'Benemid', p-(di-n-propylsulfamyl)-benzoic Acid: Inhibition of Glycine Conjugative Reactions.* (18043)

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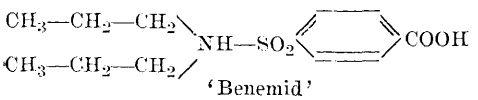
The purpose of this report is to present in general terms what seems to be a common enzymological basis for a number of physiological phenomena that have appeared to be unrelated. From this research has been developed a new compound, 'Benemid'†, p-(di-n-propylsulfamyl)-benzoic acid,



'Benemid'

synthesized by Miller, Ziegler and Sprague (1), that has interesting fundamental characteristics, and for which a diversification of clinical inquiry seems indicated.

From an analysis of the enzymic factors in the secretion of phenol red by the mammalian renal tubule *in vitro*, Beyer, Painter and Wiebelhaus(2) found that it was possible to block the utilization of energy from the phosphate cycle for phenol red secretion without impairing oxidative or transphosphorylative processes or decreasing the utilization of energy from phosphorolysis of energy-rich phosphates (*e.g.*, adenosinetriphosphate, ATP) for other essential functions of the cell or for the body as a whole. Thus it was concluded that inhibitors of this latter type blocked the ability of a definitive enzyme or enzyme system to utilize energy from phosphorolysis of energy-rich phosphates (~Ph) for a (conjugative) reaction that is essential for the ultimate secretion of phenol red. Insofar as they are comparable, these findings are in agreement with the recent similar studies of Taggart and his associates(3) relating coupled oxidation-phosphorylation reactions to phenol red secretion.

Concurrently, we have studied the characteristics of the conjugative reaction involved in the formation of p-aminohippuric acid (PAH) from p-aminobenzoic acid (PAB) and glycine, described by Cohen and McGilvery (4). They found that the conjugase required a coupled source of energy for the completion of that conjugation in the kidney or liver. A parallelism between the requirements of high energy phosphate bonds for both synthesis and secretion of PAH (which is secreted by the same renal tubular mechanism as is phenol red) has been suggested elsewhere(5).

'Benemid' has been found to be an interesting example of a class of potent inhibitors of the conjugation reaction leading to PAH synthesis. This reaction is not peculiar to PAH or hippurate formation *per se*, but it relates to the conjugation likewise of other compounds, such as p-aminosalicylate, with glycine. Inhibitors of the conjugative inactivation of endogenous or therapeutically useful agents have been referred to tentatively as anticatabolites(6).

Procedure. The system and methods of Cohen and McGilvery(4) were employed for studying the conjugation of PAB with gly-

* Presented in part before the Pharmacological Section, Fed. of Biol. Sciences, April, 1950(6).

† 'Benemid' is the trademark that has been applied to p-(di-n-propylsulfamyl)-benzoic acid by Sharp & Dohme, Inc.

1. Miller, C. S., Ziegler, C., and Sprague, J. M., to be published.

2. Beyer, K. H., Painter, R. H., and Wiebelhaus, V. D., *Am. J. Physiol.*, 1950, v161, 259.

3a. Taggart, J. V., and Forster, R. P., *Am. J. Physiol.*, 1950, v161, 167.

3b. Mudge, G. H., and Taggart, J. V., *Am. J. Physiol.*, 1950, v161, 173; *ibid.*, 1950, v161, 191.

3c. Cross, R. J., and Taggart, J. V., *Am. J. Physiol.*, 1950, v161, 181.

4. Cohen, P. P., and McGilvery, R. W., *J. Biol. Chem.*, 1946, v166, 261.

5. Beyer, K. H., *Pharmacol. Rev.*, 1950, v2, 227.

6. Beyer, K. H., Wiebelhaus, V. D., Russo, H. F., Peck, H. M., and McKinney, S. E., *Fed. Proc.*, 1950, v9, 258.

cine. The Warburg respirometer apparatus was employed, the flasks and their contents being shaken at 37°C for a period of one hour before analyzing for PAB and PAH, unless otherwise indicated in the text. The flasks contained:

Washed liver or renal cortex residue	0.6 ml
PAB, 0.02 M	0.2
Glycine, 0.15 M	0.5
Succinate, 0.154 M	0.3
Cytochrome C, 2 × 10^{-4} M	0.2
KCl, 0.5 M	0.4
$MgSO_4$, 0.154 M	0.1
K_2HPO_4, 0.3 M, pH 7.6	0.6
Adenylic acid, 0.0164 M	0.1
H_2O, with or without inhibitor added to make a total volume of 4.0 ml	

Guinea pig liver or renal cortex was homogenized in cold KCl and was washed 3 times by centrifugation. The final residue was suspended in cold isotonic KCl solution to contain 8 to 11 mg of nitrogen per ml of suspension.

