CF in PGA systems. This possible difference in mechanism of action may be of significance in the interpretation of the effects of the dihydrotriazines in other biological systems.

Studies with several of the early compounds in this series failed to demonstrate microbiological activity in systems in which thymine was substituted for PGA(1). However, experiments now in progress indicate that a number of derivatives synthesized more recently in these laboratories may exhibit significant anti-thymine activity. Details must await completion of current experiments.

Summary. Dihydropteroylglutamic acid, N^{10}-formylpteroylglutamic acid and citrovorum factor are inhibited non-competitively by a number of *1,2*-dihydro-*s*-triazines when substituted for pteroylglutamic acid in *S. faecalis* No. 8043 assay systems, but only by inhibitor:metabolite ratios considerably higher than those required for the inhibition of pteroylglutamic acid in the same microbiological system. Citrovorum factor also is similarly inhibited in *L. citrovorum* No. 8081 assay systems. The phenyl- and 3'- and 4'-halophenyl-2,2-dimethyl derivatives were the most active compounds of the series and certain correlations can be made between structure and microbiological activity vs these metabolites.

1. Modest, E. J., Foley, G. E., Pechet, M. M., and Farber, S., *J. Am. Chem. Soc.*, 1952, v74, 855.

2. Foley, G. E., and Modest, E. J., Proc. 52nd Gen. Meeting, Soc. Amer. Bact., 1952, 63, April 27-May 1, Boston, Mass.

3. Foley, G. E., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 57.

4. Sauberlich, H. E., and Bauman, C. A., *J. Biol. Chem.*, 1948, v176, 165.

5. Hutchings, B. L., Mowat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and Subbarow, Y., *J. Biol. Chem.*, 1947, v170, 323.

6. Oleson, J. J., Hutchings, B. L., and Subbarow, Y., *J. Biol. Chem.*, 1948, v175, 359.

7. Woolley, D. W., *A Study of Antimetabolites*, 1952, ed. 1, J. Wiley and Sons, Inc., New York.

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Chemical and Biological Studies on *1,2*-Dihydro-*s*-Triazines. VI. Development of Resistance by *Streptococcus faecalis* No. 8043. (20479)

G. E. FOLEY AND P. L. WATSON. (Introduced by Harry Eagle.)

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The development of resistance to various analogs of pteroylglutamic acid (PGA) has been described in human leukemia(1) and in experimental mouse leukemia(2-8). Resistance to these chemotherapeutic agents also has been reported as occurring in *Tetrahymena geleii* by Kidder, Dewey, and Parks(9), in *Streptococcus faecalis* No. 8043 by Foley(10), and more recently by Burchenal, Waring, and Hutchison(11).

Previous publications from these laboratories have described the non-competitive inhibition of *S. faecalis* No. 8043* in PGA assay systems by another class of antagonists, the *1,2*-dihydro-*s*-triazines(12,13). The purpose of the present report is to describe experiments dealing with the isolation and origin of a number of strains of *S. faecalis* No. 8043 which are resistant to inhibition in PGA assay systems by these compounds. Media, methods of preparation and assay were identical with those previously described(12,13) unless otherwise stated.

* A.T.T.C. No. 8043.

Isolation of resistant strains. When *S. faecalis*-PGA assay systems containing inhibiting concentrations of the dihydrotriazine hydrochlorides were incubated beyond the usual 18-24 hour period, growth occurred after a prolonged lag phase. Thus the minimal inhibiting concentration of 4,6-diamino-1-(3',4'-dichlorophenyl)-1,2-dihydro-2,2-dimeth-

