CXXIII. ON THE FACTORS INFLUENCING THE BLOOD-BENZIDINE REACTION AS APPLIED TO THE MICRO-DETERMINATION OF HAEMOGLOBIN.

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WE have been surprised to observe that, notwithstanding the increasing need for an accurate method of determining minute amounts of haemoglobin, the method recommended by Wu [1923] has not been more generally adopted. This author developed from the benzidine test for blood a quantitative "ultra-micro-method" by means of which the haemoglobin content of 0.0001 cc. of blood could be accurately determined.

The accuracy of the method proposed by Wu has recently been questioned by Bing and Baker [1931], who have proposed a modified procedure which, they claim, eliminates certain errors in the original method. We have recorded in this paper a critical study of the blood-benzidine reaction, particularly as affected by hydrogen peroxide concentration and temperature. Other observations having a direct bearing on the quantitative application of the reaction are discussed.

EXPERIMENTAL.

The chemical reactivity of benzidine, particularly so far as oxidation is concerned, depends on its purity and on the method of preparation. To interpret our experimental results it was essential that benzidine of known purity should be employed.

Purification of benzidine. To 100 g. finely powdered commercial benzidine in a two-litre flask were added 900 cc. boiling water and 100 cc. conc. hydrochloric acid. After boiling a short time the hot solution was filtered, using suction. The filtrate was again heated to boiling and 230 cc. conc. hydrochloric acid, in 30–50 cc. portions, were added, shaking after each addition until the grey precipitate dissolved. A permanent precipitate formed when about 150 to 175 cc. had been added. After filtering (suction) the highly coloured filtrate was discarded and the precipitate transferred to a two-litre flask. One litre of boiling water and a tablespoonful of blood-charcoal (acid-extracted) were added, and after boiling about 10 minutes the hot solution was again filtered (suction). This procedure was repeated until the filtrate was colourless. To the hot filtrate 230 cc. conc. hydrochloric acid in 30 to 50 cc. portions were added while shaking, and after cooling to 20° again filtered.

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To obtain pure benzidine, 100 g. of the hydrochloride were suspended in 600 cc. acetone, 100 cc. conc. ammonium hydroxide and 150 cc. of water. After the benzidine was completely dissolved, the solution was diluted to 5 litres with water and filtered after standing 20 minutes. The benzidine was washed free from chloride with water, and finally dried over sulphuric acid in a vacuum desiccator. The benzidine thus prepared was found to be extremely insoluble in glacial acetic acid. This difficulty was overcome by recrystallising from hot 95 % ethyl alcohol.

The benzidine solution used in all our experiments was prepared by dissolving 2 g. of the pure benzidine in 20 cc. of glacial acetic acid with the aid of heat, and, after cooling, diluting to 100 cc.

Hydrogen peroxide. All solutions of hydrogen peroxide were prepared by suitable dilution of "Superoxol." The hydrogen peroxide contents of these solutions were carefully checked by permanganate titration, using pure anhydrous sodium oxalate as the primary standard. It was not considered advisable, at least so far as our experiments were concerned, to use the commercial 3 % H_2O_2 solution, which is generally stabilised by acetanilide or by small amounts of sulphuric acid. Hydrogen peroxide forms compounds with sulphuric acid in which the peroxide is less easily decomposable than when alone.

Standard blood solution. The haemoglobin content of a sample of sheep's blood was estimated from its oxygen capacity, as determined by Sendroy's [1931] modification of the Van Slyke and Neill [1924] procedure. This blood was diluted to contain 0.05 mg. haemoglobin in 1 cc. Boric acid was used as the preservative.

General experimental conditions. Throughout the experiments, special precautions were taken in regard to the cleanliness of all glassware [see Harrison, 1930]. In carrying out the benzidine reaction the reagents were always added in the order recommended by Wu. To 2 cc. of a 2 % benzidine solution is added 1 cc. diluted blood. After carefully mixing, 1 cc. of a hydrogen peroxide solution is added. After a definite reaction time, the solution is diluted with 21 cc. 20 % acetic acid, and the intensity of the reddish-brown colour measured in a colorimeter of the Duboseq type.

The effect of the concentration of hydrogen peroxide on the benzidine reaction and on the stability of the red colour.

