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Studies in the Reaction of Amino and Imino Compounds with Sugars: the Reaction of Histidine with Glucose

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The reaction of amino acids, peptides and proteins with various sugars has been extensively investigated and reviewed (Danhey & Pigman, 1951), but the results obtained have been more often than not contradictory. These discrepancies may be due to various causes, most prominent being the failure to obtain equilibrium conditions and the likelihood that different types of reaction take place at different concentrations, pH values and temperatures. The effect of concentration of glucose or amino acid on the equilibrium constant and the degree of combination at different pH values was studied at a single temperature by Katchalsky (1941) and by Frankel & Katchalsky (1941). They used a potentiometric technique similar to that employed by Levy (1933) and by Balson & Lawson (1936), who investigated the combination of amino acids with formaldehyde. The procedure involved measurements of the decrease in pH brought about by the combination. Katchalsky (1941) interpreted his results in terms of combination between one molecule of the anionic form of the amino compound and one molecule of glucose, and he calculated the corresponding equilibrium constants for the one temperature (23°) employed. He also stated that the values of the equilibrium constants were generally independent of the concentration of both components and of pH over the range 7–9. His method of varying the concentrations was to mix different quantities of the two stock solutions (i.e. the amino acid solution adjusted to a certain pH and the glucose solution), and to keep the total volume constant. However, such a procedure cannot be considered ideal, since increase in the concentration of one component is accompanied simultaneously by decrease in concentration of the second component, and this may result in masking of opposing effects on the equilibrium constant. The fundamental work of Frankel & Katchalsky (1941) left unsolved the problem whether the reaction between amino compounds and sugars was of the same type at other temperatures. Several investigators, including Borsook & Wasteneys (1925), Euler, Brunius & Josephson (1926*a*, *b*) and Lieben & Getreuer (1932), observed that amino acid–glucose mixtures were capable of reducing methylene blue at 37° and higher temperatures. The present author has confirmed most of these observations and examined the kinetics of the glycine–glucose reduction of several indicators (unpublished work). This oxidation–reduction reaction is of physiological interest, since its mechanism may parallel some of the steps involved in the enzymic dehydrogenation of glucose. The 'browning reaction' in amino acid–sugar mixtures indicates that amino compounds and glucose may combine or react in more than one way, and it is therefore possible that the reaction

which results in the reduction of redox indicators is of a special type. It was thought desirable to investigate the amino acid–sugar combinations at several temperatures before proceeding further with the oxidation–reduction reaction. Holden & Freeman (1931) followed the combination of histidine, tyrosine, alanine, lysine, cystine, glycine and glutamic acid with formaldehyde at pH values over 7, and they stated that the reaction with histidine is the fastest. Also, Levy (1935) and Frieden, Dunn & Coryell (1943) found marked combination between histidine and formaldehyde. The extent of reaction between amino acids and formaldehyde could be indicative of the reaction between glucose and amino acids. Further, Westheimer (1937) found that, of the various amino acids examined, histidine was by far the most powerful catalyst in the pH range 4–6·5. Both Kubota (1941), working at 25°, and Ågren (1940), working at 40°, found that histidine combines with glucose, but no attempt was made by either to study the combination in detail or to calculate the equilibrium constants of the reaction. In view of this, the reaction of histidine with glucose was investigated first.

THEORETICAL

The scheme proposed here for the combination of amino acids with glucose is similar, in part, to the schemes proposed by Katchalsky (1941), by Levy (1933) and by Balson & Lawson (1936) for the combination of amino acids with glucose and with formaldehyde. It takes into consideration the reaction of glucose with both the zwitterion and the anion forms of the amino acid. Equations have been developed for the combination with one or with two molecules of glucose. The overall mechanism (Fig. 1) includes both combinations, and to make the distinction clear the equilibria associated with the second glucose combination are represented in bold type.

In Fig. 1 HR is the zwitterion form of the amino acid, G, R^-, H^+, RG^- and RGH represent glucose, the anion form of the amino acid, the hydrogen ion, the ionized form of the anion–monoglucose complex, and the zwitterion–monoglucose complex respectively. RGG^- and RGGH represent the anion–diglucose complex and the zwitterion–diglucose complex respectively.

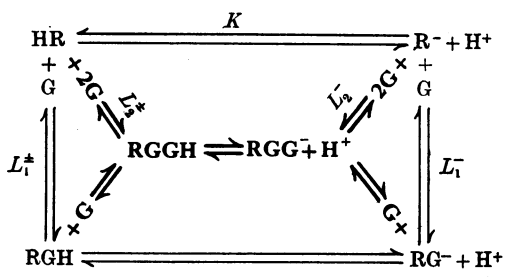


Fig. 1

Combination with one glucose molecule

The following have been taken into consideration:

