c. of the native pigment is only 20 mv., because the total absorption of light is too large. For this reason the best way is always to start with the same concentration of oxyhæmoglobin, and to use the calibration

curve for this concentration. If the percentage of oxyhæmoglobin differs more than 10 p.c. from this, a corresponding, separate calibration curve is wanted. One experiment will now be described.

3 c.c. oxalate blood from the finger, containing 16·8 g. of hæmoglobin per 100 c.c. were delivered in a 50 c.c. volumetric flask, containing 0·15*N* Na_2HPO_4 and 1 p.c. of saponin, so that the total volume was 50 c.c. After 5 min. this solution was put into a very efficient small centrifuge, so that a very clear liquid was obtained. The 1000 c.p. Pointolite lamp had already been alight for 10 min. at 3·0 amp.; the drop in potential over the 10,000 ohm fixed resistance was 835 mv. with an accuracy of 0·5 mv. when the absorption cuvette was filled with water; the same drop with darkened selenium cell was 117 mv.

10 c.c. of the prepared solution were delivered in the 10 mm. absorption cuvette; the E.M.F. over 10,000 ohm was then 710 mv. Temperature was regulated by circulation of water around the circular, metal side-wall of the absorption cuvette; in this experiment the temperature in the cuvette during the reaction was $18 \pm 0{\cdot}04°$ C. 1 c.c. of 0·9*N* NaOH solution was injected into the filled cuvette by a calibrated tuberculin syringe, and well mixed by repeated suction and pressure. The stopwatch was started at this moment, and the decreasing E.M.F. over 10,000 ohm was determined every 30 sec. The potentiometer used was the new Cambridge electrometer valve potentiometer. Afterwards the reaction of the solution in the cuvette was determined by the bubbling hydrogen electrode at pH = 11·86.

Calibration.

After completion of denaturation in the cuvette the reaction was adjusted to about pH 8 by addition of 0·5 c.c. normal acetic acid. This may cause a precipitation of the denatured hæmoglobin, which re-dissolves at once when it is well mixed. A too gradual neutralization, for instance by CO_2 bubbles, may cause a true partial reversion of denaturation [Anson and Mirsky, 1931]. If one accounts for the dilution, caused by addition of 0·5 c.c. of acetic acid to 11 c.c. of globin hæmochromogen, the absorption in the cuvette has not changed by the partial neutralization.

Mixtures of the denaturated solution at pH 8 with the original solution of oxyhæmoglobin (diluted accordingly from 10 to 11·5) are now prepared by adjusting the amount of denatured hæmoglobin at pH 8 to a volume of exactly 10 c.c., and by exchanging 1 c.c. of it for 1 c.c. oxyhæmoglobin solution, and so on. The calibration curve, giving the relation of the percentage of unchanged oxyhæmoglobin in total pigment to drop in E.M.F.

over the 10,000 ohm resistance is seen in Fig. 2. The calculation of the rate of denaturation in percentages of unchanged oxyhæmoglobin from the observed drop in millivolts is made by this corresponding calibration curve.

Results.

The constant result of the examination of human oxyhæmoglobin of normal persons, aged 7–50 years, is the fact that the reaction first proceeds rapidly, then more slowly. If we make the assumption that there

