metric increase in the amount of acetyl S CoA and CO_2 (see Table I), suggesting the reaction: FA + pyruvate + CoASH → FAH_2 + AcSCoA + CO_2. However, since pyruvate is also decomposed by non-pterine-dependent reactions, a further purification of the enzyme system is necessary before complete stoichiometry can be determined.

TABLE I

DIHYDROPTERINE FORMATION

Conditions: 1-C^{14}-pyruvate, 2.0 μM; CoA, 1.6 μM; K-succinate, *p*H 6.2, 20.0 μM in a total volume of 0.4 ml., incubated anaerobically 1 hr., 38°. Exp. I: 0.67 μM FA and 2.0 mg. protein; Exp. II: 0.5 μM teropterin and 1.6 mg. protein.

	Experiment I			Experiment II		
Omission	FAH_2[a]	AcSCoA[b]	CO_2[c]	$TerH_2$[a]	AcSCoA[b]	CO_2[c]
None	0.51	1.10	1.32	0.15	0.53	0.55
Pterine	.00	.67	1.00	.00	.38	.42
CoA	.00	.00	.12	.00	.00	.006
Pyruvate	.00	.00	.00	.00	.00	.00

[a] Calculated by assay (a). See text. [b] Determined by hydroxamic method.[5] [c] Trapped as $K_2C^{14}O_3$ and converted to $BaC^{14}O_3$.

A complete enzymatic conversion of FA to FAH_2 is shown in the accompanying figure. FAH_2 can be assayed spectrophotometrically by (a) the decrease in optical density at 365 mμ at *p*H 11, (b) the increase in optical density at 420 mμ

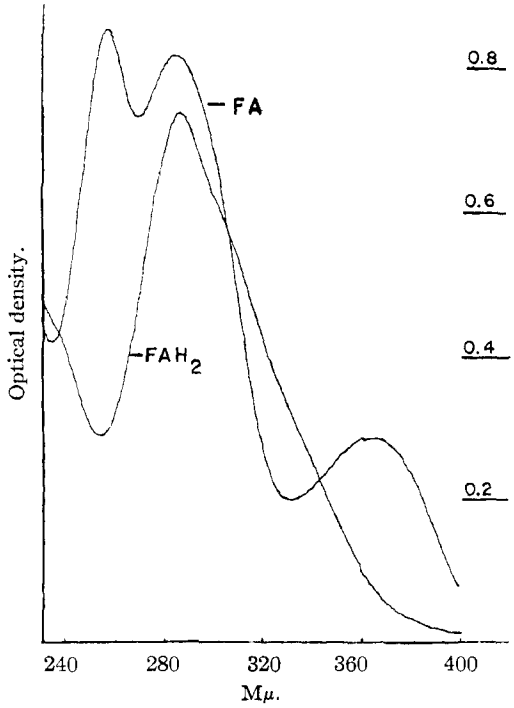

Fig. 1.—FAH_2, complete system; FA, serine omitted. The molecular extinction of FA and FAH_2, respectively, at 284 mμ, *p*H 11, is 25,200 and 22,600. Each sample was run at *p*H 11 against an enzyme blank in the reference cuvette; a Cary recording spectrophotometer was used.

following acidification, due to the formation of a yellow degradation product. Identification of FAH_2 rests on the following criteria: (I) the spectrum exhibits the expected maximum at 284 mμ, minimum at 253 mμ, and 280/340 ratio of 3.0; conversion of FA to FAH_2 also results in isosbestic points similar to those of O'Dell, *et al.*[1] (II) comparison with synthetic FAH_2,[1] which is enzymatically stable in the system, showed the same acid degradation product (assay (b)), both with respect to the time required for its optimal formation as well as the concentration of this product, as judged by the optical density at 420 mμ. (III) synthetic FAH_2 and the enzymatic product were compared chromatographically, as were their respective yellow degradation products, and found to exhibit similar R_f values in three solvent systems. FAH_4 does not fulfill criteria I, II or III. Aminopterin does not inhibit the reduction of FA to FAH_2.

(5) F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **158**, 505 (1945).