The essential feature of the modified procedure recommended by Bing and Baker is the substitution of a much more dilute (0.6 %) H_2O_2 solution for the 3 % H_2O_2 solution used by Wu. This permits an extension of the reaction time of 15 minutes, as specified by Wu, to one of 1 hour or longer, which is an undoubted convenience when many determinations are being made. It is particularly unfortunate that the experimental data justifying the use of more dilute H_2O_2 are not given.

Our results on the effect of hydrogen peroxide concentration on the development and on the stability of the red colour are given in Table I. The

Table I. *The effect of H_2O_2 concentrations on the benzidine reaction.*

Reaction time, 1 hour. Temperature, 20–22°.				
Solution No.	Percentage concentration of H_2O_2	Reading of the standard (No. 4) in mm. with the other test solutions set at 10 mm.		
		After 15 minutes	After 22 hours	
1	4.0	11.8	9.8	
2	3.0	11.1	9.8	
3	2.0	9.9	10.3	
4	1.5	10.0	10.0	
5	1.0	8.4	10.3	
6	0.75	7.6	10.2	
7	0.50	6.3	8.8	
8	0.25	5.1	7.9	

data were obtained as follows. The benzidine reaction was carried out with 1 cc. of hydrogen peroxide solutions of varying strength. The reaction was allowed to proceed exactly 1 hour (in all cases) when the solutions were diluted with 21 cc. 20 % acetic acid. After 15 minutes the comparative depth of colour was measured, with solution No. 4 (1.5 % H_2O_2) as the arbitrary standard. The test solutions were always set at 10 mm. and the standard adjusted to match. In this way the numerical value of the readings is shown as increasing with increasing intensity of colour. Using a new standard prepared exactly as solution No. 4, the relative stability of the colours was determined after 22 hours. The two standards when read against each other had practically the same depth of colour.

We can conclude that, as the concentration of hydrogen peroxide is increased from 0.25 % to 4 %, the intensity of the red colour also continues to increase. The maximum intensity of the colour consistent with the greatest stability was obtained with 1.5 % or 2 % of H_2O_2 . 0.75 % H_2O_2 gave only 76 % of the colour given by 1.5 % H_2O_2 .

Table II. *Effect of H_2O_2 concentration on the development and stability of the red colour.*

Solution No.	Percentage concentration H_2O_2	Reaction time, 1 hour. Temperature, 20–22°.			
		Reading of standard in mm. with other test solutions set at 10 mm.			
		*15 minutes	2 hours	3 hours	3 hours
1	10	11.8	10.5	8.8	7.9
2	8	12.0	11.2	9.7	8.7
3	6	11.6	10.2	9.4	8.5
4	4	11.2	11.0	10.5	10.0
5	2	10.3	10.2	9.9	9.9
6	1.5	10.0	10.3	10.2	10.2
7	1.0	8.4	8.9	9.8	10.2
8	0.5	6.8	7.4	8.4	8.9

Tube No. 6 (1.5 % H_2O_2) was used as standard in the first series of readings, i.e. after 15 minutes. A fresh standard prepared exactly as No. 6 was used for each series of readings.

* Time after dilution with 20 % acetic acid.

In Table II we have recorded the results obtained when a wider range of H_2O_2 concentrations was employed. The effect of the peroxide concentration on the stability of the red colour was determined hourly up to 4 hours. The same experimental conditions prevailed as those described above. Again 1.5 % H_2O_2 was the lowest concentration which gave the maximum depth of colour, stable for at least 4 hours. Hydrogen peroxide concentrations of 4 % or greater gave more colour, but the colour was less stable, the instability increasing with increasing H_2O_2 concentration. The intensity of the colour given by 1.0 % and 0.5 % H_2O_2 , which is initially much less than that given by 1.5 % H_2O_2 , slowly increased when the solutions were allowed to stand 4 hours.

The results of a further experiment similar to those already described, but in which the reaction time was varied, are given in Table III. By "reaction

Table III. *Effect of varying concentrations of H_2O_2 on the development and stability of the red colour.*

Reaction time varying, room temperatures prevailing.
Reading of standard in mm. with other test solutions set at 10 mm.

