The Formation of Diazotizable Amine and Hypoxanthine by a Yeast: Possible Implications in the Biosynthesis of Purines

BY N. CHAMBERLAIN AND C. RAINBOW

Department of Applied Biochemistry, University of Birmingham

SUMMARY: A diazotizable amine and hypoxanthine accumulate in cultures of Yeast 47 grown in a defined medium containing added methionine and suboptimal biotin. Amine accumulation is depressed by the presence either of added adenine, or of certain amino acids, of which aspartate, norleucine, norvaline and threonine are the most effective. The amine differs from 4-amino-5-imidazolecarboxamide and the corresponding amidine. Spectrophotometric measurements suggest that the amine lacks the completed 6-membered ring structure of the purine skeleton, but it has biological activity in lieu of adenine for the adenine-requiring Yeast 19, and of guanine for a strain of *Lactobacillus brevis*.

Hypoxanthine substitutes for adenine in the growth of Yeasts 19 and 47, and for adenine + guanine in *Lactobacillus brevis*, which suggests that hypoxanthine is a bio-precursor of both adenine and guanine. The accumulation of hypoxanthine in cultures of Yeast 47 may thus be the result of a synthetic block, conditioned by biotin deficiency, which prevents the conversion of hypoxanthine to adenine and guanine. The possible relationships between diazotizable amine and hypoxanthine are discussed.

Chamberlain, Cutts & Rainbow (1952) reported the conditions under which Yeast 47 formed pigment and accumulated a diazotizable amine in liquid medium. This paper reports a study of the constituents present in amine-containing concentrates prepared from culture filtrates of Yeast 47.

METHODS

Test organisms. Three organisms were used:

Saccharomyces cerevisiae (Yeast 47). For growth in a defined medium, Yeast 47 required added biotin and *p*-aminobenzoic acid (*p*-AB). The *p*-AB was replaceable, at least in part, by relatively large concentrations of adenine, methionine and histidine (Cutts & Rainbow, 1950).

Schizosaccharomyces octosporus (Yeast 19). For growth in a defined medium, Yeast 19 required added adenine, methionine and histidine, but not *p*-AB (Northam & Norris, 1951).

Lactobacillus brevis. This was the heterofermentative lactobacillus used by Chamberlain *et al.* (1952). For growth it required added adenine, guanine and pteroylglutamic acid (PGA).

Cultures of the yeasts were maintained as described by Cutts & Rainbow (1950). *Lactobacillus brevis* was maintained as stabs in 10 % malt wort agar supplemented with 0.1 % Difco yeast extract and 0.5 % L-arabinose.

Media. Basal medium A (for Yeast 47) was that described by Cutts & Rainbow (1950), but biotin was omitted, 0.10 μ g. *p*-AB/ml. was added and the pH adjusted to 4.8.

Basal medium B was as A, but *p*-AB was omitted and the following additions ($\mu\text{g./ml.}$) made: D-biotin (0.1), adenine (50), DL-methionine (100), L-histidine HCl (100). This medium was used for tests involving Yeast 19 and for Yeast 47 in *p*-AB-free medium.

Basal medium C (for *Lactobacillus brevis*) was as follows. Per 100 ml. final volume, in g.: glucose, 2.0; $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ (A.R.) 3.2; casein acid hydrolysate ('vitamin free', Allen and Hanbury) 1.0; $(\text{NH}_4)_2\text{SO}_4$ (A.R.) 0.3; NaCl (A.R.) 0.5; in mg.: L-cystine, 20; DL-tryptophan, 20; KH_2PO_4 (A.R.) 50; K_2HPO_4 (A.R.) 50; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (A.R.) 10; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (A.R.) 0.5; FeCl_3 (A.R.) 0.1; in $\mu\text{g.}$: thiamine (100); pyridoxin (100); nicotinic acid (100); calcium D-pantothenate (100); riboflavin (50); *p*-AB (20); PGA (10); D-biotin (0.02). Adjustment was made to pH 6.5, but, after sterilizing by heating in the autoclave momentarily to 10 lb. pressure, the pH fell to 6.2, the optimum for *L. brevis* in this medium.