This system regularly yielded a 90% or greater conjugation of PAB (4 μ moles/flask) when incubated aerobically for one hour. The observations of Cohen and McGilvery (4) with respect to the need for adenylic acid, inorganic phosphate, aerobiosis and the presence of a suitable oxidizable substrate have been confirmed. ATP, but not adenylic acid, will provide the necessary energy for the conjugation in the absence of aerobiosis.

Results. Fig. 1 illustrates the relationship of the molar concentration of 'Benemid' to the inhibition of PAB conjugation. Here it may be seen that, in the absence of 'Benemid,'

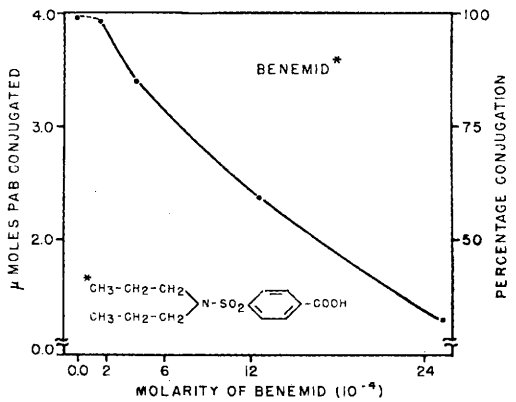


FIG. 1.

Relationship between molar concentration of 'Benemid' and extent of inhibition of p-aminobenzoic acid conjugation with glycine.

conjugation of PAB with glycine was essentially complete. The inhibitory effect of 'Benemid' on the conjugative reaction occurred without an effect on the oxygen uptake of the preparation. Likewise, it can be demonstrated that the conjugation of p-aminosalicylate (PAS) with glycine to form p-aminosalicylurate (PASU) is inhibited by 'Benemid.' However, since the conversion of PAS to PASU is not complete under these conditions it is being studied further.

'Benemid' is equally effective in the ATP-anaerobic system as an inhibitor of PAB conjugation, as is illustrated in Table I. In these experiments the duration of incubation of PAB with glycine was 30 minutes instead of one hour. Therefore, it appears that the inhibitor acts at some point beyond the oxidation/phosphorylation, or ~Ph generating, stage.

'Benemid' has no effect on the *in vitro* phosphorylation of glucose, Table II, at all concentrations of the inhibitor that have been studied (8.3 x 10^{-4} M to 5 x 10^{-3} M). The manometric method of Colowick and Kalckar (7) was employed for these studies. For each mole of phosphate transferred from ATP to glucose one acid equivalent is formed which displaces one equivalent of CO_2 from the bicarbonate buffer. Iodoacetate was used to stop the glycolytic reactions at the triose-phosphate stage. Fluoride was added to inhibit the action of phosphatases. A lyophilized powder of a dialyzed extract of dog brain was prepared in the manner described by Wiebelhaus and Lardy (8) and was used as the source of the hexokinase.

Discussion. From the data presented above and documented in other experiments it is apparent that 'Benemid' is a potent inhibitor of the conjugation of glycine with either PAB or PAS, and that the inhibition involves specifically the utilization of energy by the reversibly inhibited conjugase, rather than by prevention of the production of ~Ph or a generalized inhibition of its utilization. It

7. Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, 1943, v148, 117.

8. Wiebelhaus, V. D., and Lardy, H. A., *Arch. Biochem.*, 1949, v21, 321.

TABLE I.
Inhibition of PAB-Glycine Conjugation* by 'Benemid.'

Adenylic acid, final molarity	ATP, final molarity	Gas phase	'Benemid' final molarity	PAB conjugated, %	Inhibition of conjugation, %
		Exp. 1.			
		Air		1.6	
.0004		N_2		1.4	
.004		N_2		2.2	
.004		Air		68.7	—
.004		Air	.00125	19.8	70.2
.004		Air	.00250	14.4	79.0
	.004	Air		83.2	—
	.004	Air	.00125	37.4	55.2
	.004	Air	.00250	5.6	93.4
		Exp. 2.			
	.006	Air		59.1	
	.006	Air	.00125	53.0	10.2
	.006	Air	.00250	37.7	32.8
	.006	N_2		26.0	
	.006	N_2	.00125	22.2	14.6
	.006	N_2	.00250	18.2	30.0
.006		N_2		1.3	

* Incubated for one-half hour at 37°C. Where ATP was employed, one-half the total amount was tipped in at "0" time. The other half was tipped in from a second sidearm at 15 minutes.