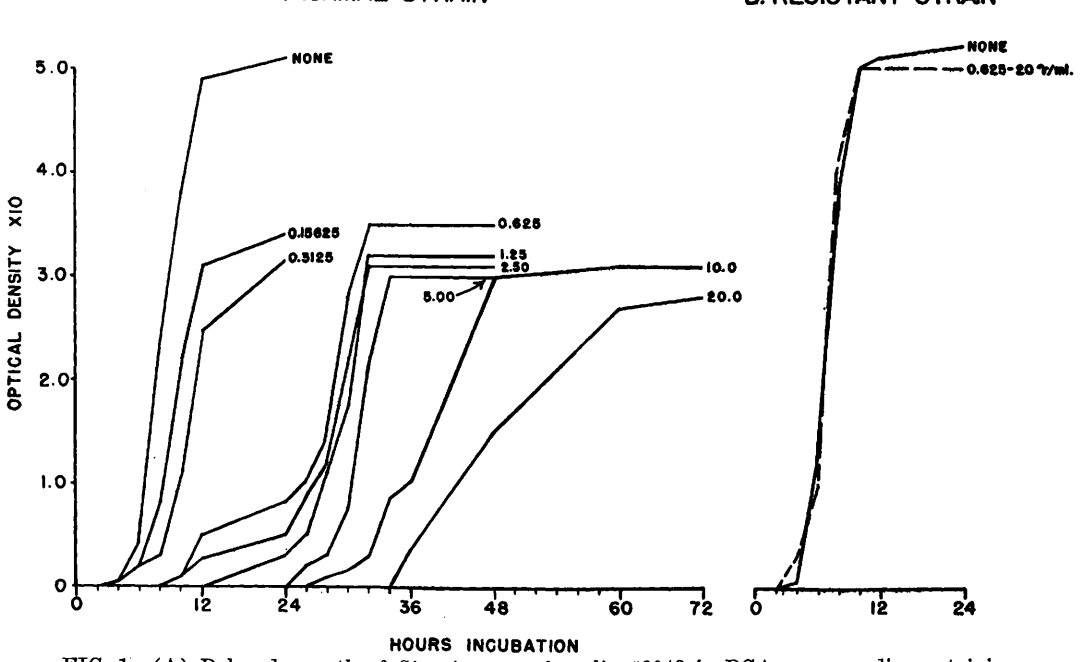


FIG. 1. (A) Delayed growth of *Streptococcus faecalis* #8043 in PGA assay media containing 0.01 γ PGA and stated concentrations of D-54 • HCl/ml. (B) Growth of D-54 • HCl-resistant strains in same medium with and without D-54 • HCl.

yl-*s*-triazine hydrochloride† (D-54 • HCl) was 0.625-1.25 γ/ml in systems containing 0.01-0.001 γ/ml PGA. However, delayed growth occurred within 72 hours in the presence of all concentrations through 20 γ/ml (Fig. 1A). No measurable growth occurred even after 108 hours incubation in the presence of 40 or more γ/ml of D-54 • HCl. When cultures obtained in this manner were exposed to 0.625-20 γ/ml of D-54 • HCl all exhibited increased resistance, the degree of resistance in each instance corresponding roughly to the concentration to which each strain had been previously exposed. All strains exhibited a normal growth curve in the presence of its corresponding concentration of D-54 • HCl (Fig. 1B).

The original assay tubes containing concentrations of more than 20 γ/ml of D-54 • HCl in which no growth had occurred after 108 hours were concentrated in the centrifuge and cultured on blood agar and PGA assay media without D-54 • HCl. Viable *S. faecalis* were recovered from assay tubes containing 40-640 γ/ml but not from those containing 1280 and 2560 γ/ml D-54 • HCl. Those strains recovered from the assay tubes containing 40-640 γ/ml were inhibited by 0.625 γ/ml of D-54 • HCl.‡ The delayed growth of cells resistant to 0.625-20 γ/ml D-54 • HCl in these experiments indicates that stock cultures of *S. faecalis* may contain cells which vary in sensitivity to the inhibitor over this entire range of concentration. The number of resistant cells probably decreases with increas-

† Two samples of this compound were used in these studies, one synthesized in these laboratories by Dr. E. J. Modest and another supplied through the courtesy of Imperial Chemical Industries, Ltd., Manchester, England.