Inocula were prepared as follows:

Yeast 47: grown up in basal medium A containing 0.1 $\text{m}\mu\text{g.}$ D-biotin/ml., other details being according to Cutts & Rainbow (1950).

Yeast 19: as for Yeast 47, but grown up in basal medium B.

Lactobacillus brevis: grown up at 28° for 16–24 hr. in basal medium C fortified with 10 $\mu\text{g.}$ of adenine and of guanine/ml. Harvesting and washing the cells was as above. One drop (c. 20 $\mu\text{l.}$) of the final suspension of the washed cells was used/3 ml. of medium to inoculate auxanograms. For tube tests, three drops of the final suspension were shaken with 6 ml. sterile 0.85 % saline and one drop of this dilute suspension used to inoculate each tube.

Tube tests (6 ml. cultures) were carried out as described by Northam & Norris (1951). Incubation was at 25° (yeasts) or 28° (*Lactobacillus brevis*).

Auxanograms were carried out in the appropriate basal medium at 28°, according to Cutts & Rainbow (1950), except that, in most cases, substances for test were applied to the agar as circular paper disks cut from papergrams with a sharp cork borer, after the position of the spot chosen for test had been located as indicated below.

Diazotizable amine was determined as described by Chamberlain *et al.* (1952), but omitting the acetylation procedure.

Chromatography. Chromatograms were run on Whatman no. 4 special chromatography paper (22½ in. long) in a glass cabinet maintained at 23–25°, the solvent being *n*-butanol + acetic acid + water (Woiwod, 1949). Test materials were applied as 10 $\mu\text{l.}$ spots, larger spots (up to 50 $\mu\text{l.}$) being obtained, if necessary, by successive applications of 10 $\mu\text{l.}$ portions, with drying between applications. Under these conditions, the run covered the length of the paper in 12½ hr., but no ill effects resulted when the papers were left for 18 hr. After runs, papers were dried in a current of warm air and compounds then detected as follows: purines by ultraviolet (UV) photography (Markham & Smith, 1949); amino acids by the ninhydrin reaction (Woiwod, 1949); diazotizable amine by the Bratton & Marshall (1939) reaction. For the latter reaction the reagents were made up in ethanolic solution to ensure rapid drying after the

application of each spray to minimize spreading of the spots. The first spray consisted of a mixture of 1 vol. aqueous 4N-HCl + 1 vol. 0.2 % sodium nitrite in 80 % (v/v) ethanol. After 3 min., 2 % ammonium sulphamate in 75 % (v/v) ethanol was sprayed on, followed rapidly by 0.1 % N-(1-naphthyl)-ethylenediamine 2HCl in absolute ethanol. Diazotizable amine showed as a pink spot after this treatment.

To examine concentrates of diazotizable amine, three identical spots were chromatographed side by side at one edge of the paper, suitable reference spots (e.g. authentic purines) being included with each run. After the run the complete papergram was examined for purines and two strips, corresponding to the outer spots of concentrate, cut from the paper for further examination, one for amino acids and the other for diazotizable amine. In this way, the positions of amino acids, UV-absorbing substances and diazotizable amine were established on the main papergram, which, being untreated by reagents, was utilizable for biological tests by auxanography.

Spectrophotometry. Measurements were made in 1 cm. quartz cells with the Unicam SP 500 quartz spectrophotometer. Distilled water was used as reference liquid, except when papergram eluates in 0.1 N-HCl were measured. In such cases, a blank eluate in 0.1 N-HCl, obtained from an unoccupied area of the papergram, was used.

'Aldehyde-free' ethanol was prepared by refluxing absolute ethanol for 1 hr. in the presence of 2:4-dinitrophenylhydrazine (1 g./100 ml.) and a trace of H_2SO_4 . The fraction which distilled at 77.5–78.5° was collected for immediate use.

