FORMATION OF AMINES BY INTESTINAL MICROORGANISMS AND THE INFLUENCE OF CHLORTETRACYCLINE*

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(Received for publication, January 10, 1955)

One explanation for the stimulus to growth exerted on animals by some chemotherapeutic agents, particularly antibiotics, is that such compounds suppress the microbial formation of toxic substances in the intestine (1–3). The evidence is indirect, being based mainly on the response of intestinal clostridia to antibacterial agents (4), and on the presence of potentially toxic substances such as amines and indole in the large intestine (5). Pure cultures of intestinal microorganisms can produce these compounds in the test tube, but there is no direct evidence to show that important amounts of "toxins" are generated within the intestinal tract. Furthermore, the complicated ecology of the intestinal microflora cannot be reproduced by the study of pure cultures.

We are indebted to Metchnikoff (6) for stimulating an intensive interest in intestinal intoxication. He assumed that bacterial action on protein produced harmful amines or "ptomaines" (7) which led to enterotoxemia; hence he concluded that any diet able to reduce putrefactive activities in the large intestine would favor the well being of the host. Early work showing the intestinal production of pressor amines (8–11) supported his concept. However, later reports suggesting that intestinal amines are oxidized rapidly by enzymes (histaminase and monoamine and diamine oxidases) in the intestinal mucosa and other tissues (12) have cast doubt on the theory. The matter is not yet settled. Mellanby (13) on the one hand reported negligible absorption of amines from the large intestine, while other studies (14, 15) have indicated that significant amounts can be absorbed. Several workers have noted that young rats and human infants possess distinctly lower amine oxidase activity in their tissues than do adults (16–21) which suggests a predisposition in the young to toxemia from amines. Possibly this accounts for the growth promoting effect of antibacterial agents when fed to young animals (22).

A number of amino acid-decarboxylating bacteria (*Escherichia coli*, *Streptococcus faecalis*, *Clostridium* spp., *Lactobacillus* spp., *Proteus vulgaris*) are known to flourish in the large intestine and produce amines from arginine, ornithine,

* This investigation was supported in part by a research grant, E-353 (C2), from the National Institutes of Health, United States Public Health Service, and by a grant-in-aid from the Graduate School, University of Minnesota, Minneapolis.

lysine, tyrosine, glutamic acid, histidine, aspartic acid, phenylalanine, valine, leucine, or diaminopimelic acid (23–26). Under suitable conditions they might be capable of evolving large doses of toxic amines within the intestinal tract. The results of the study to be reported suggest that appreciable amounts of amines actually are produced within the intestine of the rat, and that the production of decarboxylating enzymes is suppressed by the administration of chlortetracycline.[1]

Materials and Methods

Animals and Diets.—Mature female rats of the Sprague-Dawley strain, housed individually in wire-bottomed battery cages, were fed for a period of 1 week one of the following rations: (*a*) Purina "fox checkers;" (*b*) "fox checkers" + 100 P.P.M. crystalline chlortetracycline hydrochloride; (*c*) "fox checkers" + histidine hydrochloride (1 per cent) + tyrosine (1 per cent); and (*d*) "fox checkers" + histidine hydrochloride (1 per cent) + tyrosine (1 per cent) + 100 P.P.M. crystalline chlortetracycline hydrochloride. The 4 or 5 animals maintained on each ration were given both feed and water *ad libitum*. The animals were sacrificed by means of a sharp blow at the base of the skull.

Preparation of Material for Manometric Measurement of Amino Acid Decarboxylases:

(*a*) *Washed Contents of the Gastrointestinal Tract.*—Freshly collected feces and contents of different segments of the alimentary canal (stomach, duodenum, jejunum, ileum, cecum, and colon) were made into a thick brei with distilled water and the pH determined with a Beckman model H2 pH meter. The suspensions were homogenized in a Waring blendor for approximately 15 seconds and distilled water was added to give a volume of 500 ml. The material was filtered through several layers of gauze and the filtrate centrifuged at 2,500 R.P.M. in a Servall angle head centrifuge (model SP/X). The supernatant fluid, possibly containing some of the enzymes sought, was saved and the sediment was resuspended in 500 ml. of distilled water and centrifuged as before. The washing process was repeated once again and the sediment was diluted in an appropriate buffer to a concentration of 0.5 mg. nitrogen per ml. in preparation for decarboxylase measurements.