Solution No.	Reaction time	3 % H_2O_2				1.5 % H_2O_2				0.5 % H_2O_2	
		15 mins.	2 hrs.	3 hrs.	15 hrs.	15 mins.	2 hrs.	3 hrs.	15 hrs.	15 mins.	15 hrs.
1	2 hrs.	cloudy	—	—	—	13.1	12.7	12.8	11.9	9.5	10.6
2	1.5 „	cloudy	—	—	—	11.2	10.9	11.0	9.8	8.7	10.1
3	1.0 hr.	12.6	12.0	11.6	10.3	10.0	10.3	10.3	9.8	8.1	10.0
4	45 mins.	11.3	11.0	11.1	9.3	10.1	10.0	10.2	10.0	7.6	9.3
5	30 „	10.8	10.7	10.9	9.5	9.6	10.1	10.3	9.8	6.7	9.0
6	15 „	10.2	10.0	10.0	8.4	8.1	8.9	9.1	9.0	5.2	8.8

Note. A freshly prepared standard was used for each series of readings after 15 minutes, 2 hours, etc. The preparation of the standard was always the same, i.e. using 1.5 % H_2O_2 , reaction time, 1 hour, and allowing the solution to stand 15 minutes after dilution with 20 % acetic acid.

time" is meant the length of time the benzidine reaction is allowed to proceed before dilution to 25 cc. with 20 % acetic acid. The conclusion may be drawn from these results that 1.5 % H_2O_2 and a reaction time of 45–60 minutes give the maximum depth of colour, the intensity of which undergoes little or no change on standing 15 hours. The stability of the colour with 3 % H_2O_2 when the reaction time is 15–45 minutes is noteworthy.

Consideration of the data obtained from these experiments suggests that the benzidine reaction may be carried out with the greatest sensitivity, accuracy and convenience, when 1.5 % H_2O_2 with a reaction time of 1 hour is employed.

The effect of temperature on the colour development.

To study the effect of temperature, the following procedure was adopted. The benzidine reactions were carried out in 25 cc. glass-stoppered measuring cylinders. These were placed in a constant temperature water-bath. From micro-burettes were added 2 cc. 2 % benzidine solution and 1 cc. diluted blood (0.05 mg. haemoglobin) and the solutions thoroughly mixed. They were then allowed to stand until the reagents came to the temperature of the water-bath (about 3 minutes). 1 cc. of 1.5 % H_2O_2 was then added. At the end of the particular reaction time the cylinder was removed from the water-bath and 21 cc. of cold (about 2°) 20 % acetic acid were quickly added. The colorimeter readings were made 15 minutes later.

The benzidine reaction was also carried out in the same manner at 2.2° (refrigerator room).

The temperature at which the benzidine reaction is performed has evidently a marked effect upon the rate of the "initial" stage of the reaction through the blue colour to the red (Table IV). When the reaction was carried out at 2.2° the solution was still deep blue in colour after a reaction time of 1 hour. At 50° a reaction time of 2.5 minutes gave the same depth of colour as that obtained in 15 minutes at room temperatures. It appears that after the red

Table IV. *Effect of temperature on the benzidine reaction.*

Reaction time minutes	Reading of standard with other test solutions set at 10 mm.				
	20-22°	30°	40°	50°	60°
2.5	*—	—	—	7.0	—
3	—	—	—	7.6	—
5	—	5.0	7.0	8.0	7.2
10	—	6.7	—	8.2	7.4
15	7.1	7.1	8.2	8.6	7.5
20	8.3	7.8	—	8.6	—
25	8.8	8.5	—	9.4	—
30	9.2	8.5	9.2	9.4	7.5
45	10.2	9.5	9.7	9.6	Cloudy
60	10.0	10.0	9.9	9.2	—

Note. These experiments were completed on the same day, so that the same standard, using 1.5 % H_2O_2 and a 1-hour reaction time at room temperatures, were used for all readings. 1.5 % H_2O_2 was used in all the tests.

* The reaction had not proceeded to the red colour. The solution after dilution with 20 % acetic acid had a greenish-red colour.

colour has developed variations in temperature from 20° to at least 40° have very little effect on the rate at which this colour reaches its maximum intensity. The depth of colour developed after 60 minutes, using 1.5 % H_2O_2 , is the same whether the reaction is carried out at 20° or at 40°.

The results of a further experiment in which the reaction was carried out with three different strengths of hydrogen peroxide, and in which the temperature was carefully controlled, are shown graphically in Fig. 1. When the

Fig. 1. Showing the effect of varying concentrations of H_2O_2 on the rate of substrate (benzidine) oxidation.