EXPERIMENTAL

Preparation of amine concentrates

To produce diazotizable amine in quantities sufficient for experimental work, the following procedure was adopted. Two portions (each 1 l.) of medium A fortified with 0.02 m μ g. D-biotin and 500 μ g. DL-methionine/ml., were sterilized (15 lb./sq. in. momentarily) in 6 l. Erlenmeyer flasks loosely plugged with cotton-wool. Each flask was inoculated with 6 ml. of inoculum of Yeast 47 and incubated at 21° for 6 days. The pink cell crop was filtered off (Green's paper, no. 503), the filtrates (pH 4.4) combined, and a freshly prepared solution of 8 g. mercuric acetate in 150 ml. water added slowly with stirring. The precipitate was allowed to settle overnight at 4° and collected by decantation followed by centrifugation. After washing twice with water, the precipitate was dispersed in 180 ml. 0.1 N- H_2SO_4 and decomposed by a current of H_2S passed for 1 hr. Excess H_2S was removed by a current of air, a previously determined volume of barium hydroxide solution added to neutralize the H_2SO_4 and the mixed precipitates of HgS and BaSO_4 centrifuged off. The supernatant was evaporated *in vacuo* below 40° to 10 ml. and treated with 90 ml. of 'aldehyde-free' ethanol. After standing for 2 hr. at 4°, the precipitate was centrifuged off, ethanol removed from the supernatant by evaporation *in vacuo* to small volume and the final solution made up to 25 ml. This solution (pH 4.0), containing about 50 % of the amine present in the original culture

filtrate, was used in the subsequent experimental work. The most significant losses of amine in this preparation appeared to occur during the decomposition of the mercury precipitate.

Chromatography of amine concentrates

Table 1 shows the R_f values of diazotizable amine, purine and amino acids detected in amine concentrates by paper chromatography, together with the R_f values of authentic reference compounds. The predominant constituents of the amine concentrate thus revealed were:

Diazotizable amine: this spot was positionally distinct from the amino acids and purines present, with the possible exception of guanine. A faint UV-absorbing spot was visible in the amine position, which may have represented the amine itself, or very small amounts of guanine. The first possibility was regarded as the more likely from the spectrophotometric evidence (see below). No other diazotizable material was visible on the papergrams.

The UV-absorbing spot was positionally distinct from amine and corresponded closely with hypoxanthine. Its size and intensity indicated the presence of about 20 μg . hypoxanthine/30 μl . amine concentrate. There was some overlap between this spot and the large ninhydrin-positive spot ((c) below). The latter, however, was not UV-absorbing.

The large ninhydrin-positive spot corresponded in position to methionine. Methionine is precipitated by mercuric salts and would therefore be concentrated along with diazotizable amine owing to its presence in the original medium.

Table 1. R_f values of substances present in concentrates

Identical conditions for both chromatograms; temperature, 23–25°; duration of run, 12 hr.; solvent, *n*-butanol + acetic acid + water; spots: amine concentrate, 30 μl ., reference substances, 20 μg .

	Description of spot	Chromatogram		Intensity of spot
		(1)	(2)	
Amine concentrate	Ninhydrin positive	0.11	0.13	Weak
		0.15	0.17	Weak
		0.20	0.22	Weak
		0.23	0.25	Weak
		0.33	0.34	Weak
	Diazotizable UV-absorbing	0.50	0.52	Very strong
		0.36	0.38	Strong
Reference substances		0.49	0.50	Very strong
	Guanine	0.39	0.39	Strong
	Xanthine	0.42	0.42	Strong
	Hypoxanthine	0.49	0.50	Strong
	DL-Methionine	0.50	0.52	Strong
	Adenine	0.61	0.62	Strong

Neither amine nor the UV-absorbing constituent (UVAC) was detectable in concentrates prepared similarly from cultures of Yeast 47 which contained optimal biotin (1 $\text{m}\mu\text{g./ml.}$) added to the medium. With suboptimal biotin (0.02 $\text{m}\mu\text{g./ml.}$) but no added methionine, both amine and UVAC were formed,

only in much smaller amounts than when methionine was added. When methionine was not added the only source of methionine was that synthesized by the cells themselves.