(*b*) *Dried Contents of the Gastrointestinal Tract.*—The thick brei, prepared as previously described, was poured rapidly into a beaker containing 150 ml. of ice cold acetone and set in a freezer (−20°C.) for 30 minutes. The precipitate was dried on a Buchner funnel with slight suction, resuspended in cold acetone, and dried again. Further drying was accomplished by successive washings with 50 ml. volumes of a 1–1 mixture of acetone and ethyl ether and of water-free ethyl ether. The resultant powder was stored *in vacuo* over calcium chloride in a desiccator at 4°C. Dry powders of thrice-washed suspensions of feces and intestinal contents were also prepared in like fashion.

(*c*) *Mixed in vitro Fecal Cultures.*—The four media employed in this phase of the problem are listed in Table I. The inoculum was prepared by the suspension of 1 gm. of freshly voided feces from a rat fed the basal diet in 100 ml. of sterile distilled water. 5 ml. of the fecal suspension was inoculated into 250 ml. of medium. Media A and B were incubated aerobically only, while medium C, with or without 1 P.P.M. chlortetracycline, was incubated under aerobic or anaerobic conditions. Anaerobiosis was achieved in an atmosphere

[1] The trademark of Lederle Laboratories Division, American Cyanamid Co. for the antibiotic chlortetracycline is aureomycin. We are indebted to Dr. Stanton M. Hardy, Lederle Laboratories, Pearl River, New York for furnishing this antibiotic.

of 10 per cent CO_2 and 90 per cent H_2. Aerobic incubation was conducted without agitation in 2-liter Erlenmeyer flasks while anaerobic incubation was carried out in 500 ml. Erlenmeyer flasks. After a 20 hour incubation period at 37°C., the cells were harvested by centrifugation and the supernatant liquid stored at 4°C. in order to be available later for the extraction of amines. The mass of heterogeneous cells was washed twice with 250 ml. of distilled water and suspended in an appropriate buffer at a cell concentration equivalent to 0.5 mg. nitrogen per ml. In this state the resting cells were ready for manometric estimation of amino acid decarboxylases.

TABLE I

Media Used in the Manometric Study of Amino Acid Decarboxylation by Mixed Fecal Cultures

Constituent	Medium designation		
	A*	B*	C‡
	per cent	*per cent*	*per cent*
Glucose	1.0	0.1	0.05
Bacto tryptone	1.0	1.0	1.0
Bacto yeast extract	1.0	1.0	1.0
Dibasic potassium phosphate	0.5	0.7	0.6
Sodium thioglycolate	0.05	0.05	—

* Used for testing effect of pH on amine formation.

‡ Used for testing effects of oxygen tension and chlortetracycline on amine formation.

Manometric Determinations of Amino Acid decarboxylases:

Amino acid decarboxylase activity in the various preparations was determined in a Warburg apparatus using the basic techniques established by Gale and coworkers (23). To each cup was added 2 ml. of M/5 buffer containing 1 mg. of nitrogen (resting cells) or 25 mg. of nitrogen (dried cells). An additional 0.7 ml. of the same buffer was added and 0.5 ml. of a buffered M/20 solution of one of the following amino acids was placed in the side arm: histidine dihydrochloride, arginine hydrochloride, lysine hydrochloride, glutamic acid hydrochloride, and tyrosine. Buffers, composed of either sodium acetate and acetic acid or sodium acid phosphate and sodium hydroxide, were employed, depending upon whether the tests were performed below or above pH 6.

The measurement of decarboxylase action was carried out in a water bath thermostatically regulated at 30°C. The cups were flushed with nitrogen (5 per cent CO_2) gas for 5 minutes and equilibrated for 30 minutes before tipping the substrate into the main compartment. Carbon dioxide evolution was followed by observing changes in pressure every 10 minutes for a period of 1.5 hours. When the reaction was maintained at a level greater than pH 6, 0.5 ml. N/10 H_2SO_4, contained in the other side arm of the vessel—and added at the expense of a like amount of buffer solution—was tipped in at the end of the 1.5 hour period. In the case of mixed culture preparations, the determinations were made only at the reported optimal pH for the particular decarboxylase (23), while both the optimal pH and the pH found to exist in the original specimen were employed when testing preparations from feces or from the alimentary tract.