* Solutions became cloudy.

temperature is constant at 30°, increasing the concentration of H_2O_2 not only accelerates the initial stage of the reaction, but the time required for the red colour to reach an intensity equal to that of the standard is markedly shortened.

The effect of light on the colour development.

When the benzidine reaction, using 1.5 % H_2O_2 , was carried out in the dark and in the light we found no difference in the depth of colour developed after a reaction time of 1 hour.

A few experiments were carried out to determine whether the rate of the reaction was influenced by radiations in the ultra-violet. It was only possible to draw very general conclusions from these results, because of the difficulty in controlling other experimental conditions. Bernheim and Dixon [1928] have found in studying the effect of light on the reduction of methylene blue by the xanthine oxidase system with oxygen present, that a peroxide is formed during the irradiation. There was no doubt, however, that exposure of the reaction mixture to the radiations of a quartz mercury vapour lamp, set 12" directly above the solution, greatly increased the rate of the reaction. An exposure of 6 to 7 minutes gave about the same depth of colour as ordinarily develops in one hour in daylight. A cloudiness developed in the reaction mixture if irradiated for more than 7 minutes.

The effect of varying haemoglobin concentrations on the intensity of the colour.

Varying amounts from 2 cc. to 20 cc. of a dilute blood solution containing 13.5 mg. of haemoglobin in 100 cc. were measured into 25 cc. volumetric flasks. After diluting to volume, the benzidine reaction (2 cc. benzidine solution; 1 cc. 1.5 % H_2O_2 ; room temperature and reaction time 1 hour) was carried out with 1 cc. aliquots of the different blood dilutions. 21 cc. of 20 % acetic acid were then added and the solution allowed to stand 10 minutes before making the colorimeter readings. The range of haemoglobin concentration was from 0.01mg. to 0.10 mg. per cc. The fifth dilution (10 cc. of diluted blood in 25 cc.) was taken as the arbitrary standard.

In Fig. 2 are plotted the colorimeter readings of this standard when ad-

Fig. 2. Showing the relationship between the haemoglobin concentration and the intensity of the red colour.

justed to match each of the other test dilutions which were set at 10 mm. The depth of colour is directly proportional to the amount of haemoglobin present, provided the standard selected is used over a limited range of haemoglobin concentrations.

The determination of haemoglobin in small amounts of blood.

For some considerable time we have used Wu's benzidine method for the routine estimation of haemoglobin in minute amounts of rat-blood. As a result of the experimental findings already discussed, certain changes have been made in the original procedure. These modifications constitute an improvement only so far as technique is concerned.

The accuracy with which the haemoglobin content of rat-blood can be repeatedly determined is limited primarily by the error incurred in measuring small samples of blood from a free-flowing drop taken from the tail. We have used a red cell counting pipette which was accurately calibrated and found to deliver 0.00245 cc. blood from the 0.05 mark. The accuracy with which samples of blood could be procured by means of this pipette was determined as follows.

The tail was wiped clean (if the rat was anaemic the tail was immersed for a time in warm water) and the end clipped off. The sample was pipetted from the second or third drop of blood, and transferred to a 10 cc. volumetric flask containing a small amount of water with which to flush the pipette. The blood solution was diluted to 10 cc., thoroughly mixed, and the benzidine reaction carried out in the same manner as in conducting the proportionality tests. A number of samples were taken from the same and from different rats. The final colorimeter readings were all made against the same standard (0.028 mg. haemoglobin) which was adjusted to match the test solution set at 10 mm.

In all the experiments described, specially purified benzidine has been used. Quantitative determinations of equal accuracy may be made, using Merck's benzidine "suitable for detecting blood."

	Sample of blood	Colorimeter reading		Sample of blood	Colorimeter reading
Rat No. 1	1	12.0	Rat No. 2	1	9.8
	2	11.5		2	9.4
	3	11.5		3	9.4
	4	11.7		4	10.0
Rat No. 3	1	9.8	Rat No. 4	1	13.0
	2	9.8		2	12.8
	3	10.2	Rat No. 5	1	12.7
	4	10.0		2	12.2

The greatest variation in these colorimeter readings is 0.6 mm., which may be attributed almost entirely to inaccuracies in the measurement of small samples of blood by the method described. This error is equivalent to an error of 0.7 % haemoglobin in the original sample of blood.