Spectrophotometry of amine concentrates

Absorption curves were measured over the range 200–300 m μ . on solutions of (i) pure reference substances (10 μ g./ml.); (ii) as (i), but obtained by cutting the appropriate spots from papergrams and eluting with 0.1 N-HCl; (iii) the chief spots from amine concentrates, isolated from papergrams as in (ii).

The results (Table 2) confirmed the identity of UVAC with hypoxanthine and indicated that the diazotizable amine was not contaminated with detectable amounts of guanine. The reference purines and pyrimidines gave characteristic end-absorption at *c.* 200 m μ ., with peak absorption at 248–284 m μ . By contrast, diazotizable amine, methionine and histidine showed end-absorption but no peak within the latter range.

Table 2. *Values of $\lambda_{\max}$ for reference substances and constituents of amine concentrate*

Stock solutions of reference substances, 10 μ g./ml. in 0.1 N-HCl, except DL-methionine, 50 μ g./ml.; papergram extracts, 5 ml. of eluates in 0.1 N-HCl from 5 $\times$ 30 μ l. spots of amine concentrate.

	$\lambda_{\max.}$ (m μ .) Stock solutions		Papergram extracts	
	(1)	(2)	(1)	(2)
Amine concentrate:				
UV-absorbing spot	—	—	<200	248.0
Diazotizable amine	—	—	206	No peak
Reference substances:				
Adenine	204	262.5	205.5	262.5
Guanine	202.5	248.5	204	248.5
Hypoxanthine	<200	248.0	<200	248.0
Uric acid	230	283.5	—	—
Xanthine	202	265.0	—	—
Cytosine	209.5	275.5	—	—
Thymine	206	265.0	—	—
Uracil	204	258.5	—	—
DL-Methionine	204	No peak	—	—
L-Histidine HCl	210	No peak	—	—

Biological activity of constituents of amine concentrates

Auxanograms. Circles corresponding to constituents of the amine concentrate and to known reference substances (10 μ g.) were cut from papergrams and tested for biological activity as substitutes for adenine in the growth of Yeasts 19 and 47 and for adenine and guanine in the growth of *Lactobacillus brevis*. For *L. brevis*, three auxanograms were used, involving medium C supplemented (a) with 10 μ g. guanine/ml. (to test capacity to substitute for adenine), (b) with 10 μ g. adenine/ml. (to test capacity to substitute for guanine), or (c) with no addition (to test capacity to substitute for adenine + guanine).

The following substances were tested on each plate: adenine, hypoxan-

thine, guanine, xanthine, DL-methionine, diazotizable amine and UVAC. Positive responses, indicated by zones of growth visible, unless otherwise stated, after 24 hr., were observed as follows:

Yeast 47: adenine, hypoxanthine, UVAC.

Yeast 19: adenine, hypoxanthine, UVAC, diazotizable amine; guanine induced a slight response after 48 hr.

Lactobacillus brevis: plate (a) adenine, hypoxanthine, UVAC; plate (b) guanine, xanthine, hypoxanthine, UVAC and diazotizable amine; plate (c) hypoxanthine, UVAC.

These results showed that hypoxanthine and UVAC had identical biological properties in these tests. Either substituted for adenine in the growth of Yeasts 19 and 47, and for adenine + guanine in the growth of *Lactobacillus brevis*. These facts corroborate the spectrophotometric and chromatographic identification of UVAC as hypoxanthine. Diazotizable amine had activity in lieu of adenine for Yeast 19, and for guanine (but not adenine) for *L. brevis*.