Determination of Amines:

Amines were extracted from feces, intestinal contents, and culture supernates and subsequently detected by paper strip chromatography according to the methods of Block,

LeStrange, and Zweig (27). Culture supernates were concentrated *in vacuo* to a volume of 10 ml. preparatory to extraction. Feces were collected daily in a fresh state and frozen at −20°C. until 100 gm. had accumulated. In the preliminary work, aqueous extracts of feces were prepared. Because the yield of amines was poor when the latter method was used and because Koessler and Hanke (11) reported particulate matter in feces to adsorb amines, acid hydrolysis was adopted for the major portion of the work with intestinal material. This was accomplished by treating 100 gm. of feces with 1 liter of 20 per cent HCl on a heated sand bath for 20 hours. The hydrolysate was filtered and the filtrate was reduced to a volume of 100 ml. by means of vacuum distillation. A 10 ml. aliquot of the concentrate was neutralized with 8 ml. of 10/N NaOH and extraction was then initiated.

One attempt to determine amines in intestinal contents was made. This was accomplished by placing the contents of the entire intestinal tracts of 3 rats fed "fox checkers" in 250 ml. of distilled water and recovering the supernatant liquid by centrifugation. The latter was condensed *in vacuo* and amines recovered in the manner already indicated.

RESULTS

Amino Acid Decarboxylation by Feces and Contents of the Gastrointestinal Tract.—The results presented in Table II, typical of a series of tests made on specimens from rats fed "fox checkers," failed to show any significant activity of glutamic acid, arginine, lysine, tyrosine, or histidine decarboxylase to exist within the alimentary canal. Feces from rats fed the three supplemented diets were similarly inactive. For comparative purposes the tyrosine decarboxylase activity of *Streptococcus faecalis* R, determined simultaneously with the intestinal and gastric preparations, is included in the table. Additional efforts to demonstrate significant levels of amino acid decarboxylases in the rat's alimentary canal failed when: (*a*) tests were made at the pH existent in the original specimens; (*b*) various buffer eluates of acetone-ether dried powders were employed as the enzyme source; (*c*) yeast extract was added as a source of cofactor; and (*d*) the first washings of feces were tested. One difficulty not overcome was the relatively high endogenous rate of CO_2 production, particularly with powders. The possibility that an intestinal constituent might have inhibited amino acid decarboxylation may be discounted since the decarboxylation of tyrosine by *S. faecalis* R was unaffected in the presence of fecal suspensions, fecal powders, or aqueous fecal extracts.

Amino Acid Decarboxylation by Mixed Fecal Cultures.—Because essentially all previous studies of bacterial amino acid decarboxylases have been conducted with "pure cultures," it seemed desirable to investigate mixed fecal cultures. These data are portrayed in Figs. 1 to 3. Fig. 1 shows that lysine decarboxylase was considerably more active in cells grown in medium B (low glucose content, hence pH maintained near neutrality during growth) than in those recovered from medium A (high glucose content, hence low final pH). On the other hand, glutamic acid decarboxylase was equally active in cells grown under either pH, while the other three decarboxylases responded to pH in a manner opposite to that of lysine. Medium C (very low in glucose) was utilized for the studies

presented in Fig. 2 and 3 for the following reasons: (*a*) so long as the ionic strength was held below inhibitory levels, a culture containing more than 0.05 per cent glucose could not be buffered sufficiently to maintain the same pH on anaerobic incubation as it did when incubation was aerobic; and (*b*) at concentrations of glucose greater than 0.05 per cent, acid production was inhibited by chlortetracycline to the extent that the final pH was much lower than in the control culture. Medium C, therefore, avoided the complications of a pH differential between the test and control cultures when measuring the production of amino acid decarboxylases at different oxygen tensions or at low concentrations of chlortetracycline.