(*a*) *Law of Mass Action requirements*:

$$K=\frac{[R^-][H^+]}{[RH]}, \tag{1}$$

$$L_1^-=\frac{[RG^-]}{[R^-][G]}, \tag{2}$$

$$L_1^\pm=\frac{[RGH]}{[RH][G]}. \tag{3}$$

(*b*) *Requirements of electrical neutrality.* In the initial state, i.e. in the absence of glucose,

$$[R^-]_i+[OH^-]_i=[M^+]_i+[H^+]_i, \tag{4}$$

where $[M^+]$ represents any metal ions added in the course of alkali addition. Over the approximate pH range 5–9, the $[H^+]$ and $[OH^-]$ do not exceed the approximate value of 10^{-5}, and if the amino acid and alkali concentrations are much greater than this value the reduced electroneutrality equation is obtained:

$$[R^-]_i=[M^+]_i. \tag{5}$$

In the presence of glucose, when equilibrium has been attained,

$$[R^-]_e+[RG^-]_e+[OH^-]_e=[M^+]_e+[H^+]_e. \tag{6}$$

As before, this may be reduced to

$$[R^-]_e+[RG^-]_e=[M^+]_e. \tag{7}$$

If we compare only those cases in which the amino acid solution and the amino acid–glucose solution contain the same quantity of alkali added, we have

$$[M^+]_i=[M^+]_e. \tag{8}$$

Hence, combining equations (5), (7) and (8), we have

$$[R^-]_i=[R^-]_e+[RG^-]_e. \tag{9}$$

(*c*) *Total requirements.* The totals of the concentrations of the various forms of the amino acid before and after combination with glucose are equal. Thus,

$$[RH]_i+[R^-]_i=[RGH]_e+[RG^-]_e+[RH]_e+[R^-]_e. \tag{10}$$

Cancelling $[R^-]_i$ by $[R^-]_e$ plus $[RG^-]_e$, according to equation (9), followed by substitution for [RH] in terms of (1) and for $[RGH]_e$ in terms of (3) and (1) gives

$$\frac{[R^-]_i[H^+]_i}{K}=\frac{[R^-]_e[H^+]_eL_1^\pm[G]_e}{K}+\frac{[R^-]_e[H^+]_e}{K}. \tag{11}$$

Cancelling K, substituting for $[R^-]_i$ in terms of (9) followed by substitution for $[RG^-]$ in terms of (2), and subsequent cancelling of $[R^-]_e$ gives

$$[H^+]_i\{1+L_1^-[G]_e\}=[H^+]_e\{1+L_1^\pm[G]_e\}. \tag{12}$$

Rearranging, we obtain

$$\frac{[H^+]_e}{[H^+]_i}=\text{antilog }\Delta pH=\frac{1+L_1^-[G]_e}{1+L_1^\pm[G]_e}, \tag{13}$$

since $$\Delta pH=pH_i-pH_e=\log\{[H^+]_e^+/[H^+]_i\}. \tag{14}$$

If, however, the combination of glucose with the zwitterion is very small compared with that of the anion, then $L_1^\pm[G]_e \ll 1$ and equation (13) reduces to

$$\text{antilog }\Delta pH=1+L_1^-[G]_e. \tag{15}$$

The last equation is the same as that derived by Katchalsky (1941) for the combination of amino acids with glucose, on the assumption that only the anion form of the amino acid reacts with glucose. Consideration of the

Katchalsky derivation shows, however, that he assumed (*a*) that the complex RGH is completely ionized, and (*b*) that the Henderson–Hasselbalch type of equation (which strictly can apply only to one acid at a time) could be applied to both the RH and RGH dissociations as one fraction, by considering RGH absent and RG^- directly derived from RH.

Such assumptions are unnecessary, since the strict derivation given above leads to the same result. It is intended to consider the results of such assumptions and their possible cancellations in a separate publication.

Combination with one and two glucose molecules

Extension of the amino acid–glucose combination to include the combination of two glucose molecules, as shown in the overall scheme, requires the inclusion of the following equations.

(*a*) *Equilibrium requirements*:

$$L_2^- = \frac{[RGG^-]}{[R^-][G]^2}, \quad (16)$$

$$L_2^{\pm} = \frac{[RGGH]}{[RH][G]^2}. \quad (17)$$

(*b*) *Electroneutrality requirements.* Equation (5) still represents the initial state, i.e. in the absence of glucose. In the presence of glucose, when equilibrium has been attained, the simplified equation is

$$[R^-]_e + [RG^-]_e + [RGG^-]_e = [M^+]_e = [M^+]_i = [R^-]_i. \quad (18)$$

(*c*) *Total requirements.* The following equation has to be substituted for equation (9):

$$[RH]_i + [R^-]_i = [RGH]_e + [R^-]_e + [RG^-]_e + [RH]_e + [RGG^-]_e + [RGGH]_e. \quad (19)$$

Cancellation by introducing equation (18) into equation (19) gives

$$[RH]_i = [RGH]_e + [RH]_e + [RGGH]_e. \quad (20)$$

Substitution then gives

$$\frac{[R^-]_i[H^+]_i}{K} = \frac{[H^+]_e L_1^{\pm}[R^-]_e[G]_e}{K} + \frac{[R^-]_e[H^+]_e}{K} + \frac{L_2^{\pm}[R^-]_e[H^+]_e[G]_e^2}{K}. \quad (21)$$