Table 3. *Biological activity of amine concentrates*

Tube tests: growth as Spekker readings after 70 hr. Adenine (col. 6) = 100 μ g./ml. (yeasts) or 10 μ g./ml. (*L. brevis*); *L. brevis* tested in presence of (a) 10 μ g. guanine/ml.; (b) 10 μ g. adenine/ml.; (c) no purines; * indicates 10 μ g. each of adenine and guanine/ml.

	Amine concentrate (ml./6 ml. basal medium)				Adenine
	0	0.02	0.04	0.08	
	Growth (Spekker reading)				
Yeast 19	0.06	0.44	0.56	0.68	0.99
Yeast 47	0.03	0.18	0.32	0.57	1.07
<i>Lactobacillus</i> { (a)	0.01	0.01	0.02	0.02	—
<i>brevis</i> { (b)	0.01	0.38	0.60	0.85	—
{ (c)	0.01	0.16	0.31	0.49	0.94*

Table 4. *Antagonism of hypoxanthine by guanine in the growth of Lactobacillus brevis*

Tube tests; growth as Spekker readings after 47 hr.

	Added hypoxanthine (μ g./ml.)				
	0	2.5	5.0	10.0	20.0
	Growth (Spekker reading)				
Basal medium C	0.06	0.19	0.30	0.45	0.64
Basal medium C + 10 μ g. guanine/ml.	0.07	0.08	0.08	0.09	0.09

Tube tests on unseparated amine concentrate confirmed that it substituted for adenine in the growth of Yeasts 19 and 47, and for guanine or for adenine + guanine in the growth of *Lactobacillus brevis*. Although these responses were expected from the hypoxanthine (UVAC) content of the concentrate, there was an unexpected inability to replace adenine for *L. brevis* (Table 3). The effect of guanine on the growth of *L. brevis* in basal medium C supplemented with hypoxanthine was therefore studied. The results (Table 4) showed that the growth-promoting activity of hypoxanthine was antagonized by guanine.

This antagonism may be compared with that found by Fairley & Loring (1949) to operate for a mutant of *Neurospora*. The discrepancy that hypoxanthine substituted for adenine in the auxanograms, but not in the tube tests, may be explicable in terms of a concentration gradient operating in favour of the test material in the auxanograms.

*Comparison of diazotizable amine with 'carboxamide'
and 'carboxamidine'*

The properties of synthetic 4-amino-5-imidazolecarboxamide (carboxamide) and the corresponding amidine (carboxamidine), which are recognized as possible bio-precursors of purines, differed from those of diazotizable amine as follows:

(1) The peak absorptions of the coloured substances formed in the Bratton & Marshall reaction differed. Measured approximately by means of the Ilford 601-606 series of filters in the Spekker absorptiometer, the maximum absorption of the coloured solution from diazotizable amine occurred with filter 603, in contrast to filter 604 in the other cases.

Fig. 1. Ultraviolet absorption curves of solutions in 0.1 N-HCl of diazotizable amine (—); hypoxanthine (...); carboxamide(— · —); carboxamidine (---). Concentrations 10 μg./ml., except amine which consisted of 5 ml. of extract derived chromatographically from 5 × 30 μl. samples of amine concentrate.

(2) The ultraviolet absorption curves differed (Fig. 1).

(3) On chromatographing, the R_F values obtained were: amine, 0.36; carboxamidine, 0.43; carboxamide, 0.47. For the chromatographic detection of the two latter compounds, the UV photographic technique was applied, since both substances absorbed appreciably at 257 mμ.

(4) The biological activities of carboxamide and carboxamidine were distinct from those of diazotizable amine. Using small amounts of solid

substances applied to the surface of auxanograms with a platinum needle, it was shown that: neither carboxamide nor carboxamidine possessed activity as adenine substituents for Yeast 47; carboxamidine, but not carboxamide, substituted for adenine in Yeast 19; carboxamidine substituted for adenine, for guanine or for adenine + guanine in *Lactobacillus brevis*, although, in each case, the zone of growth was small compared with that induced by a roughly equal quantity of hypoxanthine. This suggested that carboxamidine was required in relatively high concentration. Carboxamide showed no capacity to substitute for purines in the growth of *L. brevis*.

