SUMMARY

1. Folic acid and pteroyl-glutamic acid have been examined for their ability to affect acid production by *Strep. lactis* R during a long period of incubation.

2. Evidence has been obtained pointing to the existence in folic acid of two factors with activity in the early and later phases of growth and acid-production.

3. Quinoxaline can act as an inhibitor of the growth of *Strep. lactis* R, and both folic acid

and pteroyl-glutamic acid can reverse this inhibition.

4. Examination of acid production in the presence of intermediate concentrations of folic acid and quinoxaline is consistent with the presence of a pterin-like structure in the component responsible for early acid production, and not in the postulated component which is responsible for later activity.

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The Folic Acid Group of Growth Factors

3. HISTIDINE AND ITS RELATIONSHIP TO 'FOLIC ACID'

By D. A. HALL, *Biochemical Laboratories, School of Medicine, Leeds*

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Since 'folic acid' was first described by Mitchell, Snell & Williams (1941) there have been repeated attempts to identify the activity of the natural preparations with compounds of known composition. On the basis of the absorption spectrum of the material, attention was drawn to the connexion between folic acid and xanthopterin (Mitchell, 1944). The work of Totter & Day (1943), Totter, Shukers, Kolson, Mims & Day (1944) and Totter, Mims & Day (1944), claiming the alleviation of nutritional cytopenia in monkeys, by the use of folic acid and to a certain extent xanthopterin, did much to establish this belief in the identity of the two compounds. Mitchell & Williams (1944), however, showed that xanthopterin could not replace folic acid in the growth of *Strep. lactis* R nor was folic acid as effective as xanthopterin in fish anaemia. It was also shown that of a large group of compounds with a high nitrogen content, only thymine was able to replace folic acid for *Strep. lactis*. This fact has been proved and examined by

both Stokes (1944) for folic acid and Kreuger & Peterson (1945) for vitamin B₁₂, the former claiming that thymine does not directly replace folic acid, but that a thymine-like compound is the product of a reaction in which folic acid plays a part. On the other hand, in view of certain of his results, and those reported by the present author in a previous paper (Hall, 1947a) it appears more likely that if thymine is directly connected with folic acid, it may more readily be supposed to be a precursor than a product of folic acid activity. Chattaway (1944) examined a number of pyrimidine derivatives as growth factors for *L. casei*, and showed that orotic acid was active in this respect. It has also been shown (Hall, 1947a) that orotic acid, although not active alone for *Strep. lactis*, showed synergism with folic acid, as also did thymine. This tended to support the other possible conclusion that the active pyrimidine derivatives do not replace folic acid, but are in effect offering an alternative route for metabolism.

Many workers have shown that in addition to folic acid, both *L. casei* and *Strep. lactis* R require the presence of a selection of purines and pyrimidines (Snell & Peterson, 1940; Feeney & Strong, 1942; Snell & Mitchell, 1941), from which it would appear that heterocyclic nitrogen compounds play a considerable part in the nutritional requirements of these two organisms. Of other heterocyclic nitrogenous bases having biological activity, histidine is unique in that there are indications that it may act as a precursor for purines in mammalian metabolism (Degan, 1938). Although the evidence in this respect is rather confused, it appeared desirable to examine the action of histidine on the growth and acid production of *Strep. lactis* R to determine whether there is a similar connexion between it and purine or pyrimidine metabolism in the case of this organism.

METHODS

To obtain all the experimental results below, methods were employed as previously reported (Hall, 1947*a*, *b*). In all cases the organism employed was *Strep. lactis* R, although preliminary experiments had shown that similar effects could be observed on the addition of histidine to cultures of *L. casei*. The interpretation of the latter results was, however, more difficult, owing to the more complex growth requirements of *L. casei*. The medium and conditions of inoculation and incubation were identical in all respects to those employed during the investigation of the utilization of folic acid by *Strep. lactis* R and when the period of incubation was extended above the normal period of 18 or 24 hr. the method described by Hall (1947*b*) was used.