So far we have been primarily concerned with the empirical standardisation of the benzidine reaction, so that it might be applied to comparative haemoglobin determinations in rat's blood. Haemoglobin estimations on the same sample of oxalated blood have been made by the colorimetric method and by oxygen capacity determinations. Since the colorimetric method is based on the oxygen capacity determinations, a comparison of the results obtained by the two methods is of no significance, so far as the absolute accuracy of the colorimetric method is concerned. However, since new standards have to be prepared from time to time, the relation of one series of colorimetric determinations to another depends upon the accuracy with which the oxygen capacity determinations check with the colorimetric method.

g. haemoglobin in 100 cc. blood			
	Sample of blood	Colorimetric method	Calc. from O ₂ capacity [Sendroy, 1931]
Human blood	*1	14.3	13.93 } Average 14.23 } 14.08
	2	13.8	
	3	13.7	
	4	13.7	
Sheep-blood	1	13.9	13.97
	2	13.6	
	3	14.2	
Rat-blood No. 3.	1	14.0	14.4
	2	14.0	
Rat-blood No. 4	1	10.1	9.64
	2	9.9	

* Aliquots of the same sample of blood.

The effect of heating the diluted blood.

Three 10 cc. aliquots of a diluted blood (13.5 mg. haemoglobin in 100 cc.) were measured into 25 cc. volumetric flasks. Two of the flasks were placed in a constant temperature water-bath at 60°. One was removed at the end of 30 minutes and cooled to room temperature, while the other remained in the water-bath for 1 hour. The contents were all diluted to 25 cc., then thoroughly mixed and the benzidine reaction was carried out on a 1 cc. aliquot (0.054 mg. haemoglobin).

No.	Treatment of diluted blood	Colorimeter readings in mm. of No. 1 as standard with other test solutions set at 10 mm.
1	Unheated	10.0
2	Heated for 30 mins. at 60°	9.1
3	Heated for 1 hour at 60°	9.0

These observations have been repeated independently by both authors with different samples of human, sheep- and rat-blood. About 10 % of the peroxidase activity is destroyed by heating the diluted blood at 60° for 30 minutes. The activity is unchanged when the sample is held at the same temperature for 1 hour.

GENERAL DISCUSSION.

To those familiar with the blood-benzidine reaction, it is evident that the quantitative application is only possible when the use of each reagent has been standardised. In this way only can those conditions be maintained which are necessary to preserve a constant peroxidase activity of the blood. The amount of benzidine transformed into the red oxidation product in a given time should then be directly proportional to the quantity of peroxidase (haemoglobin) present.

The greatest difficulty encountered in controlling the reaction, so that it may form the basis of a colorimetric procedure which is free from objections, is to

establish the optimum concentration of hydrogen peroxide. According to Wu, when 3 % H_2O_2 is used, the peroxidase reaction reaches a maximum in about 25 minutes, the "haemoglobin being all destroyed by the peroxide." The depth of colour, after 25 minutes, slowly increased, "due to direct oxidation of the benzidine by the peroxide alone." No explanation is offered as to how the cessation of peroxide activity (indirect oxidation) was differentiated from the commencement of the direct oxidation of the benzidine by the hydrogen peroxide. Even more indefinite and confusing is the statement of Bing and Baker that "We feel these better results (using 0.6 % H_2O_2) are due in part to a lessened tendency for the destruction of the haemoglobin activity by stronger peroxide solutions, and in part to the avoidance of the direct oxidation of the benzidine."

It is at once obvious that if these suggestions are tenable they preclude any possibility of developing an accurate quantitative colorimetric method based on the benzidine blood reaction. We can find no evidence to support the supposition that the benzidine may be directly oxidised by the excess hydrogen peroxide, or that the peroxidase activity of oxyhaemoglobin may be destroyed by stronger (presumably 3 %) peroxide solutions when benzidine is also present. The destruction of the peroxidase activity of dilute blood by hydrogen peroxide in the absence of benzidine is no evidence that the same reaction may take place in the presence of a readily oxidised substance. On the contrary, Willstätter and Stoll [1918] have shown that the catalytic function of oxyhaemoglobin, in contrast with the true plant peroxidase, increases pronouncedly with rising hydrogen peroxide concentration. Oxyhaemoglobin acts as a peroxidase without accelerating the destruction of hydrogen peroxide to any detectable extent [see Kuhn and Brann, 1926]. Again, our own observations on the increase in colour intensity with increasing concentrations of hydrogen peroxide from 0.5 % to 10 % (see Table II), while difficult to interpret, certainly do not support the above contentions. For the sake of clarity the first series of readings recorded in Table II are plotted in Fig. 3.