‡ A single colony originally exposed to 40 γ/ml was found to be resistant to 10 γ/ml D-54•HCl, suggesting in this instance the chance occurrence of cells resistant to this concentration in the original inoculum or the appearance of resistance as the result of chance cell division which occurred in the inhibited culture. Repeated attempts to duplicate this observation have been unsuccessful; resistant cells have not appeared in assays containing more than 20-25 γ/ml D-54•HCl.

TABLE I. Survival of *Streptococcus faecalis* #8043 in PGA Media* Containing D-54 • HCl.

D-54 • HCl, γ/ml	Expected survivors† (most probable No.)	Observed survivors, pour plates	Survivors per 1×10^8 cells	Mean frequency‡
.625	127	200	.027	1 in 4.82×10^7
1.25	57	90	.012	1 " 1.07×10^8
2.50	21	30	.004	1 " 3.00×10^8
5.00	—	10	.0013	1 " 7.50×10^8
10.0	—	5	.00065	1 " 1.50×10^9
20.0	—	5	.00065	1 " 1.50×10^9
40.0-2560	—	0	0	

* PGA, .01 γ/ml.

† The disparity between expected and observed survivors is due to differences in the ability of these broth and agar media to initiate growth from a single unit, *i.e.*, a pair or a chain of cells of *Streptococcus faecalis*.

‡ In standard suspensions of 7.5×10^9 cells/ml.

ing concentrations, as evidenced by the progressively longer lag phase. The isolation of these resistant strains could be explained on the basis of simple selection or alternately by adaptation by a few cells to these concentrations of D-54 • HCl.

The frequency of cells resistant to increasing concentrations of D-54 • HCl in standard inocula prepared from the stock strain of *S. faecalis* was determined by dilution experiments(14) and by actual count of the survivors at increasing concentrations of inhibitor in agar pour plates. The number of survivors observed at increasing concentrations of inhibitor in agar checks reasonably well with the expected number as estimated from dilution data and the frequency of resistant cells decreases with increasing concentrations of D-54 • HCl (Table I). Representative samples of the surviving colonies were fished from these agar plates to PGA assay broth containing 0.625 γ/ml D-54 • HCl, incubated and studied in duplicate serial assays against D-54 • HCl. All but one of the 60 strains so studied failed to exhibit resistance to concentrations of inhibitor significantly greater than that to which it had been originally exposed. The single exception, a colony fished from agar containing 0.625 γ/ml D-54 • HCl required 48 hours to grow in subculture and when assayed was found to be resistant to 500 γ/ml D-54 • HCl.

Resistant strains isolated from the stock culture in this manner exhibited increasing resistance when carried in subculture in the presence of increasing concentrations of inhibitor, the resistance of the survivors increasing *pari passu* to the concentration to which the strains had been exposed. By serial passage in this manner, resistance was increased progressively and a culture completely resistant to 1000 γ/ml of D-54 • HCl was obtained after 14 serial passages (Fig. 2). Data on the frequency of resistant cells in the stock culture (Table I) indicate that it is unlikely that cells exhibiting this degree of resistance were contained in the original inocula. This order of resistance therefore must have appeared as the consequence of the selective growth of cells in the original inocula which either by serial mutation or adaptation became progressively more resistant to the inhibitor. The apparently step-wise appearance of increasing levels of resistance (Fig. 2) actually reflects the level of resistance attained at the fortuitous time of sampling and the development of resistance probably does

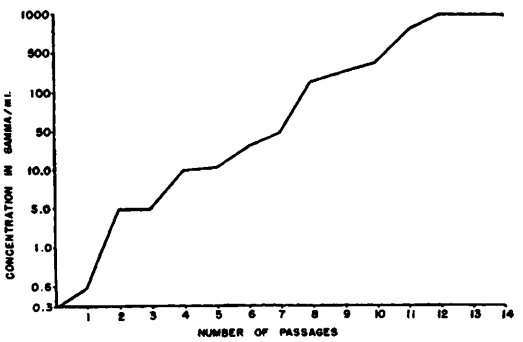


FIG. 2. Development of resistance in *Streptococcus faecalis* #8043 by serial passage in PGA assay media containing D-54 • HCl.