Histidine was added as an aqueous solution of *l*-(–)-histidine monohydrochloride, rendered neutral to bromothymol blue with NaOH before use. It was introduced into the medium either before sterilization or aseptically just before inoculation. All values given below for histidine content of the various cultures refer to the amount of histidine hydrochloride added. Owing to the fact that *Strep. lactis* R will not grow on a basal medium made from an amino-acid mixture without the addition of other uncharacterized factors besides folic acid, the histidine content of the casein-hydrolysate had to be taken into account. Analyses of the casein hydrolysate by an adaptation of the method of Kapeller-Adler (1934; cf. Chattaway, 1945) showed that within narrow limits, the histidine content of the basal medium amounted to 0.1 mg. of histidine/ml. Samples of *l*-(–)-histidine monohydrochloride were obtained from various sources, and some of them purified by recrystallization from 50% (w/v) aqueous ethanol. No differences in physiological activity were observed between crude and recrystallized specimens. Figures for the optical rotation of the purified material in a concentration of 2% (w/v) in *N*-HCl show a value for $[\alpha]_D^{20}$ of +8.8°.

Folic acid (a '7.7% concentrate' kindly supplied by Prof. R. J. Williams) was invariably added in aqueous solution prior to sterilization, which was carried out in steam at 15 lb./sq.in. for 10 min.

A sample of synthetic pteroyl-glutamic acid (Angier, Boothe, Cosulich, Fahrenbach, Hutchings, Hultquist, Kuh,

Mowat, Northey, Seager, Semb, Sickels, Smith, Stokstad, Subbarow & Waller, 1945, 1946) was also used in certain experiments, being added to media in the required dilutions in aqueous solution before autoclaving.

RESULTS

The effect of histidine in the absence of folic acid

Various concentrations of *l*-histidine monohydrochloride were added to a series of tubes before autoclaving, and the results for titrations after 18, 40 and 64 hr. are shown in Fig. 1. The corresponding

Fig. 1. The effect of histidine on the acid production of cultures of *Strep. lactis* R when grown in the absence of folic acid. Curves relating to different periods of incubation.

values for a culture containing no added histidine, but 0.01 μ g. of folic acid/ml. are 4.55, 12.70 and 13.70 ml. 0.05 *N*-acid/10 ml. of medium, and for a culture with no addition 0.60, 2.95 and 4.40 ml. By a comparison of these figures with the curves in Fig. 1, it can be seen that when added in large quantities histidine gave the same degree of acid production as folic acid. The addition of 0.5 mg./ml. of histidine hydrochloride increased the acid production above that observed in the blank, but it was not until a concentration of 80 mg./ml. was reached that an increase in acid production comparable with that promoted by 0.01 μ g. of folic acid was obtained. Histidine therefore, although potentially active as a substitute for folic acid, is 8×10^5 times less active at high concentrations. This ratio did not however remain constant over the whole range of concentrations. The rate of increase of acid production which was brought about by an increase in histidine concentration was such that when the concentrations of histidine and folic acid which separately induce identical degrees of acid

production are plotted against one another on a logarithmic scale (Fig. 2) the curves are linear in form. This indicates that the relationship between the molecular concentration of histidine (x) and of folic acid (y) (the molecular weight of folic acid being taken as 400 on the basis of the evidence of Mitchell & Williams, 1944) has the form of the equation $x = y^n$. The complex linear relationship between the log concentrations of the two additions indicates that there are two values for n , one of which is applicable at low concentrations, the other at high. These two values calculated from the slopes of the respective curves in Fig. 2, are 0.15 and 0.30, while the value for the comparison of histidine and pteroyl-glutamic acid which gives only one straight line over the considerable range of concentrations examined, is 0.60.

Fig. 2. The relationship between the log molar concentrations of histidine and two growth factors required to produce identical effects in the acid production of *Strep. lactis*.

The large concentrations of histidine, which were required to produce activity comparable to that of folic acid, may indicate that the activity is due to an impurity not chemically detectable but detectable by growth response. Experiments were carried out to determine whether such an impurity could be responsible for the activity.

Adequate acid production was obtained by the inclusion of 2.5 mg. of histidine hydrochloride/ml. of medium, and this concentration was employed to estimate the effect of steam sterilization on the acid production caused by such an addition. Pairs of tubes containing this concentration, added respectively before and after sterilization, were inoculated and incubated. Marked differences in turbidity and acid production were invariably observed. Typical figures for titrations carried out after 18 hr. on three separate pairs of tubes are given in Table 1. These figures indicate that when

heated with the medium, histidine becomes active, being entirely inactive and in certain cases significantly inhibitory when unheated. As yet the component of the medium which may be considered responsible for the production of increased activity by reacting with the histidine during autoclaving has not been identified, since apparently none of the components of the medium has the power of activating histidine when autoclaved separately with this amino-acid. Adenine, guanine and uracil increased the activity slightly but not to the same extent as the complete medium.