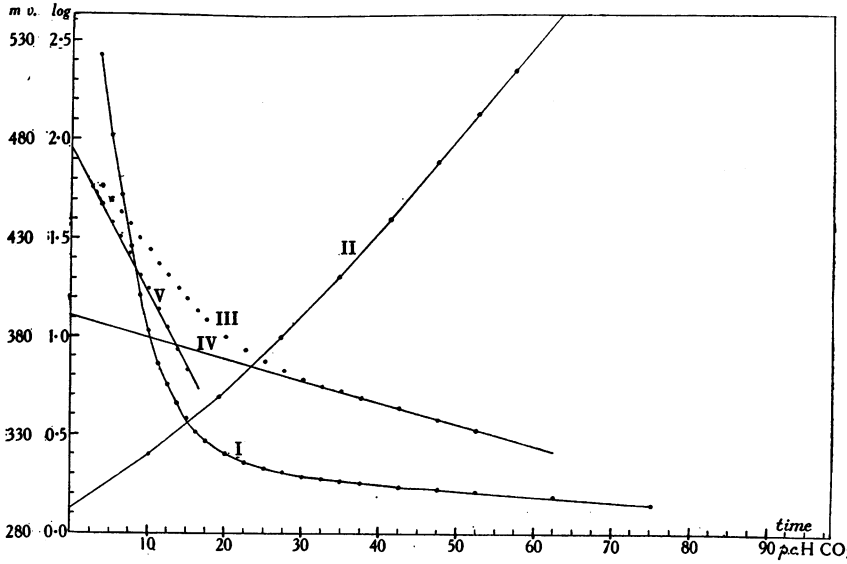

Fig. 2. Rate of alkali denaturation of the hæmoglobin of R. B., in 1·0 p.c. solution. Description in text, p. 384. I. Observed drop in millivolts. II. Calibration curve. III. Log percentage of unchanged C_1+C_2. IV. Extrapolation of C_2. V. Log C_1. Vertical axis, millivolts and log percentage unchanged hæmoglobin. Horizontal axis, time in minutes and percentage unchanged hæmoglobin (for II).

is no appreciable change in *p*H value, or other secondary reactions affecting the velocity constant during the course of the main reaction, the diminution in the reaction velocity may be attributed to the existence of a resistant form of hæmoglobin. Control experiments show that the change in *p*H is negligible. In an experiment similar to the one described, the *p*H was determined every minute by the bubbling hydrogen electrode; 1 min. after the addition of 0·9 *N* NaOH 1 c.c. *p*H was 11·83, 1 min. later 11·82, 2 min. later 11·82, 4 min. later 11·81, 6 min. later 11·80, 10 min. later 11·79, 15 min. later 11·79, 20 min. later 11·79.

TABLE I.

t min.	mv.	Percentage unchanged C_1+C_2	Log unchanged C_1+C_2	Extrapol. log C_2	Extrapol. C_2	By subtraction C_1	Log C_1	Calibration: Percentage unchanged hæmoglobin	Calibration: mv.
0	710·0	100·0	2·000	1·108	12·80	87·20	1·941	0	292
1·0	562·0	68·7	1·836	1·080	12·00	56·70	1·754	10·0	320
1·5	522·0	58·6	1·768	1·068	11·70	46·90	1·671	19·0	349
2·0	482·0	49·3	1·693	1·052	11·30	38·00	1·580	27·1	380
2·5	452·0	43·1	1·634	1·040	10·96	32·14	1·507	34·4	411
3·0	426·0	37·6	1·575	1·027	10·64	27·00	1·431	40·9	441
3·5	401·0	32·0	1·505	1·012	10·52	21·50	1·312	46·9	470
4·0	383·0	27·8	1·444	1·000	10·00	17·80	1·250	52·2	494
4·5	366·0	23·4	1·369	0·988	9·73	13·67	1·137	57·0	516
5·0	355·0	20·5	1·312	0·972	9·38	11·12	1·046		
5·5	345·0	17·7	1·248	0·960	9·12	8·58	0·934		
6·0	338·0	15·6	1·193	0·947	8·85	6·75	0·830		
6·5	331·0	13·5	1·130	0·932	8·55	4·95	0·695		
7·0	327·0	12·2	1·086	0·920	8·32	3·88	0·589		
8·0	320·0	10·0	1·000	0·891	7·78	2·22			
9·0	316·0	8·6	0·934	0·862	7·28	1·32			
10·0	313·0	7·55	0·878	0·838	6·89				
11·0	311·0	6·8	0·832	0·810					
12·0	309·0	6·1	0·785	0·785					
13·0	307·5	5·7	0·756						
14·0	306·5	5·35	0·728						
15·0	305·5	5·0	0·699						
17·0	304·0	4·4	0·644						
19·0	302·5	3·85	0·586						
21·0	301·2	3·35	0·525						
25·0	299·0	2·3	0·362						
30·0	296·0								
40·0	292·5								
50·0	292·0								

In Table I and Fig. 2 the result of the experiment described is analysed from this point of view. Calculations are made on the assumption that the hæmoglobin was composed of two kinds, with different rates of denaturation, the concentration of the type with the largest resistance against alkali denaturation being C_2, and of the other form C_1. Experimentally found is the amount of C_1+C_2 which is not denatured after a given time; the logarithms of these concentrations, plotted against time, show that from 12 min. after the beginning of the reaction the points fall on a straight line. This is interpreted as the monomolecular denaturation reaction of C_2, and extrapolation of log C_2 to $t=0$ gives 12·8 p.c. as the initial concentration of C_2. Subtraction of the extrapolated corresponding values of C_2 from C_1+C_2 gives the true rate of denaturation of C_1, which also seems to proceed as a monomolecular reaction.