TABLE II

Decarboxylation of Amino Acids by Contents of the Rat's Alimentary Tract

Source of preparation	Glutamic acid		Arginine		Lysine		Tyrosine		Histidine	
	P*	S‡	P	S	P	S	P	S	P	S
Stomach	0§	0	0	0	0	0	0	0	0	0
Jejunum	0	0	0	0	0	0	0	0	0	0
Ileum	0	6	0	10	0	0	0	2	0	12
Cecum	0	8	7	5	0	0	0	11	0	9
Colon	5	5	9	5	0	0	0	6	8	5
Feces (unwashed)	0	—	0	—	0	—	0	—	0	—
Feces (washed)	18	10	20	8	20	0	17	0	21	0
S. faecalis	—	—	—	—	—	—	548	302	—	—

Each Warburg cup contained a powder or a suspension in 2.7 ml. M/5 sodium acetate–acetic acid and 0.5 ml. M/20 amino acid solution in the same buffer. The reaction was measured in an atmosphere of N_2 at 30°C. and at pH values of 4.5 (histidine, glutamic acid), 5.2 (arginine), and 5.5 (tyrosine). Lysine decarboxylase was determined at pH 4.5 (resting cells) or pH 6.0 (powders).

* Acetone-ether–dried powders (25 mg./cup).

‡ Washed suspensions (1 mg. N/cup).

§ µl. CO_2 evolved in 1 hour.

The data in Fig. 2 show clearly that aerobiosis inhibits the synthesis of amino acid decarboxylases. Cells grown aerobically in medium C produced glutamic acid and arginine decarboxylase but failed to produce decarboxylases for lysine, tyrosine and histidine; when incubated anaerobically the cells lacked only histidine decarboxylase. Fig. 3 reveals that chlortetracycline in an aerobic culture inhibited the production of arginine decarboxylase. Mixed fecal cells grown anaerobically in the presence of chlortetracycline (*cf.* Fig. 3) had neither tyrosine nor lysine decarboxylase activity and possessed lowered decarboxylase potency toward arginine and glutamic acid.

Chromatographic Detection of Amines in Intestinal Material and Mixed Fecal

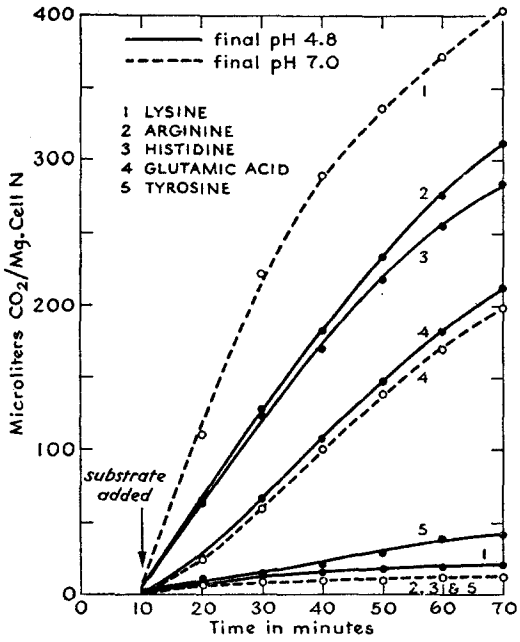

FIG. 1. Effect of pH on amino acid decarboxylation by washed cells from fecal cultures. See caption under Table II for description of flask contents. Cells were harvested from medium A (final pH 4.8) or medium B (final pH 7.0) after a 20 hour period of aerobic incubation at 37°C. (see Table I for composition of media).

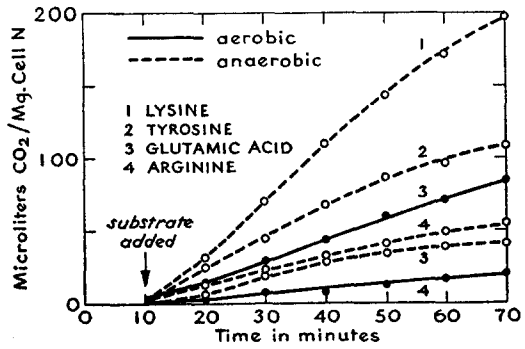

FIG. 2. Effect of oxygen tension on amino acid decarboxylation by washed cells from mixed fecal cultures. See caption under Table II for description of flask contents. The cells were grown in medium C (*cf.* Table I) at 37°C. and harvested after 20 hours. Aerobically incubated cells possessed no measureable decarboxylase activity with lysine, tyrosine, or histidine; anaerobically incubated cells were devoid of histidine decarboxylase.