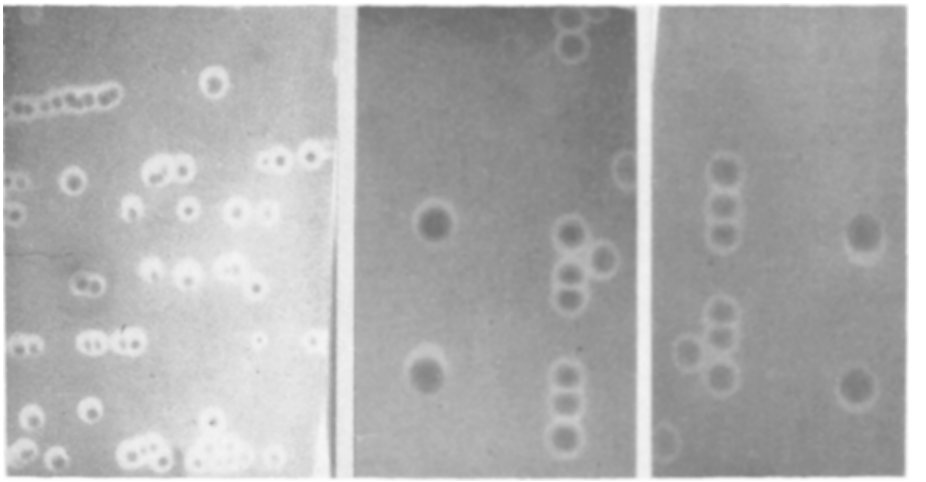


FIG. 3. Colony morphology of dihydrotriazine resistant variants: comparison with stock culture of *Streptococcus faecalis* #8043 (right). 24 hr culture on 5% horse blood agar, ×1.5.

not proceed in steps of these magnitudes.

These dihydrotriazine-resistant mutants have been carried through several generations in the laboratory in media with and without inhibitor with no significant loss of resistance during the past 9 months. Thus far the only recognizable difference between the mutant and parent strains other than their behavior with respect to inhibitors is in colony size, the resistant strains grow in uniformly smaller colonies which otherwise resemble those of the parent strains (Fig. 3).

Origin of resistance. Luria and Delbrück (15) developed a method of variance analysis to test the 2 hypotheses, mutation and adaptation generally postulated for the development of bacterial resistance. The appearance of dihydrotriazine resistance was analyzed by duplicate studies on 3 series of independent and replicate cultures derived from the stock culture. Standard inocula containing 7.5×10^9 cells were prepared from stock cultures and 0.1 ml of a 1×10^6 dilution (circa 750 cells) was inoculated into each of 21 tubes of PGA assay broth containing 0.01 γ/ml PGA. After 24 hours incubation at 37°C, 0.1 ml samples of each of 20 cultures was plated in PGA assay agar containing 1.25 γ/ml D-54 • HCl. A series of 15 replicate plates were prepared from the remaining culture in the same manner. After incubation at 37°C for 24 hours, the surviving colonies were enumerated in the usual manner. The results of these experiments are summarized in Table II.

Statistical analyses indicate that variation in the frequency of resistant cells appearing in independent cultures initiated from minimal inocula are greater than the chance variations observed in replicate plates of the same parent culture. These results are compatible with the hypothesis that resistance to D-54 • HCl appears as the result of spontaneous mutations(15).

Specificity of resistance. Resistant strains derived from these experiments with D-54 • HCl exhibited cross-resistance against a number of related aldehyde and ketone derivatives of the dihydrotriazine series, suggesting that resistance is related to the basic molecular configuration rather than to any of the particular substitutions mentioned (Table III). However, the D-54 • HCl resistant mutants so far studied have exhibited no cross-resistance with certain chloro- and dichloro-substituted 2,4-diamino-pyrimidines§ which also exhibit potent anti-PGA activity(16) and the PGA analogs 4-aminopteroylglutamic acid and 4-amino-N^{10}-methylpteroylglutamic acid (a-methopterin).‖

§ These compounds were made available through the courtesy of Dr. G. H. Hitchings, the Wellcome Research Laboratories, Tuckahoe, N. Y.