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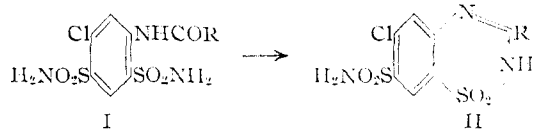
RECEIVED FEBRUARY 20, 1957

BENZOTHIADIAZINE DIOXIDES AS NOVEL DIURETICS

Sir:

Organic mercurials and carbonic anhydrase inhibitors have proven clinically useful as diuretic agents,[1] but both have certain undesirable properties. The toxicity and poor oral absorption of organic mercurials and the tendency to create electrolyte imbalance, the early and frequent development of refractoriness and the low level of potency of carbonic anhydrase inhibitors have proved serious limitations in the treatment of conditions associated with fluid and electrolyte retention. This communication concerns a new type of highly active compounds that are orally effective and possess the favorable biological properties common to both classes of the forementioned drugs.

While studying aromatic sulfonamides in our laboratories, an unexpected high order of activity has been observed with benzenedisulfonamides, particularly with the benzene-1,3-disulfonamides. Certain substituents on the benzene ring augment the activity; chlorine, amino, or acylamino have marked enhancing effects. However, further study of the chemistry of compounds of this type (I) wherein an acylamino group occupies a position *ortho* to one of the sulfamyl groups has led to novel compounds of still greater interest. Ring closure



occurs between the sulfamyl group and the adjacent acylamino group to yield a benzothiadiazine dioxide (II). Cyclization has been accomplished with a variety of acyl derivatives, both aliphatic and aromatic, including a series of homologous acyl compounds (I) where the acyl group ranges from formyl (R = H) to hexanoyl (R = C_5H_{11}). Ring closure is especially facile in the formyl derivatives and the resulting benzothiadiazines are of particular biological interest.[2]

(1) *Cf.* R. W. Berliner and J. Orloff, *Pharmacol. Rev.*, **8**, 137 (1956).

(2) We are indebted to Drs. K. H. Beyer, J. E. Baer and their associates for the biological data.

These compounds exhibit an order of inhibition of carbonic anhydrase previously observed only among the heterocyclic sulfonamides and in animals promote the renal excretion of sodium. In addition, however, they produce also a marked increase in chloride excretion and cause a diuresis not unlike that observed with organic mercurial compounds. One compound of this type, 6-chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide (II, R = H) has been selected for clinical trial and assigned the generic name of chlorothiazide. Preliminary results[3] in man substantiate the important pharmacological findings observed in our laboratories.

Chlorosulfonation of *m*-chloroaniline at 150° in the presence of sodium chloride[4] yielded 6-amino-4-chlorobenzene-1,3-disulfonyl chloride (III) which with ammonium hydroxide gave 6-amino-4-chlorobenzene-1,3-disulfonamide (IV) (m.p. 251–252°; *Anal.* Calcd. for $C_6H_8ClN_3O_4S_2$: C, 25.22; H, 2.82; N, 14.71. Found: C, 25.48; H, 2.81; N, 14.68). Reaction of III with acetic anhydride afforded 6-acetylamino-4-chlorobenzene-1,3-disulfonyl chloride (m.p. 137–139°; *Anal.* Calcd. for $C_8H_6Cl_3NO_5S_2$: C, 26.21; H, 1.65; N, 3.82. Found: C, 26.39; H, 1.77; N, 3.79) which with alcoholic ammonia gave a mixture of 6-acetylamino-4-chlorobenzene-1,3-disulfonamide (I, R = CH_3) (m.p. 261–262° dec.; *Anal.* Calcd. for $C_8H_{10}ClN_3O_5S_2$: C, 29.31; H, 3.08; N, 12.82. Found: C, 29.49; H, 3.25; N, 12.85) and 6-chloro-3-methyl-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide (II, R = CH_3) (m.p. 332° dec.; *Anal.* Calcd. for $C_8H_8ClN_3O_4S_2$: C, 31.02; H, 2.60; N, 13.57. Found: C, 31.27; H, 2.53; N, 13.50) that was separated by recrystallization from water. When heated with formic acid under reflux, IV was converted to 6-chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide (m.p. 342.5–343° dec.; *Anal.* Calcd. for $C_7H_6ClN_3O_4S_2$: C, 28.43; H, 2.05; N, 14.21. Found: C, 28.65; H, 2.23; N, 14.11).