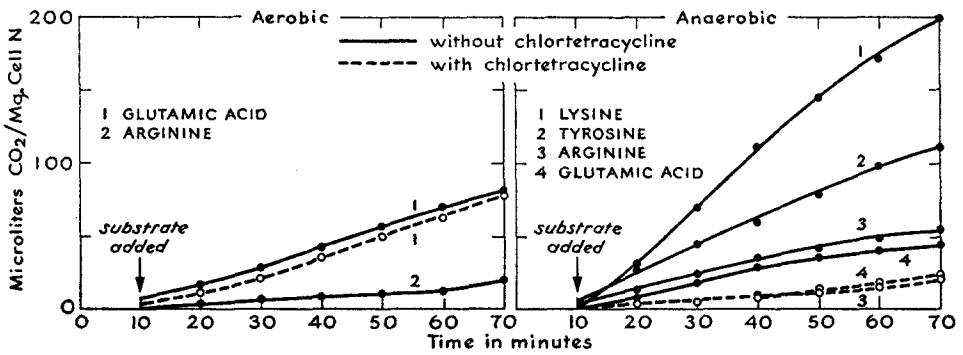

FIG. 3. Effect of chlortetracycline on amino acid decarboxylation by washed cells from mixed fecal cultures. See caption under Table II for description of flask contents. The cells were grown aerobically or anaerobically in medium C (*cf.* Table I) at 37°C. and harvested after 20 hours. Chlortetracycline was added to the medium at a level of 1 μg./liter. Control cells lacked the decarboxylases indicated under Fig. 2. Aerobically incubated cells grown in the presence of chlortetracycline could not decarboxylate lysine, tyrosine, histidine, or arginine; anaerobically incubated cells exposed to the antibiotic during growth were inactive toward tyrosine, lysine and histidine.