*Effect of amino acids and purines on the accumulation
of amine in culture filtrates*

Tube tests were used to study the effects of added amino acids and purines on amine formation in an otherwise suitable medium. The results (Table 5) showed that aspartate, threonine and norvaline strongly depressed amine formation, as did norleucine in the early stages. Increased amine formation after 192 hr. in the presence of glutamate, lysine and serine was associated with higher final levels of growth, amine formation in terms of unit dry weight of cells being less than, or equal to, the control. There was some suggestion that, after 96 hr., histidine stimulated amine formation.

Table 5. *Effect of amino acids and purines on amine formation*

Tube tests in basal medium A containing 500 μ g. DL-methionine/ml. and 20 μ g. D-biotin/ml. Growth and amine as Spekker readings. Amino acids tested at 500 μ g. (as L-isomer), purines at 50 μ g./ml.

	Growth		Amine		Amine/unit dry-wt. cells	
	96 hr.	192 hr. Spekker readings	96 hr.	192 hr.		
Basal medium plus						
No additions	0.33	0.39	0.13	0.11	0.66	0.45
Amino acids:						
L-Aspartic acid	0.40	0.41	0.03	0.02	0.12	0.08
L-Glutamic acid	0.47	0.59	0.12	0.20	0.36	0.45
L-Histidine	0.31	0.43	0.13	0.16	0.67	0.56
L-Lysine	0.54	0.64	0.18	0.17	0.47	0.35
DL-Norleucine	0.40	0.59	0.07	0.12	0.27	0.27
DL-Norvaline	0.37	0.54	0.05	0.08	0.21	0.19
DL-Serine	0.37	0.52	0.12	0.13	0.51	0.29
DL-Threonine	0.36	0.43	0.07	0.07	0.32	0.24
Purines:						
No additions	0.50	—	0.25	—	0.72	—
Adenine	0.56	—	0.10	—	0.24	—
Guanine	0.50	—	0.24	—	0.69	—
Hypoxanthine	0.50	—	0.25	—	0.72	—
Uric acid	0.50	—	0.16	—	0.46	—
Xanthine	0.51	—	0.23	—	0.64	—

The effect of adenine in depressing amine formation (Table 5) was reported by Chamberlain *et al.* (1952). Of other purines tested, uric acid exerted a slight depressant effect, but added guanine, xanthine and hypoxanthine did not affect amine formation.

The partial prevention of amine formation by norleucine was probably due to competitive interference with methionine utilization (cf. Porter & Meyers, 1945; Lampen & Jones, 1947; Harding & Shive, 1948). For concentration ratios of DL-norleucine to DL-methionine between 0.5 and 2.0, when the concentration of norleucine was increased fourfold from 250 $\mu\text{g./ml.}$, the concentration of amine formed remained fairly constant. Since Yeast 47 did not require added methionine for growth when *p*-AB was present, presumably then synthesizing its own requirements, the norleucine methionine antagonism should be overcome eventually in favour of methionine, as was found to be the case (Table 5).

Since no similar competitive interference with methionine utilization was demonstrable for norvaline and aspartate, the prevention of amine accumulation by these amino acids was not explicable on the above grounds.

DISCUSSION

In view of the accumulation of hypoxanthine as well as diazotizable amine in cultures of Yeast 47 grown in defined medium containing methionine and sub-optimal biotin, some modification of the scheme proposed by Chamberlain *et al.* (1952) is necessary. In particular, it seems that the block caused by biotin deficiency occurs after completion of the purine skeleton (as hypoxanthine), the essential systems affected being either or both of those by which hypoxanthine is transformed to adenine and guanine. The results with *Lactobacillus brevis*, which cannot accomplish the inter-conversion adenine $\rightleftharpoons$ guanine, indicate that hypoxanthine (or a derivative) is a common precursor of adenine and guanine. The same may be true for Yeasts 19 and 47, but less evidently so, because both organisms convert adenine to guanine (via hypoxanthine?), only the reverse process being impaired.