In Fig. 3 a number of determinations on the hæmoglobin of R. B. are collected; *p*H and temperature during each experiment were constant but varied slightly in the separate examinations, as it is recorded in the legend. The denaturation velocity of C_1 is not designed here, but it was represented by a straight line in each case. Our interpretation is that the alkali denaturation of human oxyhæmoglobin is going on as two monomolecular reactions with different velocity constants. The values of these constants can of course be calculated, but the main object of this paper is the demonstration of the existence of two kinds of hæmoglobin in adult human blood. It is seen, that in the blood of R. B. the log of the concentration of the resistant form is found between 0·93 and 1·3, giving the amounts of resistant hæmoglobin as 8·5 to 20 p.c. of the total amount.

In the blood of other persons the results were quite similar, with a tendency in children to show higher values for the resistant form. The examination of the blood of the umbilical vein in new-born children is described by lines A and B in Fig. 3. In confirmation of former researches [von Krüger, 1927; Bischof, 1926; Haurowitz, 1929] the amount of resistant hæmoglobin in neonati is about 80 p.c. (similar determination in six cases).

Hæmoglobin of normal rabbit's blood, and the blood of horse, pig and ox, examined in the same way, but at *p*H 12·50–*p*H 13 has always given one straight line, indicating the presence of only one kind of hæmoglobin. How this may be altered in experimental anæmia is a subject under research now.

Checks have been made with known mixtures of hæmoglobin of adults and of new-born children. A typical example is given in Fig. 4. Here,

line I gives the rate of denaturation of adult hæmoglobin (pH 12·20, t 17·7° C.), line II the rate of denaturation of new-born's hæmoglobin at

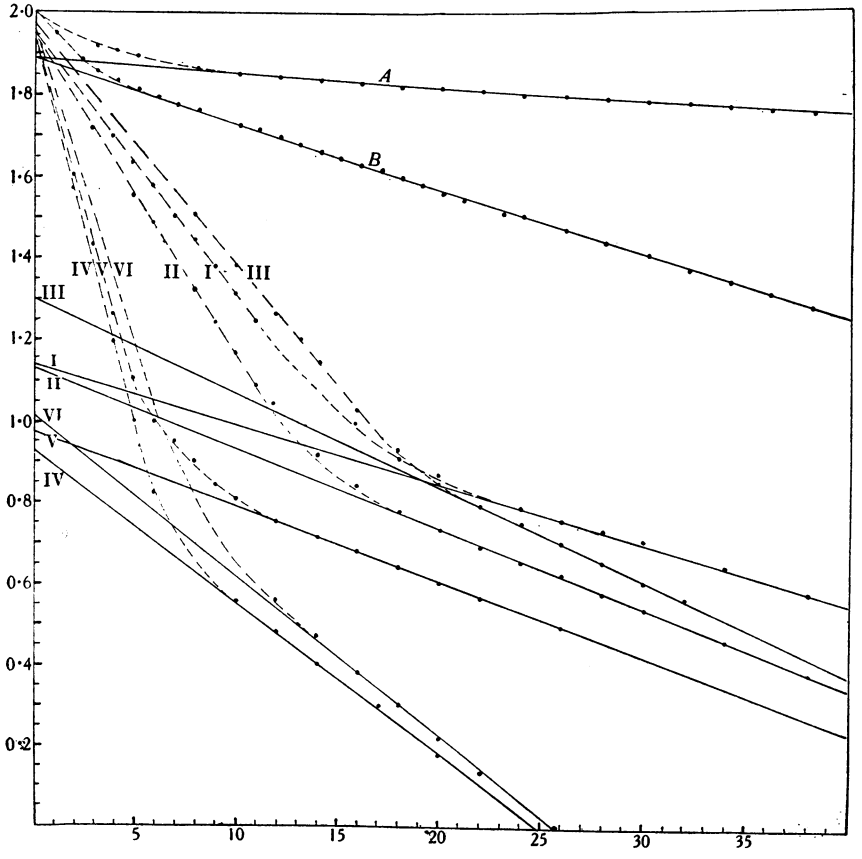

Fig. 3. Rate of alkali denaturation of the hæmoglobin of R. B., in 1·0 p.c. solution:

I. Sept. 4, 1933. pH = 11·80, t = 18·2° C.
II. „ 5, „ pH = 11·80, t = 18·0° C.
III. „ 10, „ pH = 11·80, t = 17·2° C.
IV. „ 14, „ pH = 11·86, t = 18·6° C.
V. „ 21, „ pH = 11·84, t = 18·5° C.
VI. „ 28, „ pH = 11·84, t = 18·8° C.