Cancellation of K, substitution for $[R^-]_i$ and rearrangement gives

$$[H^+]_i[R^-]_e\{1 + L_1^-[G]_e + L_2^-[G]_e^2\} = [H^+]_e[R^-]_e\{1 + L_1^{\pm}[G]_e + L_2^{\pm}[G]_e^2\}. \quad (22)$$

Cancelling $[R^-]_e$ and rearranging, we obtain

$$\frac{[H^+]_e}{[H^+]_i} = \text{antilog } \Delta pH = \frac{1 + L_1^-[G]_e + L_2^-[G]_e^2}{1 + L_1^{\pm}[G]_e + L_2^{\pm}[G]_e^2}. \quad (23)$$

Only if both monoglucose–zwitterion and diglucose–zwitterion combinations are negligible compared with the glucose anionic combinations does equation (23) reduce to

$$\text{antilog } \Delta pH = 1 + L_1^-[G]_e + L_2^-[G]_e^2. \quad (24)$$

Equations (13), (15), (23) and (24) represent the various possibilities in the overall scheme of combination.

To facilitate calculation the assumption will be made that the glucose concentration at equilibrium is practically the same as that initially introduced when the glucose concentration is many times greater than that of the amino acid. This assumption is reasonable, particularly when the value of the equilibrium constant is small, as was found in the histidine–glucose combination.

EXPERIMENTAL

Materials. L-Histidine monohydrochloride of analytical quality (L. Light and Co., Colnbrook, Bucks.) was employed throughout; D-glucose and sodium hydroxide were also of analytical quality (British Drug Houses Ltd. and Hopkin and Williams Ltd.). Solutions of NaOH were carbonate-free.

All solutions were made in hard-glass distilled water (conductivity 2–2·6 reciprocal MΩ). All reaction vessels kept in the thermostats were of hard glass. The vessels were initially thoroughly cleaned with 'permanganic acid', followed by many washings with hot tap-water and several washings with distilled water and steaming for several minutes.

Procedure. Experimental procedure used was to follow the decrease in pH caused by the reaction in histidine–glucose solutions of different initial pH values. Constant-volume techniques were employed throughout. A control tube containing amino acid and sodium hydroxide was made up to a certain volume with distilled water. Other tubes containing identical quantities of histidine and sodium hydroxide, but different quantities of glucose, were made up with distilled water to the same volume as the control. Several such sets were made, each with the same histidine concentration, but different initial pH, so as to enable a titration curve to be obtained from pH 5 to 9. These experiments were repeated with other histidine concentrations. Some sets were duplicated. The reaction was studied generally under anaerobic conditions, but occasionally also under aerobic conditions.

pH *measurement.* The pH measurements were carried out with lithium-glass electrodes, at 20°, 30°, 40° and 50°; temperature was controlled within ±0·04°. The pH values of the control were measured as well as those of the tubes containing different quantities of glucose; the pH measurements were usually followed for over 6 weeks, and in some cases over 3 months. Sterility was ensured by addition of toluene to, and frequent shaking of, each tube. The pH measurements were carried out in the various thermojackets developed by the author (Lewin, 1953). These required less than 1 ml. of solution for individual pH measurements. Glass electrodes were standardized with both borate and phthalate buffers; occasionally phosphate was used for a further check. Agreement was usually within ±0·01 pH. Checks on the glass-electrode standardizations were carried out frequently during each set of measurements; reproducibility was within the above limits. In the few cases when certain glass electrodes did not obey the strict hydrogen-electrode function, but showed reproducible and strict dE/dpH linearity, calibration curves were used to ensure accurate determinations (Lewin, 1955).

Corrections to pH *values.* Balson & Lawson (1938) have maintained that the negative pH shift obtained on the addition of glucose to an amino acid solution was due solely to the acid dissociation of glucose and not to any combination between them. However, Frankel & Katchalsky (1938) have shown that this is not the case and that in the approximate pH range 7–9 any negative shifts due to the acidity of glucose are quite small; Katchalsky (1941) went further and assumed that over this pH range glucose

behaves as a non-electrolyte, and he consequently ignored such dissociation. In the present investigation, the glucose solutions used for making up the histidine–glucose mixtures were not initially adjusted either to the initial pH of the histidine solution or to the subsequent, first steady pH-state value. The titration of glucose shows, however, that after about pH 8 the slope of the curve increases noticeably, and therefore it was deemed necessary to assess the effect of the acid dissociation of glucose on the depression of pH values and to correct these correspondingly. The value of the correction depends on the relative values of the buffering capacity of the histidine and glucose solutions at the concentrations used and on the pH when the steady pH states have been attained, and not on the initial pH. Calculations showed the correction values to be generally well below 0·01 pH. Since the experimental variation was usually within 0·02 pH, and since the variation in the value of the equilibrium constant given (i.e. ±0·2) more than covers this variation and made allowance when somewhat greater variations were obtained, such corrections are well within the overall experimental error. They were therefore considered negligible. This applies to the various data quoted in this paper, with the exception of the solutions with an initial pH value of 9·12 at 20°, where the calculated values of the corrections at the lower glucose concentrations were outside experimental error, i.e. about 0·04 pH. In this case, the corrections were applied.