Table 1. *The effect of steam sterilization on histidine activity*

(10 ml. quantities of medium were treated with 25 mg. of *L*(-)-histidine monohydrochloride either before or after sterilization for 10 min. at 15 lb./sq.in. pressure. Acid production is measured in terms of ml. of 0.05 N-NaOH/10 ml. medium after 18 hr. incubation at 30°).

Treatment	Acid production		
	1	2	3
Heated	1.95	2.00	1.90
Unheated	0.45	0.40	0.50
Blank	0.80	0.60	0.80

Further evidence against the suggestion that the activity was due to an impurity is the fact that the *d*- and *l*-isomers were equally active. Acid production figures for pairs of cultures containing 5 mg./ml. of *d*- or *l*-histidine hydrochloride consistently fell around the following values after 18 hr. incubation: Blank 0.04 ml.; *d*-histidine 1.07 ml.; *l*-histidine 1.10 ml.

The effect of histidine and folic acid added simultaneously

The simultaneous addition of histidine and folic acid to the basal medium produces a variety of responses according to the concentrations employed.

Eight series of tubes were employed in a typical experiment containing folic acid in concentrations varying from 0.1 to 0.0001 μ g./ml. and to each series histidine hydrochloride was added in concentrations varying from 0 to 5.0 mg./ml. The titration figures for the 8 series of tubes after 18 hr. are given in Table 2. Low concentrations of histidine had the power of inhibiting the growth and acid production of the organism when added simultaneously to certain sub-threshold concentrations of folic acid. When this threshold value of 0.001 μ g./ml. of folic acid was exceeded, however, the inhibition was reversed and normal, or at higher concentrations enhanced, growth and acid production occurred. Even in the absence of folic acid histidine was inhibitory if administered in concentrations of less than 0.5 mg./ml. Since the basal medium contained

approximately 0.1 mg. of histidine/ml. the effect of complete absence of the amino-acid cannot be assessed, but minimal acid production occurred when the concentration of added histidine reached

Table 2. *Acid production by Strep. lactis R in the presence of histidine and folic acid*

(Acid production was measured in terms of ml. of 0.05 N-NaOH/10 ml. medium to return to pH 7.4 after incubation for 18 hr. at 30°.)

L-(-)-Histidine HCl (mg./ml.)	Additions of folic acid (μ g./ml.)					
	0	0.0001	0.001	0.005	0.01	0.1
	Acid production (ml.)					
0	0.70	0.80	1.05	1.55	2.65	4.70
0.1	0.50	0.60	1.10	1.60	2.70	5.10
0.2	0.45	0.55	1.15	1.65	2.75	5.35
0.4	0.60	0.75	1.30	1.80	2.95	5.60
0.5	0.75	0.95	1.40	1.85	3.05	5.75
1.0	0.90	1.15	1.65	2.20	3.45	6.16
2.5	1.15	1.30	1.70	2.35	3.90	7.30
5.0	1.30	1.60	2.75	3.05	5.30	8.90

a level between 0.2 and 0.4 mg./ml. The inhibition which was observed at concentrations around this level was apparent over the whole of the period of incubation, and was not an increase in the lag phase followed by a period of enhanced activity, as occurs under other conditions (see below).

When greater concentrations of histidine were added simultaneously with folic acid, inhibition occurred for the first 24 hr. but thereafter enhanced growth and acid production supervened, until ultimately full utilization of the glucose supply took place. Fig. 3 shows a series of curves relating histidine additions up to 80 mg./ml. to acid production, in cultures containing 0.001 μ g. of folic acid/ml., after varying periods of incubation.

The inhibition observed in the presence of low concentrations of histidine was different from that produced by high concentrations, since the latter caused only an increase in the lag phase. Such a retardation of the onset of growth and acid production could be observed on the addition of a concentration of sodium chloride equivalent to that present in the cultures from the neutralization of

the histidine hydrochloride. This increased lag was only apparent when folic acid was present in the medium (cf. Fig. 1).

Fig. 3. The acid production induced by the addition of high concentration of histidine hydrochloride to cultures containing 0.001 μ g. of folic acid/ml. of medium.