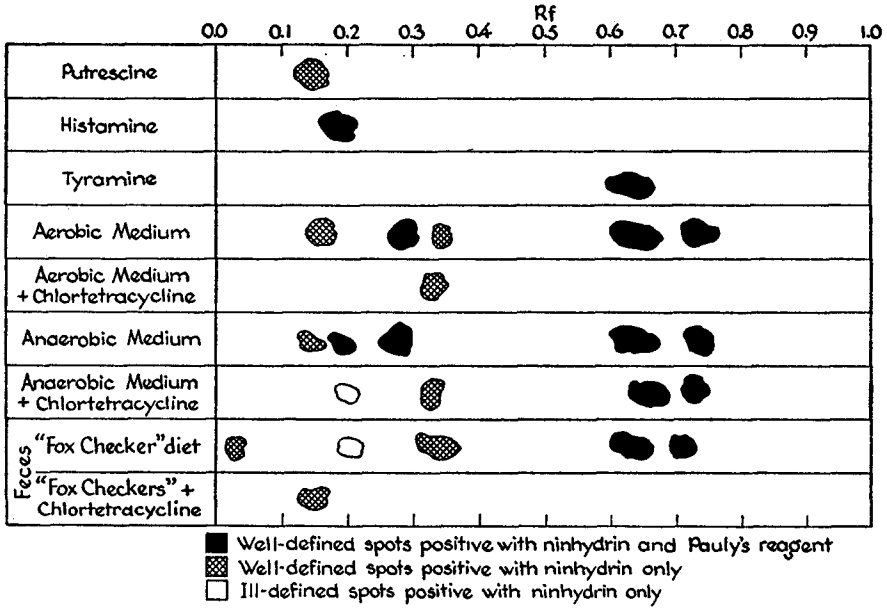

FIG. 4. Amines present in feces and mixed fecal cultures as demonstrated by paper chromatography. The crude fecal cultures were prepared as described in the caption under Fig. 3. The procedure used to extract amines from the culture supernates and acid hydrolysates of feces is described by Block, LeStrange, and Zweig (27). The amines were recovered from experimental material by primary and secondary extractions from alkaline solutions with peroxide-free ether and *n*-butanol. After conversion to the hydrochloride form, the amines were subjected to ascending paper chromatography on Whatman 4 paper strips (4 × 50 cm.). The water-poor phase of a butanol–acetic acid–water (40–10–50 per cent) mixture served as the solvent, while the water-rich phase was placed in the bottom of the chamber. Ten drops of the amine-containing butanol extract was dried near the end of the paper strip. After equilibration for 14 hours at room temperature with the aqueous phase of the solvent, the water-poor phase of the solvent was pipetted into the glass trough. 7 hours later the strip was dried at room temperature and developed by spraying with either a 0.2 per cent alcoholic solution of ninhydrin or a reagent (27) giving Pauly's reaction (specific for imidazoles and phenols).

Cultures.—The results of chromatography of amines in intestinal contents of the rat and in mixed fecal cultures are presented in Fig. 4. The first three rows represent the standards, *viz.* putrescine, histamine, and tyramine. Rf values varied ±0.02 for the first two amines and ±0.04 for tyramine. The sensitivity of the method to the three standard amines was determined to be less than 10 μg. The next four rows of spots indicate amines present in mixed fecal cultures grown, aerobically or anaerobically, in the absence or presence of chlortetracycline on medium C. The last two rows show the effects of chlortetracycline feeding upon amines in acid-hydrolyzed rat feces. With respect to the cultures, it should be noted that chlortetracycline was better able to supress amine formation under aerobic conditions of incubation than under anaerobic conditions. The effects of oral administration of chlortetracycline on fecal amines was striking: only one amine—putrescine—was detected in rats fed the antibiotic-supplemented "fox checker" diet, while the control animals evidenced some 5 amines in their feces. Spots with Rf values of 0.03, 0.33, and 0.74 are probably agmatine, ethanolamine, and ephedrine, respectively, according to the data of Bremner and Kenten (28). The amine yielding a spot with Rf 0.28 could not be identified. The two ill defined spots, positive with ninhydrin and giving a negative Pauly's test (*cf.* rows 7 and 8 of Fig. 4) are probably histamine, since the application of trace amounts of histamine to the paper strip was found to result in a spot of this type. Although such results are not presented in Fig. 4, the suppression by chlortetracycline of fecal amines in rats fed a ration supplemented with tyrosine and histidine (ration described as (*c*)) was not marked: agmatine, putrescine, tyramine, and ephedrine were found in feces from animals fed diet *c*, while putrescine, ethanolamine, tyramine, and ephedrine were present in the analogous chlortetracycline-supplemented diet (ration described as (*d*)).

Because of the relative ease with which amines were found in feces of the rat, the failure to detect a significant level of activity of five amino acid decarboxylases in feces and other contents of the alimentary canal attests to the insensitivity of the manometric method employed.

DISCUSSION

In the experiments with mixed fecal cultures environmental factors apparently influenced not only the enzymic constitution of the population, but also the relative numbers of the different species. Cells grown at a near neutral reaction (medium B) were better able to produce lysine decarboxylase than when cultivated under acidic conditions (medium A), an observation not consistent with the fact that lysine decarboxylase activity is favored by acidic conditions (23). Presumably medium B supported heavier growth of lysine decarboxylase–producing bacteria than medium A. By comparable reasoning one can explain the relative insensitivity to pH of glutamic acid decarboxylase

formed in the mixed cultures. It should be noted that Virtanen and Laine (29) found some strains of *E. coli* to produce a lysine decarboxylase characterized by optimal activity at pH 7, although Gale (19) questions this.

In assessing factors which influence intestinal amine formation *in situ*, it is perhaps noteworthy that anaerobiosis was superior to aerobiosis for promoting amine formation by mixed fecal cultures since it is assumed that conditions within the lumen of the lower bowel are anaerobic and the redox potential is low. Therefore, it would seem that the oxidation of amines by the microflora must be minimal in the lower intestine except for those amines which are in intimate contact with the amine oxidase–rich intestinal mucosa.