‖ We wish to express our appreciation to the Lederle Laboratories, Pearl River, N. Y., for generous samples of these compounds.

TABLE II. Comparison of Frequency of Colonies of *Streptococcus faecalis* #8043 Resistant to 1.25 γ/ml D-54 • HCl in Independent and Replicate Cultures.

Culture	Colony counts: Individual cultures	Colony counts: Replicate cultures
1	16	7
2	5	8
3	0	10
4	10	9
5	80	10
6	6	18
7	59	9
8	10	7
9	16	10
10	20	11
11	17	19
12	0	5
13	11	15
14	12	7
15	32	7
16	37	
17	18	
18	72	
19	0	
20	30	
Mean	22.6	10.1
Variance	544.15	15.55
X^2	458.49	21.49
P	Much less than .001	.05

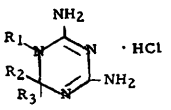
Such data represent a Poisson distribution; thus variance equals the mean. Chi square is defined as $\left(\frac{\text{observed} - \text{expected}}{\text{expected}}\right)^2$ and equals $\left(\frac{\text{observed} - \text{mean}}{\text{mean}}\right)^2$.

Discussion. Bacterial resistance to a number of inhibitors and lethal agents; bacteriophage(15), penicillin(17-19), sulfonamides (20), irradiation(21), and streptomycin(19) appears to originate from spontaneous mutations occurring in heterogeneous cell populations independently of the specific agents. Evidence for this view has been based largely upon results obtained with variance analyses as developed by Luria and Delbrück(15). Additional supportive evidence is afforded by the isolation of indirectly selected members of the populations such as those described by Burnet(22) and more recently by Lederberg and Lederberg(23) which exhibit specific resistance without previous exposure to the specific lethal agent or inhibitor. In the present studies, the results of variance analyses are compatible with the hypothesis that dihydrotriazine resistance also originates from spontaneous mutations which occur in the stock culture in the absence of inhibitor and which grow out selectively in its presence.

In the present experiments, a) the decreasing frequency of survivors at increasing concentrations of inhibitor, b) the correlation between the resistance of survivors and the concentrations of inhibitor to which they had been exposed, and c) the slow emergence of growth in cultures containing low concentrations of inhibitor suggest a specific adaptive process similar to that suggested by Eagle *et al.*(24) as a possible explanation for the development of slightly increased resistance to antibiotics on exposure to low concentrations. According to this interpretation, the varying degrees of resistance to minimal concentrations of inhibitor as exhibited by the stock culture may represent specific environmental adaptations occurring in susceptible cells. Sevag and Rosanoff(25) similarly concluded from their experiments with *Micrococcus pyogenes* var. *aureus* in complete and deficient media that resistance to streptomycin represents a specific adaptation by a sensitive cell. Gibson and Gibson(26) and more recently Eagle *et al.*(24) have suggested that the hypotheses of mutation and adaptation are not necessarily mutually exclusive, *i.e.*, within the spectrum of resistance of a "normal" culture the appearance of resistance to minimal concentrations of inhibitor may be adaptive, while the few highly resistant cells which appear represent spontaneous mutations.

The cross-resistance exhibited by these mutant strains with other derivatives of the dihydrotriazine series resembles the cross-resistance among the sulfonamides(27) and the analogs of PGA. Cross-resistance among the latter compounds has been observed in acute lymphoid mouse leukemia(2-8) and *T. gelei*(9) as well as in *S. faecalis*(11). The lack of significant cross-resistance with 4-APGA, a-methopterin, and the 2,4-diaminopyrimidines suggest an intimate relationship between resistance and the basic molecular configuration of the selective agent. However, studies with *E. coli*(21,28) and other

TABLE III. Cross-Resistance vs. Related Dihydrotriazines Exhibited by D-54 • HCl-Resistant Mutants of *Streptococcus faecalis* #8043.