Although at the end of 18 hr. it appeared that histidine could raise the acid production above the level obtained with folic acid alone, it is apparent from the upper curves of Fig. 3 that the effect was transitory and was merely an increase in the initial rate of acid production, since the ultimate acid production was independent of the histidine concentration.

The reversal of quinoxaline inhibition

Tubes were prepared containing, in addition to the normal constituents of the basal medium, quinoxaline (2.5 mg./ml.) and histidine hydrochloride in concentrations from 2.5 to 10 mg./ml. The figures of the titration of the acid produced by such cultures after varying periods up to 143 hr. are given in Table 3. It will be seen that these concentrations of histidine were inadequate for complete reversal of the inhibition by quinoxaline, but a similar partial reversal was observed with sub-optimal concentrations of folic acid. The inclusion of greater concentrations of histidine with the

Table 3. *The reversal of quinoxaline inhibition by histidine*

(Acid production is measured in terms of ml. of 0.05 N-NaOH/10 ml. medium required to bring to pH 7.4 after incubation for the periods indicated at 30°.)

Additions (mg./ml.)	Time of incubation (hr.)				
	18	40	66	120	143
	Acid production (ml.)				
2.5 quinoxaline	—	0.15	1.75	3.35	4.15
2.5 quinoxaline + 10 histidine HCl	1.50	3.70	5.25	6.60	6.60
2.5 quinoxaline + 5 histidine HCl	1.15	2.65	3.80	5.35	5.35
2.5 quinoxaline + 2.5 histidine HCl	0.45	2.20	3.55	4.60	4.60
10 histidine HCl	1.60	5.20	8.20	11.15	13.35
5 histidine HCl	1.30	2.85	6.95	8.15	9.95
2.5 histidine HCl	1.15	3.60	5.85	7.35	8.85

object of completely reversing the inhibition would be inconclusive owing to the inhibition produced by the sodium chloride content (cf. Fig. 3). With concentrations up to 10 mg. histidine HCl/ml., however, there was a steady increase in reversal of the inhibition by quinoxaline.

DISCUSSION

It appears possible that folic acid can be replaced by histidine as a growth factor for *Strep. lactis* R. There is, however, some evidence to show that although histidine is the source of the activity, the amino-acid itself is not active—it is in fact inhibitory when added aseptically after sterilization of the medium, and even after heating with the medium, if added in small enough quantity. This may be due to the inadequacy of the active material formed under these conditions, to counteract the effect of the unchanged histidine. The evidence of Fig. 1 may indicate an equilibrium reaction between histidine and some component or components of the medium, the concentration of the final product being directly proportional to the concentration of added histidine.

The rate of increase of activity of histidine with concentration when compared with folic acid, as indicated by the slopes of the curves in Fig. 2, throws some light on the relationship between the factor produced from histidine and the folic and pteroyl-glutamic acids. The values for n of 0.15 and 0.30 indicate that below a certain level of folic acid a hundredfold increase in concentration of this preparation is required to produce an effect equal to a twofold increase in histidine concentration. Above this level the comparable factor is four. Evidence in favour of increased folic acid activity may be obtained by a comparison of histidine and pteroyl-glutamic acid, which when examined over the same range of histidine concentration as for folic acid (above) does not show any similar abrupt change, the value for n being constant over the whole range at 0.60, i.e. a hundredfold increase in pteroyl-glutamic acid concentration being equivalent to a sixteenfold increase in histidine. This may indicate that the synthetic pteroyl-glutamic acid maintains a constant activity ratio towards histidine, whereas the possibly complex (cf. Hall, 1946a) folic acid increases in relative activity when present in concentrations of more

than 0.01 μ M. These observations answer the question of the origin of the activity associated with histidine, for if the activity were due to the presence of either folic acid or pteroyl-glutamic acid, the activity ratio would be unity. It is, therefore, reasonable to assume that neither of these two substances is responsible.

Since the suggestion of Stokes (1944) that thymine does not replace folic acid, but supplies preformed the product of the folic acid utilizing reaction, it was of interest to see whether the activity associated with histidine might agree with this suggestion. From the quinoxaline data it would appear that histidine is replacing folic acid as such or at least is supplying a product of the quinoxaline-inhibited step in the metabolism. It may equally well, however, be offering an alternative route for metabolism of glucose. If not identical, this route is similar, since the total amount of acid produced is independent of the presence of histidine, and lactic acid is always the sole product. The other observations, however, tend to indicate that a reaction product is formed by histidine with some component of the medium under the condition of sterilization.