A positive effect of chlortetracycline on amino acid decarboxylation was noted as follows: (*a*) except for glutamic acid decarboxylase, a suppression of amino acid decarboxylase synthesis by mixed fecal cultures; (*b*) a reduction in the number of amines formed by mixed fecal cultures; and (*c*) a reduction in the number of amines present in feces of rats given a ration containing the antibiotic. The nature of chlortetracycline's effect on amine formation cannot be deduced from the data presented. It is important to note, however, that the presence of the antibiotic (0.1 μg./ml.) in the Warburg cup had no measurable influence on the capacity of either resting or dried cells derived from mixed fecal cultures to decarboxylate the 5 amino acids studied.[2] Evidently the synthesis rather than the action of these decarboxylases is impeded by the antibiotic.

Although the low concentration of chlortetracycline in the mixed fecal cultures had no appreciable effect on final pH, Gram-negative cells predominated in the control cultures while Gram-positive cells gained ascendency in the presence of the antibiotic. Likewise, pronounced changes are seen in the intestinal microflora upon the administration of chlortetracycline (31). Because sublethal levels of chlortetracycline alter mixed microbial populations, it is impossible to ascertain whether the pronounced effects of chlortetracycline on amine formation in such a system reflect a specific metabolic interference or are a result of the selectivity of the environment. The net effect of either alternative could be represented by an altered capacity of the component cells to decarboxylate amino acids. The inability to decide which way chlortetracycline acted to reduce the production of amines clearly demonstrates the difficulty inherent in resolving alterations, at a metabolic level, of a microbic ecological system.

If the well known ability of chlortetracycline (and other antibiotics) to stimulate animal growth (1–3, 31) is directly related to the observation of this study that the oral administration of chlortetracycline reduces the intestinal

[2] In view of the discovery by Zeller, Van Orden, and Vögtli (30) of a "guanidine-decomposing enzyme" which hydrolytically splits guanidine compounds into an amine and urea, amino acids should not be considered to serve as the sole precursors of amines.

content of amines, the sympatheticomimetic amines may have an adverse, albeit "subclinical," nutritional effect in animals under "normal" conditions. Moreover, tissues of young animals, especially rats, are lacking in, or possessed of low amine oxidase function (12). Certainly amine toxicity can be induced by alteration of the diet. For instance, excess dietary tyrosine is extremely toxic to young rats, presumably as a result of vasoconstriction caused by the overwhelming amount of tyramine formed in the intestine (20, 32). The capacity of chlortetracycline to decrease the production of amines in the intestine together with the observations of others that antibiotics suppress the intestinal formation of indole (33) and ammonia (34, 35) lends support to the hypothesis that antibiotics stimulate the growth of animals by lowering the output of toxic substances within the intestinal canal.

SUMMARY

Although gastric and intestinal contents from rats failed to show amino acid decarboxylase activity when tested against five different amino acids (glutamic acid, arginine, lysine, tyrosine, and histidine), the feces contained at least seven different amines, some known to be pharmacologically active. Putrescine, histamine, and tyramine were identified by means of paper chromatography in both intestinal material and mixed fecal cultures; four other spots were found, three of which had Rf values similar to agmatine, ethanolamine, and ephedrine. The formation of lysine and glutamic acid decarboxylases was not enhanced by an increased acidity during growth while increased oxygen tension was inhibitory to amino acid decarboxylase synthesis in these fecal cultures. The feeding of chlortetracycline to rats, or its presence at a very low concentration in media in which the mixed cultures were grown, reduced the capacity of intestinal microorganisms to produce amines. Cells from mixed fecal cultures grown in the presence of chlortetracycline lacked or contained but weak amino acid decarboxylase activities. The action of the enzymes themselves was unaffected by the presence of the antibiotic in the Warburg cup during assay. The results suggest that amines formed within the intestinal tract might be toxic to the rat, and that chlortetracycline accelerates animal growth by suppressing their production.

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