It is necessary to emphasize that the above calculations were based on the titration curve of freshly prepared glucose solutions, which were used throughout this investigation. Old (e.g. 6-month-old) glucose solutions, which were kept under toluene and which showed no signs of attack by micro-organisms, give titration curves which require more than twice the amount of sodium hydroxide required for fresh solutions to reach a given pH under identical conditions.

RESULTS

Several consecutive steady pH states have been noted, as is described below in section (*e*) (3). The results given in sections (*a*), (*b*), (*c*) and (*d*) are concerned only with the first steady pH state.

(*a*) *Dependence of* ΔpH *on the initial* pH. In Fig. 2 are shown the titration curves of 0·026 M L-histidine monohydrochloride, at constant volume, with and without glucose at 40°. It will be seen that over the pH range 5–6·5 the presence of glucose does not cause depression in the pH values of the histidine–sodium hydroxide mixtures. This indicates that no histidine–glucose combination takes place over this pH range. Above pH 6·5 the titration curves diverge, and over the approximate pH range 7·6–8·8 the pH depressions are practically independent of the initial pH. At higher pH, however, ΔpH increases considerably. There, however, the second nitrogenous group present in the histidine may begin to combine with the glucose, thereby giving rise to greater pH depressions.

The pH in the (no glucose) control tubes remained steady over the period during which the pH

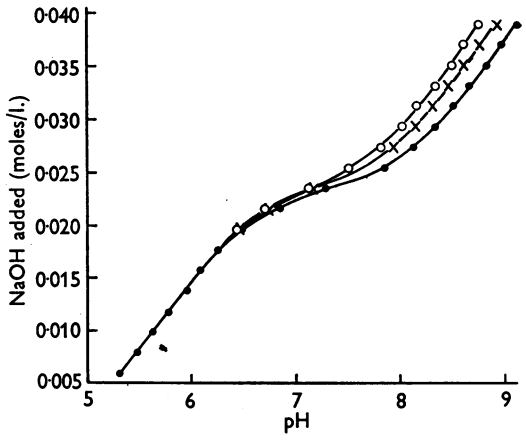


Fig. 2. Effect of glucose on the constant-volume titration curve of L-histidine monohydrochloride (0·026 M) with sodium hydroxide at 40° in the first steady pH state. ●, Histidine; ×, histidine plus 0·2 M glucose; ○, histidine plus 0·4 M glucose.

Table 1. *Variation of* pH *of* 0·016 M L-*histidine monohydrochloride solutions* (*containing various quantities of alkali*) *with temperature*

55 ml. of 0·2 M histidine–HCl mixed with 0·49 N-NaOH (see col. 1); each soln. diluted with water to give 0·016 M histidine.

NaOH added (ml.)	pH at 20°	30°	40°	50°
22·95	8·47	8·26	8·05	7·81
24·5	8·70	8·50	8·30	—
26·5	—	8·72	8·50	8·25
28·5	9·12	8·92	8·68	—
30·5	—	9·08	8·85	8·61

measurements were taken. The pH values in the series of tubes containing the 0·2 and 0·4 M glucose dropped continuously, after mixing, and attained, within a day, values which remained steady over the subsequent 3–4 days; the latter are the pH values recorded in the respective curves. Similar results were obtained for the dependence of the ΔpH on the initial pH, at other temperatures. However, the curves were now found to have been displaced towards the higher pH values as the temperature was lowered. Thus, for 0·3 M glucose concentration, at 20°, at an initial pH of 7·60 the ΔpH obtained was only 0·09, but this increased to 0·32 at initial pH 8·47, the latter ΔpH value remaining the same as far as an initial pH 8·80. This displacement parallels the displacement of the histidine-titration curve with temperature which is given in Table 1.

(*b*) *Dependence of* ΔpH *on the histidine concentration and the glucose concentration in the first steady* pH *state.* (1) ΔpH and histidine concentration: the

ΔpH was found to be practically independent of the histidine concentration at 20°, but some increase of the ΔpH was found with decreasing amino acid concentration at higher temperatures (see Table 2).

It is pertinent to note here that histidine solutions are remarkably good buffers with pH values that are hardly affected by dilution (see Table 3).

(2) ΔpH and glucose concentration: the pH depressions increased with rise in glucose concentration at all histidine concentrations and temperatures examined. In Fig. 3 the antilogarithm of the pH depression is plotted against the glucose concentration at various initial pH values and temperatures. The plots are mainly linear, having an intercept of unity on the ordinate. However, at the highest glucose concentration (1·2M) and particularly at the highest initial pH, positive deviations outside experimental error are found [e.g. curve (*a*) at initial pH 9·12]. On the other hand, below pH 8 lower slopes are found (which is in agreement with the smaller pH depression values obtained for the intermediate range at 40°; see Fig. 2) which give, however, straight lines over the entire glucose concentration range. See Fig. 3 (*f*) for initial pH 7·81 at 50°.