Whatever the process by which this active substance is formed from histidine, it appears clear that it is playing an important role in the metabolism of glucose by *Strep. lactis* R. While it may bear no structural relationship to folic acid, it appears to maintain in operation a metabolic process not unlike that involving folic acid.

SUMMARY

1. The effect of histidine on the growth and acid production of *Strep. lactis* R, both alone and in the presence of folic acid has been examined.
2. Indications have been obtained of a reaction product between histidine and some component of the medium formed during autoclaving, which has activity similar to a member of the 'folic acid group' of growth-factors.
3. This material is neither folic acid nor pteroyl-glutamic acid.

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The Purification of Azoprotein Antibodies by the Dissociation of Specific Precipitates

By F. HAUROWITZ, SH. TEKMAN, MÜRVET BILEN AND PAULA SCHWERIN
Department of Biological and Medical Chemistry, University of Istanbul

(Received 9 November 1946)

Antibodies can be separated from a part of the serum proteins by electrophoresis or by the fractional precipitation with ammonium sulphate. Purification by these or similar non-specific methods is limited by the fact that some of the serum proteins have properties almost identical with those of the antibodies. The separation of antibodies from such proteins is rendered possible, however, by the application of specific methods. These involve the precipitation of the antibody by the homologous antigen and the dissociation of the antibody complex into its components. The main difficulty consists in finding conditions, under which only the antibody is dissolved, while the antigen remains in the precipitate. This difficulty does not arise if cellular or bacterial antigens are used (Heidelberger & Kabat, 1938). Soluble antigens have been made insoluble by coupling them with stroma (Landsteiner & van der Scheer, 1936) or by adsorbing them on charcoal particles (Svensson, 1943). The antibody can be split off from these particles by dilute acetic acid. On the other hand, antibodies to the specific polysaccharides of pneumococci are extracted from the precipitate by lime water or baryta, while Ca or Ba compounds of the polysaccharide remain undissolved (Felton & Bailey, 1926; Heidelberger & Kabat, 1938).

Our own attempts to purify anti-azoproteins were based originally on the experience that azoproteins are precipitated by acids, while globulins (antibodies) remain dissolved (Haurowitz, 1936a). Accordingly we have attempted splitting of antibodies from the antigen-antibody precipitates by dilute acids.

EXPERIMENTAL

Preparation of antigens and immunization. Pseudo-globulin from beef or sheep serum was prepared as described previously (Haurowitz, Sarafyan & Schwerin, 1941). The protein (2 g.) was coupled with 2 mmol. of anthranilic, sulphanilic, arsanilic acid or *p*-aminobenzylamine (Haurowitz, 1936b, 1942). The azoproteins were purified by re-precipitation and dialysis and were mixed with 1% of solid NaCl. They were injected into rabbits subcutaneously as described in the papers mentioned. The immune sera were stored at -12° and thawed out shortly before the addition of the antigen.

Precipitation of anthranilic acid-azoglobulin by buffer solutions. A 5% solution of this azoprotein (0.2 ml.) was mixed with 2 ml. of the standard buffer solution of Teorell & Stenhagen (1938) and adjusted to the intended pH by the addition of the required amount of 0.1 N-HCl. The volume was adjusted to 2.5 ml. by the addition of water and the mixture kept at room temperature for 24 hr. The tubes were then centrifuged and the amount of azoprotein in the supernatant solution determined colorimetrically. The amount of precipitated azoprotein was obtained as the difference between the total and the dissolved azoprotein. The results are shown by the following figures:

pH:	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Precipitated azoprotein (mg.):	0	4.2	5.4	6.2	0	0	0

Precipitation of the antibodies. Different amounts of the immune sera, varying from 3 to 55 ml., were precipitated by the addition of solutions containing from 1 to 3% of the homologous antigen. The antigen solution was added either in a single dose or fractionally, in amounts of 0.05–3.0 mg., until no further distinct precipitate was formed (Haurowitz, 1942). Some of the sera (Table 1, no. 263) were precipitated in both ways in parallel experiments. The precipitates obtained by the fractional addition of the antigen were