Structure

Compound	R_1	R_2	R_3	Normal strain, 50% inhibition, γ/ml	Resistant strains, no inhibition at, γ/ml
D-19 • HCl	4′-carbethoxyphenyl	methyl	methyl	.375	1000
D-20 • HCl	4′-chlorophenyl	"	"	.095	1000
D-23 • HCl	phenyl	"	"	.10	1000
D-53 • HCl	2′,4′-dichlorophenyl	"	"	150.0	1000
D-55 • HCl	3′,4′-dichlorophenyl	H	phenyl	.62	100
D-56 • HCl	2′,5′-dichlorophenyl	methyl	methyl	3300.0	3000
D-57 • HCl	2′,4′-dichlorophenyl	H	phenyl	130.0	500
D-60 • HCl	4′-chlorophenyl	"	ethyl	2.0	1000
D-63 • HCl	3′,4′-dichlorophenyl	"	"	.4	100
D-65 • HCl	4′-bromophenyl	methyl	methyl	.12	1000
D-67 • HCl	3′,4′-dichlorophenyl	H	"	.325	100
D-68 • HCl	2′-chlorophenyl	methyl	"	141.0	1000
D-69 • HCl	3′-chlorophenyl	"	"	.325	100
D-76 • HCl	3′,4′-dichlorophenyl	H	*n*-propyl	.950	100
D-82 • HCl	2′-bromophenyl	methyl	methyl	1640.0	3000
D-54 • HCl	3′,4′-dichlorophenyl	"	"	.10	1000

All media contained .01 γ/ml PGA.

microorganisms(29) indicate that resistance to a number of apparently unrelated agents may appear following exposure to a single selective agent. Experiments are now in progress on the elucidation of the mechanism of dihydrotriazine resistance and on the continued search for mutants exhibiting cross-resistance with other PGA antagonists.

Summary. A number of strains have been isolated from stock cultures of *S. faecalis* No. 8043 which are resistant to inhibition by the *1,2*-dihydro-*s*-triazines in pteroylglutamic acid assay systems. The strains studied so far exhibit cross-resistance with related aldehyde and ketone derivatives of the dihydrotriazine series but not with 4-aminopteroylglutamic acid, 4-amino-N^{10}-methylpteroylglutamic acid or certain 2,4-diaminopyrimidines.

The results of variance analyses were compatible with the conclusion that dihydrotriazine resistance originates from spontaneous mutations independently of the inhibitor. However, the possibility that resistance to minimal concentrations of inhibitor may be an adaptive change could not be excluded.

1. Farber, S., *Blood,* 1952, v7, (Suppl.).

2. Law, L. W., and Boyle, P. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 599.

3. Burchenal, J. H., Robinson, E., Johnston, S. F., and Kushida, M. N., *Science,* 1950, v111, 116.

4. Law, L. W., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 340.

5. Burchenal, J. H., Webber, L. F., Meigs, G. M., and Biedler, J. L., *Blood,* 1951, v6, 337.

6. Burchenal, J. H., Johnston, S. F., and Waring, G. B., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 348.

7. Law, L. W., *Blood,* 1952, v7, (Suppl.), 160.

8. Burchenal, J. H., *Blood,* 1952, v7, (Suppl.), 166.

9. Kidder, G. W., Dewey, V. C., and Parks, R. E., Jr., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 88.

10. Foley, G. E., *Blood,* 1952, v7, (Suppl.), 165.

11. Burchenal, J. H., Waring, G. B., and Hutchison, D. J., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 311.

12. Foley, G. E., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 57.

13. ———, *ibid,* 1953, v83, 62.

14. Halvorsan, H. O., and Ziegler, N. R., *Quantitative Bacteriology,* ed. 1938, Burgess Pub. Co., Minneapolis, Minn.

15. Luria, S. E., and Delbrück, M., *Genetics,* 1943, v28, 491.

16. Hitchings, G. H., Rollo, I. M., Goodwin, L. G., Coatney, G. R., *et al., Trans. Royal Soc. Trop. Med. and Hyg.,* 1952, v46, 467.