The straight-line relationship obtained over the approximate initial pH range 8–9 is in agreement with equation (15) and shows that the first reaction is primarily in terms of one glucose molecule combining with one molecule of the anionic form of histidine.

Table 2. *Effect of histidine concentration on the* pH *depressions* (ΔpH) *in histidine–glucose solutions, at various temperatures*

Histidine concn. (M)	Glucose concn. (M)	ΔpH at 20°	30°	40°
0·067	0·3	0·31	0·27	—
0·033	0·3	0·32	0·28	—
0·022	0·3	0·31	0·27	—
0·016	0·3	0·32	0·29	0·25
0·067	0·6	0·49	0·44	0·35
0·033	0·6	0·50	0·45	—
0·022	0·6	0·50	0·44	0·39
0·016	0·6	0·51	0·47	0·43

Table 3. *Effect of dilution on the* pH *of histidine solutions*

Histidine concn. (M)	pH at 20°	30°
0·067*	8·46	8·23
0·033	8·46	8·24
0·022	8·46	8·24
0·016	8·47	8·25

* Made by adding 91·8 ml. of 0·490N-NaOH to 220 ml. of 0·2M histidine monohydrochloride soln. and diluting to 660 ml. with distilled water.

(*c*) *Variation of the equilibrium constant with temperature.* The equilibrium constant values calculated from the slopes of the lines in Fig. 3 are given in Table 4. The experimental accuracy limits impose a maximum variation of ± 0·2 on the values in the table.

The decrease in the value of the equilibrium constant with temperature shows the histidine–glucose reaction to be exothermic. Inspection of Table 2 shows that the ΔpH, and therefore the equilibrium constant, decreases with increasing histidine concentrations at the higher temperatures, but this phenomenon was not studied further.

(*d*) *Heat of reaction.* Plotting the logarithm of the equilibrium constant (obtained at 0·016M histidine concentration) against the reciprocal of the absolute temperature, we obtain the graph of Fig. 4. The plot is in the nature of a curve which can be broken up into a practically straight line over the temperature range of 20–30–40°, and a line

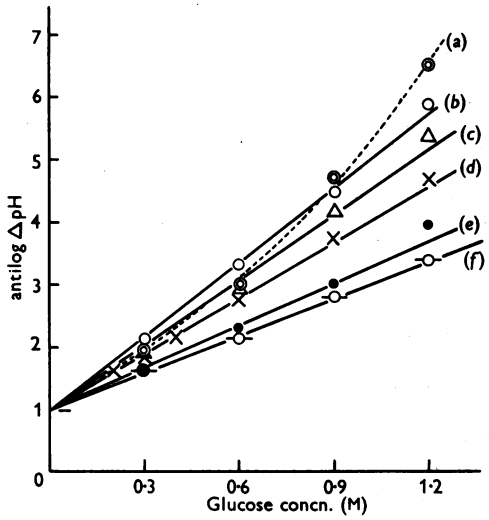


Fig. 3. Variation of the antilogarithm of ΔpH of the first steady pH state of the histidine–glucose reaction, with glucose concentration at various temperatures. Histidine concn. 0·016M. (*a*) ◎---◎, 20°, initial pH 9·12; (*b*) ○—○, 20°, initial pH values 8·47, 8·58, 8·70; (*c*) △—△, 30°, initial pH values 8·49, 8·72, 8·92; (*d*) ×—×, 40°, initial pH values 8·30, 8·50, 8·68, 8·85; (*e*) ●—●, 50°, initial pH values 8·23, 8·54; (*f*) –○– – –○–, 50°, initial pH 7·81.

Table 4. *Variation of the equilibrium constant (first steady* pH *state) of the histidine–glucose combination with temperature (histidine concn.* 0·016M)

Temperature	20°	30°	40°	50°
Equilibrium constant	3·9	3·5	3·0	2·3

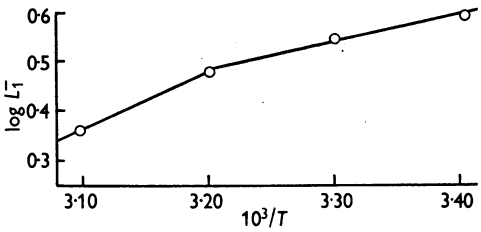


Fig. 4. Variation of the logarithm of L_1^- (the equilibrium constant of the monoglucose–mono-anionic histidine combination) with the reciprocal of the absolute temperature, for the first steady pH state. Histidine concn. 0·016 M.

joining the two points at 40° and 50°. The value of the heat of reaction calculated for the 20–40° range is $2{\cdot}6 \pm 0{\cdot}5$ kcal., and for the 40–50° range $5{\cdot}2 \pm 0{\cdot}9$ kcal.

This variation in the heat of reaction shows that the nature of the combination changes in the higher temperature region. The correct value for the heat of reaction which predominates over the higher temperature range is probably greater than the value obtained for the range 40–50°.

(*e*) *Effect of time on the* pH *and* ΔpH *values recorded.* (1) The approach to equilibrium: the time taken to attain 'equilibrium' in the glucose–histidine solutions varied with temperature. About 5–6 days were required at 20°, 1–2 days at 30°, 12–24 hr. at 40°, and about 2 hr. at 50°.