As the formation of adenine and guanine from hypoxanthine involves amination, it is interesting to note that Lichstein & Umbreit (1947) reported the deaminases of aspartic acid, serine and threonine to be specifically activated by biotin. In the case of aspartic acid, the reverse (amination) reaction was also activated by biotin. It is possible to explain our biotin-conditioned synthetic block in the light of these findings. Thus if aspartate (and/or threonine) are amino group donors in the amination of hypoxanthine to adenine (and possibly guanine), then failure to synthesize aspartate by amination (a biotin-dependent reaction) would explain hypoxanthine accumulation in our experiments.

Assuming that amine and hypoxanthine are intermediates related by a simple biosynthetic chain, the following scheme is possible:

In such a sequence in the cell, the actual substances transformed might be derivatives (e.g. ribosides or ribotides) of the amine and purine molecules

indicated. The evidence that diazotizable amine is intermediate in purine biosynthesis is: (a) the circumstances under which amine accumulates, the prevention of that accumulation by added adenine and other indirect evidence (see also Chamberlain *et al.* 1952); (b) the biological activity of amine as a substituent for adenine (Yeast 19) and guanine (*Lactobacillus brevis*). There are, however, a number of objections to such a scheme. Thus, amine did not substitute for adenine in the auxanograms of Yeast 47, whereas hypoxanthine did. Again, if it is permissible to attempt an interpretation of the sequence of events in *L. brevis* in terms of the scheme, the failure of amine to substitute for adenine + guanine, whereas it will do so for guanine, is not immediately explicable if hypoxanthine (which will substitute for adenine + guanine in *L. brevis*) is intermediate between amine and guanine. A further obstacle is to explain the accumulation of two intermediates in the same chain as a result of biotin deficiency. Possibly biotin is also concerned with the conversion of amine to hypoxanthine, in which case the step must be insensitive to biotin deficiency relative to the processes responsible for utilizing hypoxanthine.

Following from the above, it is interesting that amine appears to lack the complete purine ring structure. The spectrophotometric evidence suggests that the pyrimidine ring is lacking, since amine solutions do not show the peak absorption between 245 and 285 $m\mu$, characteristic of the purines and pyrimidines examined. Carbon-6 of the purines is known to be donated by CO_2 (Sonne, Buchanan & Delluva, 1946) the fixation of which is known, in other contexts, to be associated with biotin-requiring systems. It is thus possible that amine may lack carbon-6 of the purine skeleton.

If biotin is not implicated between amine and hypoxanthine in the above scheme, it is necessary to suppose that this conversion is freely reversible, so that the accumulation of hypoxanthine, resulting from biotin-deficiency, would lead to a parallel accumulation of amine.

If amine is not in the biosynthetic chain which yields hypoxanthine, it could arise by a side reaction from hypoxanthine and, in turn, react with cell constituents to form the red pigment always found in cultures in which amine accumulated:

This hypothesis is favoured because, taken together with the suggested roles of aspartate and threonine as amino group donors for the conversion of hypoxanthine to adenine and guanine, it provides a simple explanation of the prevention of amine accumulation by added aspartate and threonine, the

presence of these amino acids permitting normal amination of hypoxanthine, which thus does not accumulate.

The observations of Friedman & Gots (1952) may also be relevant to this explanation. They showed that hypoxanthine and adenine could be reduced to diazotizable amines. Hypoxanthine yielded a mixture of two amines, one of which was carboxamide; the other, and that from adenine, resemble our amine sufficiently in their UV absorption spectra to suggest that they may be related substances.

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