A mixture of 2 cc. of our benzidine solution and 1 cc. 3 % H_2O_2 , when allowed to stand at room temperatures for 2 hours, developed only a faint yellow tinge. There is no reason to suspect that direct oxidation of the benzidine by 3 % H_2O_2 is any more likely to occur when the dilute blood is present. The values plotted in Fig. 3 represent the amounts of substrate (benzidine) oxidised by varying amounts of H_2O_2 in a given time (1 hour) with a constant quantity of the catalyst present. The amount of substrate oxidised in 1 hour increases with increasing H_2O_2 concentrations, and reaches a maximum with 8 % H_2O_2 .

To form the basis of an accurate colorimetric method, the optimum concentration of H_2O_2 is considered to be that which gives the maximum depth of colour consistent with greatest stability. Under the conditions of our experiments this was found to be 1.5 % hydrogen peroxide.

The literature contains ample evidence to support the conclusions that the leucocytes contain a true (thermolabile) peroxidase. At such large dilutions

as those employed above, one would conclude the true peroxidase activity to be quantitatively negligible compared with the pseudo-peroxidase (thermostable) activity of oxyhaemoglobin. 10 % of the peroxidase activity of diluted blood is destroyed by heating at 60° for 30 minutes. Whether this is due to the destruction of a thermolabile peroxidase or to some change in the oxyhaemoglobin molecule remains to be determined. Kuhn and Brann [1926] have

Fig. 3.

shown that relatively unimportant chemical alterations in the haemoglobin molecule markedly change the peroxidase activity. When the opportunity presents itself we plan to repeat our observation on the effect of heating the diluted blood, using pathological bloods with very high leucocyte contents, such as occur in leucaemias. Delhougne [1929] has reported that the true peroxidase number of blood as determined by the method of Bach and Zubkova [1921] is sharply increased by vigorous exercise and in pathological conditions.

SUMMARY.

1. Certain factors influencing the blood-benzidine reaction have been studied, with the following results.

(a) The amount of benzidine oxidised in 1 hour by hydrogen peroxide in the presence of a fixed amount of the catalyst (haemoglobin) increases with increasing hydrogen peroxide concentration. A maximum is reached with an 8 % solution of hydrogen peroxide. The maximum amount of the red oxidation

product which is stable for at least 12 hours, under the conditions of the blood-benzidine reaction, is given by a 1.5 % solution of hydrogen peroxide.

(b) The initial reaction, through the blue colour to the red, is markedly accelerated by increasing temperature. The rate at which the red colour reaches its maximum intensity is not influenced by variations in temperature from 20° to 40°.

(c) Light has no effect on the colour development. Radiations from a quartz mercury vapour lamp greatly increase the rate of the reaction.

(d) Heating diluted blood at 60° for 30 minutes destroys about 10 % of its peroxidase activity. The inactivation is not increased by heating for 1 hour at 60°.

(e) When the reaction is carried out with a 1.5 % solution of hydrogen peroxide and a reaction time of 1 hour at room temperatures, the peroxidase activity within the range of dilutions studied, is proportional to the concentration of haemoglobin.

2. The benzidine reaction, modified in accordance with the results summarised above, has been applied to the estimation of haemoglobin in minute amounts of blood. The accuracy of these estimations depends primarily upon the accuracy with which small samples (*e.g.* 0.0025 cc.) can be measured.

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REFERENCES.

- Bach and Zubkova (1921). *Biochem. Z.* **125**, 283.
Bernheim and Dixon (1928). *Biochem. J.* **22**, 113.
Bing and Baker (1931). *J. Biol. Chem.* **92**, 589.
Delhougne (1929). *Deutsch. Arch. klin. Med.* **165**, 213.
Harrison (1930). Chemical methods in clinical medicine, p. 437.
Kuhn and Brann (1926). *Ber. deutsch. chem. Ges.* **59**, 2370.
Sendroy (1931). *J. Biol. Chem.* **91**, 307.
Van Slyke and Neill (1924). *J. Biol. Chem.* **61**, 523.
Willstätter and Stoll (1918). *Liebig's Ann.* **416**, 21.
Wu (1923). *J. Biochem. (Japan)*, **2**, 189.