17. Demerec, M., *Proc. Nat. Acad. Sci. U. S.,* 1945, v31, 16.

18. ———, *Ann. Missouri Botan. Garden,* 1945, v32, 131.
19. ———, *J. Bact.,* 1948, v56, 63.
20. Oakberg, E. F., and Luria, S. E., *Genetics,* 1947, v32, 249.
21. Witkin, E. M., *Genetics,* 1947, v32, 221.
22. Burnet, F. M., *J. Path. and Bact.,* 1929, v32, 15.
23. Lederberg, J., and Lederberg, E. M., *J. Bact.,* 1952, v63, 399.
24. Eagle, H., Fleischman, R., and Levy, M., *J. Bact.,* 1952, v63, 623.
25. Sevag, M. G., and Rosanoff, E. I., *J. Bact.,* 1952, v63, 243.
26. Gibson, M. I., and Gibson, F., *Nature,* 1951, v167, 113.
27. Schmith, K., *Acta Path. et Microbiol., Scand.,* 1943, v20, 563.
28. Saz, A. K., Eagle, H., and Toal, J. N., *J. Bact.,* 1952, v63, 513.
29. Julius, H. W., *Ann. Rev. Microbiol.,* 1952, v6, 411.

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Effect of Food and Water on Thyroid Uptake of Radioiodine (I^{131}) in Rats. (20480)

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Based on the avidity of the thyroid gland for iodine, diagnostic tests using tracers of radioactive iodine have been devised for studying patients with thyroid disease. Many variations of these tests have been described in the last few years, ranging from measurements of early accumulation rate over the thyroid gland to 24-hour uptake studies(1-4). The accumulation rate of iodine in the thyroid gland is in part dependent upon the plasma level of iodine. Therefore, alterations in plasma concentration may produce changes in the accumulation rate of iodine in the thyroid. Since plasma levels might be affected by variation in gastrointestinal or renal activity, and since no standard procedures having to do with restricted amounts of food or fluid are instituted during routine thyroid studies, it seems advisable to study the effects of these substances on the total thyroid accumulation of a tracer dose of radioactive iodine.

Method. Male albino rats, C. F. Wistar strain, were used. The animals weighed from 120 to 150 g at the onset of the experiment. For a period of 4 weeks prior to the study, the animals were placed on distilled water and an iodine-poor diet. The diet was similar to that used by Levine and Remington(5) in their studies on goiter production in rats.† It was found necessary to use an iodine-poor diet for this experiment since stock diet contains sufficient stable iodine to completely saturate the rat thyroid. The animals were separated into 8 groups of 3 animals, and were given single intraperitoneal injections of 0.1 μc of radioactive iodine per g of body weight. All animals were placed in metabolism cages and their excreta collected throughout the entire period of the experiment. Groups 1-A and 1-B received no water or diet food for 36 hours prior to the administration of the I^{131} and none during the period of the experiment. Groups 2-A and 2-B received diet food and water up to the time of injection but received none after the injection of I^{131}. Groups 3-A and 3-B received diet food and water up to the time of injection, but only distilled water after the injection. Groups 4-A and 4-B received diet food and water *ad lib.* prior to and following the injection of I^{131}. Animals in A groups were sacrificed at the end of 6 hours, and those in B groups at the end of 24 hours. The tissues to be studied were removed and dissolved in 30% potassium hydroxide. The excreta and carcasses were similarly treated. The radioactivity of aliquots of the various tissues were compared with standards. Measurements of gamma ray activity were made

* Most of the work performed under Atomic Energy Commission Contract W-31-109-eng-78 with Western Reserve University.

† This diet is the Rachitogenic Diet II (with added irradiated yeast) supplied by the Nutritional Biochemical Co. It contains ground gluten 20%, ground whole yellow maize 76%, calcium carbonate 3%, and sodium chloride 1%.