(3) R. V. Ford and C. L. Spurr, "Electrolyte Excretion Patterns Due to Chlorothiazide, A New Orally Effective Diuretic Agent," Presented at the Southern Society for Clinical Research, New Orleans, January 26, 1957.

(4) O. Lustig and E. Katscher, *Monatsh.*, **48**, 87 (1927).

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RECEIVED FEBRUARY 18, 1957

CAROTENE, 3-C^{14}- AND 4-C^{14}-LEUCINE

Sir:

3-C^{14}- and 4-C^{14}-*d,l*-leucines were synthesized by conventional methods from carboxyl labeled isobutyric acid and carbonyl labeled acetone, respectively, and incorporated into culture media in which *Phycomyces blakesleeanus* was grown. The culture medium consisted of glucose (2.5% w./v.), asparagine (0.125%), and 3-C^{14}-leucine or 4-C^{14}-leucine (0.125%), together with inorganic salts and thiamine.[1] Thirty petri dishes were inoculated with an active spore culture of *P. blaskesleeanus*. Cultures were grown at room temperature for 7 days, harvested and extracted.[1] The extract was saponified, chromatographed and the carotene crystallized without carrier, with yields 7 and 9 mg., respectively. Both were recrystallized with resulting yields of 5.8 and 6.0 mg.

The activities of medium and carotene were determined in triplicate. Samples were burned in a Pregl furnace and the CO_2 was precipitated from sodium hydroxide as $BaCO_3$. The carbonate was plated on 1-inch copper planchets and counted in a gas-flow counter. Counts were recorded on a Tracer-Lab Autoscaler. Activities were corrected for self-absorption.

The presence of leucine in the culture medium visibly stimulates production of β-carotene by the mold, but the incorporation of leucine carbon into carotene is insignificant for 1-C and limited for 2-C.[1] The loss of the carboxyl carbon was to be expected, but the limited incorporation of 2-C made it impossible for the leucine to provide the whole of the requisite C_5 repeating unit in the carotene molecule.

From media in which 6.4% of the total carbon is represented by leucine carbon, we find an enrichment of 3.6-fold for the 3-C, and of 8.7 for 4-C, in the carotene. The specific activities of the leucines and carotenes are shown below, in cts. per min. per mg. $BaCO_3$, together with the activities of the media determined by independent combustion and by calculation based on combustion of the leucine alone. The activities are the averages for

Specific activity	3-C^{14}-Leucine	4-C^{14}-Leucine
Leucine	1442	2350
Medium calcd.	94.0	149
Medium exptl.	96.8	159
Carotene	343	1392

the six carbons of leucine and for the forty of carotene. The true activities of carbons 3 and 4 are six times greater and the extent to which they are incorporated is given by the expressions 343/1442 × 40/6 or 1.59, and 1392/2350 × 40/6 or 3.96, respectively.

Up to this point, we have introduced no assumptions. In all our work, the primary carbon source has been glucose which comprizes *ca.* 90% of the carbon of the medium. With labeled glucose, the specific activity of the carotene is less than that of the glucose, and the extent to which the glucose carbon is discriminated against varies with the non-glucose carbon source, *e.g.*, yeast autolysate,[2] glycine.[3]

If the assumption is made that formation of carotene derivable from non-leucine sources is unaffected by the leucine, the labeled carotene derived from leucine has been diluted by an equal quantity of inert endogenous carotene.

The figures 1.59 and 3.96 may therefore be multiplied by a factor of *ca.* two. This makes it possible for the 4-C of leucine to appear eight times in

(1) C. O. Chichester, T. Nakayama, G. Mackinney and T. W. Goodwin, *J. Biol. Chem.*, **214**, 515 (1955).

(2) C. O. Chichester, P. S. Wong and G. Mackinney, *Plant Physiol.*, **29**, 238 (1954).

(3) C. O. Chichester, T. Nakayama and G. Mackinney, *J. Biol. Chem.*, **221**, 819 (1956).