(2) The pH of histidine solutions under aerobic and anaerobic conditions: the pH of histidine solutions in absence of glucose which had been initially adjusted to certain pH values were steady for some time, but eventually dropped. The time interval during which the solutions retained their original pH values depended upon whether aerobic or anaerobic conditions were employed, and to some extent on the temperature. Under anaerobic conditions, the pH was steady for longer periods. Thus, at 20°, 0·016 M histidine solution adjusted to pH 8·46 retained this value for over 5 weeks under both aerobic and anaerobic conditions. Thereafter the pH of both solutions decreased, almost stepwise, the anaerobic solution by 0·2 pH, the aerobic one by 0·23 pH; the values then remained constant over the next month. Somewhat similar results were obtained at higher temperatures, except that the pH decreases occurred earlier and that the aerobic drop occurred earlier still and was noticeably greater than the anaerobic. Thus, with a solution, at 50°, of the same composition as that employed at 20° (initial pH 7·81) it was noted that under aerobic conditions the pH dropped on the fourth day to 7·72, and under anaerobic conditions the solution retained its initial pH value for over 13 days. The cause of these changes has not been investigated in this Laboratory. However, Holden & Freeman (1931) and Wadsworth & Pangborn (1936) have observed that alkaline solutions of amino acids display a gradual loss of amino nitrogen. In this work and in the present investigation toluene was added as a preservative, and it was suggested (French & Edsall, 1945) that this behaviour may be due to contaminating organisms. It is, however, difficult to accept this suggestion, since the presence of such micro-organisms should be accompanied by phenomena usually associated with bacterial attack; the histidine solutions in this investigation remained perfectly clear, and acquired a yellow tint after standing for some time. It would seem more likely that some deamination process, or some internal rearrangement blocking the amino group, takes place on long standing. The yellow tint acquired was noticeable in histidine hydrochloride solutions; it was bright yellow in histidine solutions adjusted to alkaline pH after standing for some time even at temperatures below 0°. However, no such colour development could be detected if glucose was present, even after 8 months, at temperatures below 0°. This striking phenomenon is significant in that it also shows the complete absence during that time of any 'browning reaction', at such temperatures.

(3) Steady pH states: the pH measurements were continued long after the histidine–glucose solutions had reached their first steady pH values (from which the equilibrium constants were calculated). It was found that these pH values remained steady for about 4 weeks at 20°, about a week at 30°, 60–90 hr. at 40° and 5–8 hr. at 50°. Subsequently, the pH of the solutions began to fall and, eventually, again reached steady values. This second steady pH state lasted for approximately the same time as the corresponding original equilibrium or first steady pH state. Subsequently, a third pH drop occurred, resulting in a third steady pH state. Further consecutive steady pH states were found. With 20° measurements, the exact values of the ΔpH of the second and subsequent steady pH states could not be evaluated, as the pH drop of the histidine solutions (in absence of glucose) took place at about the same time. But with increase in reaction temperature, although the duration of the glucose–histidine steady pH states was smaller, the duration of the steady pH readings of the corresponding histidine solutions did not decrease as much. This enabled several steady pH states to be noted at the higher temperatures (see Fig. 5). Similar results were obtained at 30° and 40°. It was also observed that the duration of some of the later steady pH states was greater than the earlier ones. Further, the development of brown colour was noted as beginning at the end of the second or the start of the

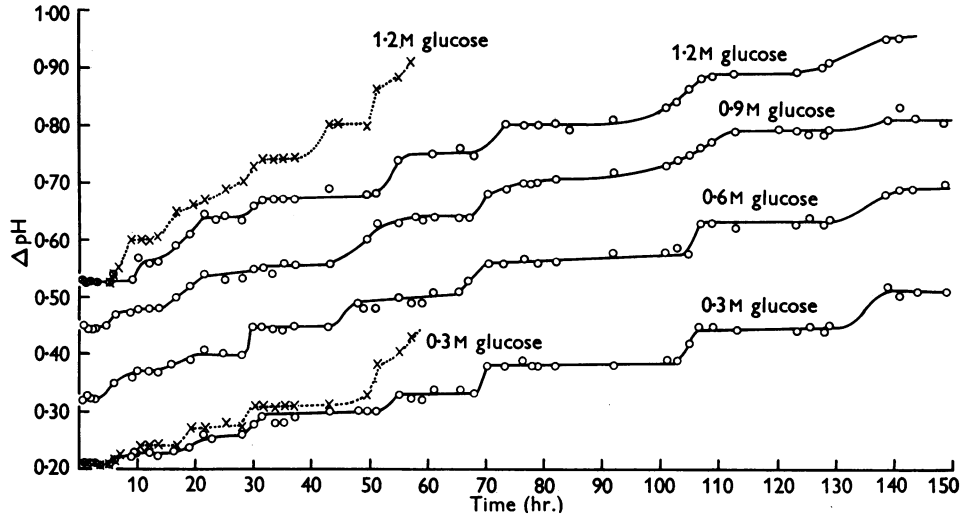


Fig. 5. Steady pH states at 50°. Variation of the pH depressions (ΔpH) with time in glucose–histidine solutions at 50°. Histidine concn. 0·016M. Initial pH 7·81. O—O, Anaerobic conditions; × - - - ×, aerobic conditions.

third steady pH state; its intensity increased greatly with the passage of time. In order to test the dependence of the browning on various factors, visual comparisons were made of a large number of solutions observed simultaneously. The brown colour was found to be intensified with increase in initial pH, increase in glucose concentration and rise in temperature. These results parallel the findings of many workers including Mohammed, Frankel-Conrat & Olcott (1949) with the combination of other amino acids with glucose. It was also noted that browning was less pronounced under aerobic than under anaerobic conditions.

