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Microbiological Activities of Iodoorotic Acid and Iodouridine.* (20953)

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In a previous paper it was shown that neither 5-iodoorotic acid-I^{131} nor 5-iodouridine-I^{131} is concentrated by the regenerating liver of the rat or by normal or certain neoplastic tissues of rats or mice(1). For several species of microorganisms, however, certain halogenated pyrimidines have been described as antimetabolites(2-4) or have exerted limited activity as growth factors(2). In fact, 5-bromouracil appears to be incorporated into the nucleic acids of *Streptococcus faecalis*(5), and to interfere markedly with the utilization not only of exogenous thymine, but also of a related compound formed endogenously in the presence of derivatives of folic acid(6). These results with bacteria suggested a reinvestigation of the effect of iodoorotic acid and iodouridine on the growth of certain microbial species.

Experimental. 5-Iodoorotic acid-I^{131} and 5-iodouridine-I^{131}. These compounds were synthesized by previously published methods (1). *Determination of radioactivity.* The concentration of I^{131} in the cells was determined with the aid of a well-type scintillation counter constructed by MacIntyre(7). Since I^{131} emits highly penetrating gamma radiations, the cellular concentrations of I^{131} could be determined directly without fractionation or isolation. *Apparent metabolic activity of iodoorotic acid.* Increasing amount of iodoorotic acid were added to 2 series of assay tubes (final volume: 10 ml); of these, each tube of one series was supplemented with 50 μg of orotic acid. The tubes were autoclaved at 120°C for 15 minutes, inoculated with *L. bulgaricus* 09, and incubated for 72 hours at 37°C. Growth was measured turbidimetrically in a Klett-Summerson photoelectric colorimeter using filter No. 66, or by titration with 0.1 N NaOH.

TABLE I. Effect of Iodoorotic Acid on Growth of *L. bulgaricus* 09.

Iodoorotic acid, mg	0.1 N acid/10 ml media*	
	No addition	Plus orotic acid (50 μg)
	ml	ml
1.0	16.1	15.6
.10	12.9	15.5
.01	5.9	8.6
0	3.5	7.8

* Acid-production after 72 hr growth.

The *results,* presented in Table I, indicate that iodoorotic acid not only did not antagonize the growth of *L. bulgaricus* 09 in the presence of orotic acid, but, in the absence of the latter, enabled maximal growth to be attained.

Activity of autoclaved versus sterile-filtered solutions of iodoorotic acid. The growth of

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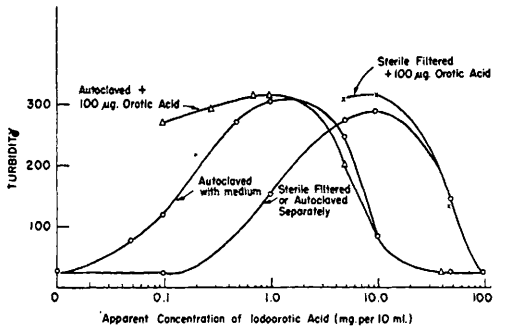

FIG. 1. Comparison of growth (45 hr growth at 37°) of *L. bulgaricus* 09 in media supplemented with iodoorotic acid which was autoclaved in the medium, or in an aqueous solution, or sterile-filtered and added aseptically.

L. bulgaricus 09 was observed: 1) when, as in the first experiment, increasing quantities of iodoorotic acid were autoclaved with the media, 2) when an aqueous neutralized solution of iodoorotic acid was autoclaved for 15 minutes at 120°C and then added aseptically to the previously autoclaved basal medium, and 3) when a neutralized aqueous solution of the iodinated pyrimidine was sterile-filtered and varying amounts were added aseptically to the autoclaved basal medium.

The 3 series of tubes were inoculated with *L. bulgaricus* 09 and incubated for 45 hours at 37°C. The results which were obtained are shown in Fig. 1.

The growth-promoting effect of 0.32 µmole of orotic acid was equalled by 0.55 µmole of iodoorotic acid which had been autoclaved with the medium, while of that which had been sterile-filtered or autoclaved as an aqueous solution, 3.58 µmoles were required. At higher concentrations the growth-promoting activity of iodoorotic acid disappeared and was replaced by a complete inhibition of growth; this effect was seen regardless of the manner of pre-treatment of the iodinated pyrimidine.