A. Hæmoglobin in 1·0 p.c. solution of new-born child pH = 12·00, t = 19° C.
B. Hæmoglobin in 1·0 p.c. solution of new-born child pH = 13·00, t = 19° C.

Vertical axis, log percentage unchanged hæmoglobin; horizontal axis, time in minutes.

the same pH and temperature, and line III the denaturation velocity of a mixture of 80 p.c. of I and 20 p.c. of II. It is seen that I indicates the presence of 7·1 p.c. of the resistant type, II gives 93 p.c. of this kind of

hæmoglobin and from III 19·2 p.c. is found; calculation from I and II gives 18·6 p.c. Further, it is seen that one cannot say that the resistant type of adult human hæmoglobin and the new-born's human hæmoglobin are identical. It must be the object of a further study to compare the velocity constants of denaturation of various human hæmoglobins of normal and pathological cases more carefully. In this paper the demonstration of two kinds of hæmoglobin in human blood was intended.

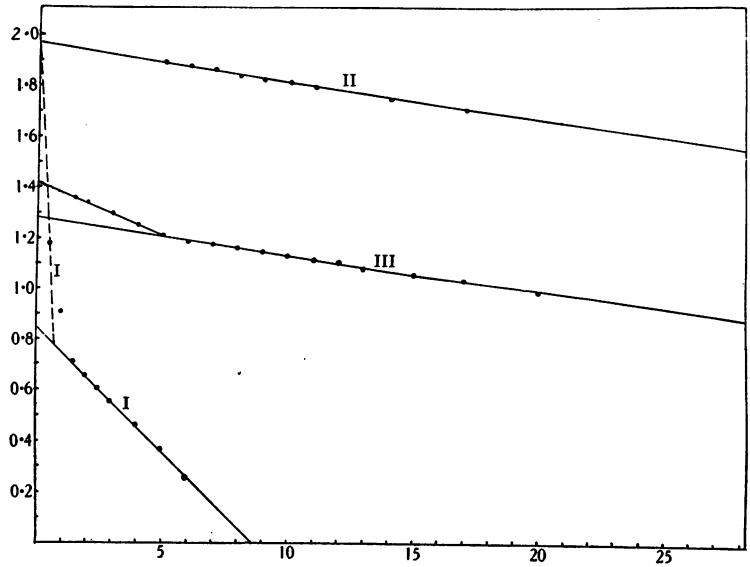

Fig. 4. Denaturation rates of adult hæmoglobin (I), new-born's hæmoglobin (II), and of a mixture of 80 p.c. I and 20 p.c. II. Description in text (p. 385). Vertical axis, log percentage unchanged hæmoglobin in total mixture; horizontal axis, time in minutes.

Discussion.

It appears to be generally accepted that, if an eventual difference in various hæmoglobins is present, this specificity must be located in the globin part of the molecule. Evidence for this supposition is growing continuously; apart from the physico-chemical methods of difference in absorption spectra, of crystal form, of solubility and of differences in isoelectric zone and rate of alkaline denaturation, we have the direct chemical analyses of globin. The researches of Schenck [1930] demonstrate a constant difference in the amount of certain amino-acids in the globins of various species. In human blood, especially, the arginine content of globin is constantly higher in the blood of new-born children than

in adult blood. In hypochrome cases of anæmia with normal regeneration this juvenile type seems also to be present; in pernicious anæmia it is missing.

Schenck also shows that the chemical differences of human globins correspond to their different resistances to hydrolysis by pepsin HCl.

Unsolved is the question whether human hæmoglobin is changing from one form to the other, or whether there are two, originally different forms (*e.g.* the resistant "fœtal" type and the adult form). The sharp distinction which can be found in the rates of denaturation suggests the existence of two independent kinds, not connected by transitional types.

Summary.

The rate of alkali denaturation of hæmoglobin, studied by means of a sensitive photo-electric arrangement, was determined in human hæmoglobin. It is shown that, in normal blood, alkaline denaturation is proceeding as two monomolecular reactions with greatly differing velocity constants. This indicates the presence of two kinds of hæmoglobin in human blood.

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