It was noted that the ΔpH obtained in the first steady pH state was the same whether aerobic or anaerobic conditions were employed. But rise in temperature associated with increase in initial pH value (in the region approaching pH 9 and over), and increased glucose concentration, resulted in the ΔpH obtained aerobically being noticeably greater than the corresponding anaerobic result. This suggests that aerobic oxidation of the histidine–glucose complex takes place and forms acidic products. Further, in the subsequent steady pH states, all the aerobic pH values are greater than the corresponding anaerobic ones (the plots are parallel) and the difference increases with the age of the solution. It was also observed that whereas the ΔpH values of the various anaerobic steady pH states were reproducible, within experimental error, the corresponding ΔpH values of the second and subsequent aerobic steady pH states, though retaining their original shape and parallelism to the anaerobic states, could not be reproduced within the same limits of accuracy.

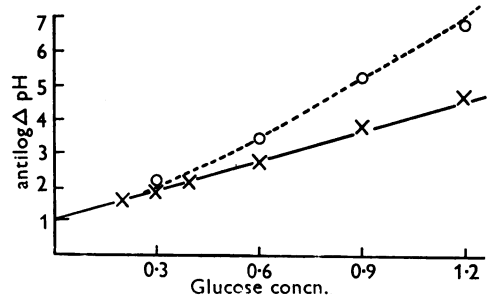


Fig. 6. Variation of the antilogarithm of the pH depression of the histidine–glucose reaction in the first and second anaerobic steady pH states, with glucose concn., at 40°. × — ×, First steady pH state; O - - - O, second steady pH state (0·016M histidine).

The pH depressions of the anaerobic second steady pH state [ΔpH_2 = (initial pH) − (pH value of the second steady pH state)] showed an independence of the initial pH similar to that found for ΔpH_1. Thus, at 30° at initial pH 8·50, 8·72, 8·92 and 9·07, for a given glucose concentration at the lower glucose concentrations, ΔpH_2 was practically independent of the initial pH, but at the higher glucose concentrations, and particularly at the highest initial pH, positive deviations were found; over a lower pH range, i.e. below pH 8·24, lower ΔpH_2 values were obtained. Similar results were obtained at 40°.

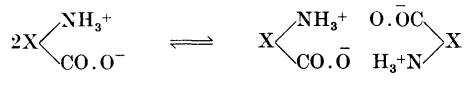
The plot of the antilogarithm of ΔpH_2 against the glucose concentration at 40° is given in Fig. 6.

DISCUSSION

First steady pH *state.* The straight-line relationship obtained for the plot of the antilogarithm of ΔpH_1 against the glucose concentration is in agreement with equation (15) and therefore with the formulation of one glucose molecule combination with one molecule of anionic histidine. This formulation does not, however, decide what forms of glucose, i.e. whether aldehydic or glycosidic, or both, take part in the combination or whether it is the amino or imino group which reacts with the glucose. Combination takes place over the range where the imino group is responsible for the buffering activity of histidine solutions, and this might suggest a mechanism of combination between the imino group and glucose. However, Kubota (1941) and Ågren (1940), working with solutions at pH 7·4, showed that there is a decrease in the amino content when histidine and glucose solutions are mixed. This indicates that at least part of, if not all, the combination proceeds via the amino group.