When iodoorotic acid was autoclaved in the medium in a concentration of 100 mg/10 ml of media, the solution turned a deep brown and a white precipitate formed upon cooling. No precipitate or darkening of the media was observed at this concentration of iodoorotic acid when the compound was sterile-filtered or autoclaved in an aqueous solution prior to its addition to the basal medium. The white precipitate was removed from the media by filtration, washed with 50% alcohol, then with 95% alcohol, and finally with ether, recrystallized from 50% ethyl alcohol. The spectral relationship of the compound to iodoorotic acid and orotic acid, shown in Fig. 2, suggests its identity with orotic acid. The maximum and minimum values in 0.01 N NaOH were 285 and 247 mµ, respectively (orotic acid 285 and 247 mµ), and in aqueous solution 277 and 242 mµ, respectively (orotic acid 277 and 240 mµ). As shown in Fig. 3, the growth-promoting activity of the compound for *L. bulgaricus* 09 was essentially identical with that of orotic acid.

Growth of L. bulgaricus 09 on iodoorotic acid-I^{131}. *L. bulgaricus* 09 was used to inoculate 2 one-liter flasks each of which contained 500 ml of media. The first was supplemented with sterile-filtered iodoorotic acid (165 mg/liter; 2 x 10^7 c.p.m./liter) and the second with orotic acid (20 mg/liter) plus radioactive potassium iodide-I^{131} (14 x 10^7 c.p.m./liter). The cells were harvested after 48 hours and washed 2 times with 50 volumes of 0.9% NaCl. The radioactivity contained within the cells was determined in the manner previously described. The washed cells which were grown on iodoorotic acid-I^{131} contained a total of 742 c.p.m., while those grown on KI^{131} and orotic acid had 972 c.p.m. That the activity contained in the cells grown on radioactive iodoorotic acid, as well as that observed with the cells grown on KI^{131}, could

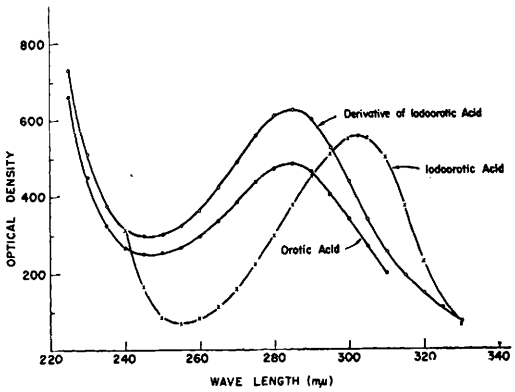

FIG. 2. Alkaline spectra of orotic acid, of iodoorotic acid, and of product formed during autoclaving of iodoorotic acid in medium.

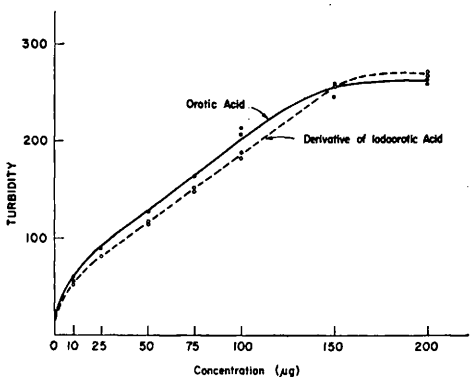

FIG. 3. Comparison of growth of *L. bulgaricus* 09 in a medium supplemented with orotic acid or a compound formed during autoclaving of iodoorotic acid in the medium.

be accounted for by the presence of iodide ion, was indicated also by the fact that more extensive washing of both types of cells removed all radioactivity.

Effect of iodide on growth of L. bulgaricus 09. Potassium iodide was added in increasing quantities to assay tubes, each of which contained 50 μg of orotic acid. After inoculation with *L. bulgaricus* 09, growth was permitted for 48 hours at 37°C. The inhibition of growth as measured turbidimetrically, which began at 4 mg/10 ml, is shown in Table II. This inhibition was not reversed by orotic acid even in concentrations of 1000 μg/10 ml media.

Metabolic activity of iodouridine and iodoorotic acid for L. casei and S. faecalis. Neither iodoorotic acid nor iodouridine, in concentrations of from 0.01 to 1.0 mg/10 ml of media, was able to support the growth of *L. casei* or *S. faecalis* in the usual basal media devoid of pteroylglutamic acid, thymine or thymidine; furthermore, the iodinated compounds were not inhibitory when growth was supported by any of these growth factors.

TABLE II. Effect of Potassium Iodide on Growth of *L. bulgaricus* 09.*

Amt of KI (in 10 ml), mg	Turbidity
40.	23 21
10.	77 84
4.0	116 122
1.0	153 156
.40	153 156
.10	153 155
0	153 157

* Medium supplemented with 50 μg orotic acid/10 ml. Turbidity, in Klett units, after 48 hr growth at 37°.

Discussion. The results indicate that iodoorotic acid is neither a growth factor nor an antagonist of the growth factor activity of orotic acid for *L. bulgaricus* 09. The pronounced growth-promoting effect of iodoorotic acid autoclaved in the microbial assay medium may be explained by a dehalogenation of iodoorotic acid with the subsequent formation of orotic acid.

When the amount of iodoorotic acid autoclaved in the medium was sufficient to yield a concentration of orotic acid greater than the solubility of that compound at room temperature, there precipitated from the medium upon cooling a substance with the properties of orotic acid. This precipitation is consistent with the fact that at pH 5.7 orotic acid is only about one-sixth as soluble as is the halogenated compound. Further, the ultraviolet absorption spectrum of the compound formed from iodoorotic acid was not significantly different from that of orotic acid. That the apparent growth-promoting activity of iodoorotic acid for *L. bulgaricus* 09 was not due to the utilization of the halogenated pyrimidine *per se* was shown by studies with iodoorotic acid-I^{131} in which the radioactive iodine was shown to behave in the same manner as KI^{131} and to be washed from the bacterial cells in a manner indistinguishable from the iodide ion. The inhibition observed with high concentrations of iodoorotic acid may have resulted from the increased iodide concentration formed by the dehalogenation of the compound. Since orotic acid is not capable of reversing the inhibition due to potassium iodide, the antagonism is considered to be of the non-competitive type. When autoclaved in the medium approximately 60% of the iodoorotic acid was converted to orotic acid, whereas an apparent conversion of only about 8% occurred when the aqueous solution of the iodinated pyrimidine was added to the medium following sterile-filtration or autoclaving. Although heating iodoorotic acid in the medium greatly accelerated the dehalogenation of this compound, the mechanism of the

dehalogenation has not been investigated further.

Summary. 1. 5-Iodoorotic acid and 5-iodouridine *per se* are not utilizable for the growth of *S. faecalis* or *L. casei,* while in media supplemented with thymine, thymidine or pteroylglutamic acid, the growth of these organisms is not inhibited by iodoorotic acid or iodouridine. 2. Although 5-iodoorotic acid appeared to support the maximal growth of *L. bulgaricus* 09, evidence was presented that this was attributable to the release of orotic acid from the iodinated derivative. 3. High concentrations of 5-iodoorotic acid inhibited the growth of *L. bulgaricus* 09, but this could not be reversed by orotic acid. This inhibition was attributed to the formation of iodide ion, since potassium iodide in similar concentrations produced a comparable inhibitory effect on the microorganism.

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Enzymatic Hydrolysis of Acyl Naphthylamines.* (20954)

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The literature of enzymes hydrolyzing aryl-substituted acylamides such as acetanilid and phenacetin is very scanty(1-4). In the course of attempts to develop a more accurate quantitation of these enzymes, N-acetyl naphthylamines were tried as substrates because of the high color intensity of dyes obtainable from naphthylamines by azo-coupling. It was soon found that extracts of many organs will hydrolyze acetyl naphthylamines at a low rate. Taking a lead from Birnbaum and coworkers (5), and from Rao and associates(6) who found that chloroacetyl amino acids are hydrolyzed much faster than the corresponding acetyl and glycyl derivatives, chloracetyl naphthylamines were tried next as substrates. They were found to be hydrolyzed by all tissues examined 30 to 100 times faster than the corresponding acetyl compounds. The activity of some tissues was high enough to permit its localization by a histochemical method.

Experimental. Chloroacetyl naphthylamines were prepared by acylating α- and β-naphthylamine with chloroacetyl chloride in an acetone-pyridine medium. The compounds were recrystallized twice from 60% methanol. The corrected melting points are: chloroacetyl α-naphthylamine, 161°C; chloroacetyl β-naphthylamine, 113°C. 0.03 M stock solutions in acetone were prepared of both substances. For use, one ml of the stock solution was blown into 100 ml of 0.04 M buffer. Both the stock solutions and the buffered substrate mixture were found to be stable at room temperature for several weeks. The enzymes used were 1:10 homogenates of fresh human tissues in saline, cleared by the freezing-thawing method(7), and human serum. One ml of enzyme dilution was incubated with 5 ml of buffered substrate for

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