At first sight it would appear that any zwitterion combination over the pH region examined is negligible, since the latter combination should according to equation (13) result in the line curving somewhat downwards. However, any small second glucose molecule combination with the anionic form of histidine would cause an upward curvature of the line. Over the range given, it is possible that the magnitudes of L_2^- and $L_1^{\pm}$ are such that the two opposing effects are cancelled. Increase in initial pH value decreases the zwitterion concentration, and increase in glucose concentration favours anionic-diglucose combination. It may therefore be significant that at the highest initial pH values examined [see Fig. 3, curve (*a*); 20° results at initial pH 9·12], and to a smaller extent at the highest glucose concentrations at lower initial pH values, there is a distinct upward trend. The line drawn through (*a*) is a curve having, over the lower glucose concentration range, lower slope than the straight line obtained for the intermediate pH range (*b*), but thereafter curves upwards. The lower slope might possibly be explained on the assumption that the equilibrium constant of any combination between the anionic glucose (the proportion of which increases with increasing pH) and anionic histidine is, owing to the possession of similar charges, far smaller than that of the undissociated glucose with anionic histidine. This results in an overall smaller combination and therefore smaller ΔpH_1. However, with increase in glucose concentration, the increased diglucose–anionic histidine combination results in increased ΔpH_1 values which indirectly lead to a further increase in the overall value of the pH depression by lowering the equilibrium pH (first steady pH state) and therefore resulting in decreased proportion of anionic glucose. The combined effects of both these factors are likely to result in considerable increase in the value of the slope. The upward trend in the slope at the highest glucose concentration is noticeable, but only to a small extent at lower initial pH values. However, this trend may not necessarily be due to a diglucose–anionic histidine combination since other factors may be concerned such as: (*a*) combination with a second nitrogenous group; (*b*) increasing association of the hydroxyl groups of glucose with histidine; (*c*) change of medium, with considerable increase in glucose concentration, which may result in increased equilibrium constant values; (*d*) increase in the relative proportions of the reacting forms of glucose. Thus, assuming that in aqueous solution equilibrium exists between the various anhydrous forms and the hydrated form, and assuming that only the anhydrous forms react with the amino or imino groups, then since an increase in glucose concentration eventually results in a relative decrease in the water concentration, the concentration of the anhydrous forms increases to a greater extent than the overall increase in the concentration of the glucose, thus resulting in increasing combination and thereby increasing slope.

The increase in ΔpH obtained on dilution of histidine at 40° (see Table 2) can be explained in terms of association of single zwitterions to form polyzwitterions, with increase in concentration, such as



On dilution the equilibrium is displaced to the left, particularly with rise in temperature, and assuming that the polyzwitterions combine with glucose to a smaller extent than the monozwitterions do, this results in increasing combination.

The increase in the heat of reaction of the first steady pH state with rise in temperature indicates a change in the nature of the reaction. Since the plot of the antilogarithm of ΔpH_1 against the glucose concentration still gives straight lines at the highest temperature employed (50°) it suggests that the reaction is still essentially a monoglucose–mono-anionic histidine combination. It is therefore possible that this change is due to some temperature effect on the equilibrium between the various forms of glucose or on an equilibrium between any forms participating in the suggested zwitterionic association.

The lower slopes obtained in the intermediate pH region merit further investigation. But as inspection of Fig. 2 shows, the pH depressions

decrease at lower initial pH values and this results in decreasing experimental accuracy. Consequently, this range was not investigated in detail.

Second steady pH *state.* The apparent independence of ΔpH_2 of initial pH in the pH range examined, and the upward curving of the plot of the antilogarithm of ΔpH_2 against the glucose concentration, are interpretable by a formulation involving the co-existence of monoglucose and diglucose combinations with the anionic form of histidine, since a 'best' hyperbolic curve could be drawn (see Fig. 6) in contact with the experimental points obtained for the second steady pH state. This curve could be interpreted in terms of equation (24), and, assuming the value of L_1^- not to have changed, the approximate value of 2 is obtainable for L_2^- at 40°. The time interval between the first and second steady pH states may be explained as being due to some steric hindrance which disappears as an internal rearrangement within the complex takes place. In the combination between histidine and formaldehyde Neuberger (1944) showed that there are two distinct stages, and it has been suggested (Levy, 1935) that an internal rearrangement involving a hydrogen-ion shift takes place. It is therefore possible that a similar shift takes place in the histidine–glucose combination leading to the change from the first steady pH state to the second. However, it is also possible that this change or subsequent changes from one steady pH state to the others may be due to other causes such as the conversion of glucose into a mixture containing glucose, fructose and mannose, the latter two possibly combining more strongly than glucose with histidine. Alternatively, or additionally, combination or bridging may take place via the hydroxyl groups of the glucose molecule with histidine. Combinations between polyhydric alcohols and amino acids are well known to lead eventually to the 'browning reaction'. Browning could be visibly noted at the end of the second steady pH state. It is hoped that further work, already in progress, will shed more light on these possibilities.

SUMMARY

1. The combination of histidine with glucose has been examined with varying concentrations of histidine (0·067 to 0·016 M) and glucose (up to 1·2 M) over the pH range 5–9 and at 20°, 30°, 40° and 50°, both aerobically and anaerobically, by measuring the pH depressions which follow the mixing of the solutions.

2. A series of subsequent steady pH states having successively greater pH depression values was found.

3. No combination was found within the approximate pH range 5–6·5. In the first steady pH state, over the approximate pH range pH 8–9, the results are in agreement with one glucose molecule combining with one molecule of anionic histidine.

4. The equilibrium constants of the first steady pH state decreased with rise in temperature, thus showing the reaction to be exothermic.

5. The pH depressions obtained for the first steady pH state were the same whether aerobic or anaerobic conditions were employed. However, under conditions involving both high initial pH values and high glucose concentrations, the aerobic pH depressions were somewhat higher in value. In subsequent steady pH states, particularly at the higher temperatures, the aerobic pH depressions were noticeably higher.

6. Browning was noted as starting at the end of the second or the beginning of the third steady pH state. Its intensity was found to increase with rise in initial pH, glucose concentration and temperature, but to decrease in presence of air.

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