TABE II.
Lack of an Effect of 'Benemid' on Enzymatic Transphosphorylation Between ATP and Glucose.*

Glucose final molarity	ATP final molarity	'Benemid' final molarity	CO_2 liberated μl
	.008		32
.01	.008		398
.01	.008		409
.01	.008	.00083	403
.01	.008	.00083	398
.01	.008	.00167	397
.01	.008	.00167	391
.01	.008	.005	388
.01	.008	.005	391

* For each mole of phosphate transferred from ATP to glucose one acid equivalent is formed and a molar equivalent of CO_2 is displaced from the bicarbonate buffer. Gas phase was 95% N_2 — 5% CO_2 mixture. The system was incubated at 37°C. Duration of the experiment was 30 min.

is our present impression that glycine and perhaps other conjugative mechanisms are implicated fundamentally in certain processes of secretion; the "catabolism" of essential metabolites, possibly some hormones, certain therapeutically useful agents, and in some other as yet ill-defined reactions.

The inhibition of PAB conjugation with glycine by 'Benemid' cannot be considered as an effect on peptide bond formation because of the lack of an α-amino group adjacent to the PAB-carboxyl group that is involved in the amide formation. Obviously, PAH is not a typical dipeptide since the α-amino group is not repeated in the molecule. In long-term chronic toxicity experiments, 'Benemid' had no effect on protein metabolism(9). The disposition of 'Benemid' by the body has been described elsewhere(6,10).

Clinical substantiation of the "anticatabolite" inhibition of PAS (p-aminosalicylate) conjugation with glycine by 'Benemid' has been indicated by Boger, Gallagher and Pitts (11) who reported that the coadministration of 'Benemid' with PAS increased the plasma concentration attained from a given dose of the latter compound. 'Benemid' inhibits the renal tubular secretion of penicillin(5,12), but is itself reabsorbed almost entirely by the tubules rather than being eliminated by

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10. Tillson, E. K., Schuchardt, G. S., Pitt, A. A., Gass, R. W., and Beyer, K. H., *Fed. Proc.*, 1950, v9, 320.

11. Boger, W. P., Gallagher, M. E., and Pitts, F. W., *J. Phila. Gen. Hosp.*, 1950, v1, 51.

12. Beyer, K. H., Russo, H. F., Tillson, E. K., Gass, S. R., and Schuchardt, G. S., to be published.

them(6,12).

Summary. 'Benemid', p-(di-n-propylsulfamyl)-benzoic acid, inhibits the conjugase responsible definitively for the over-all reaction involved in the conjugation of glycine with p-aminobenzoic acid. It was demonstrated that the compound did not decrease the availability of energy derived from the phosphate cycle, which is a coupled component of the conjugative reaction, since 'Benemid' did not inhibit glucose phosphorylation by phosphorylase plus preformed ATP.

The implication of such conjugative reactions was discussed and several pharmacological and clinical applications of the anti-catabolite hypothesis were indicated.

Received June 22, 1950. P.S.E.B.M., 1950, v74.

Labelling in the Glucose Deposited as Starch during Photosynthesis. (18044)

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We have extracted radioactive starch from detached tobacco leaves which were permitted to carry on photosynthesis in an atmosphere of 5% $C^{12}O_2$ containing about 0.25 mc of $C^{14}O_2$. Prior to the experiment plants were kept in darkness to deplete their initial starch reserves. The extracted starch was hydrolized, and on addition of ordinary glucose, as a carrier, radioactive glucose was crystallized. The technic of the experiments was essentially that of Putman *et al.*(1). Radioactive glucose obtained was fermented with *Lactobacillus casei* and degraded according to Wood *et al.*(2). The results are shown in Table I.

From the results of the Experiment 1, it is evident that if after 24 hours of preliminary starvation photosynthesis is permitted to take place for a long time, then C^{14} is spread fairly evenly in all positions.

It has been shown by Benson *et al.*(3) that in the initial stages of photosynthesis C^{14} appears in glucose at first in the 3 and 4 positions and only later spreads to others. It was thought that an extension of the period of darkness prior to photosynthesis may accentuate this tendency, and that under these conditions either the bulk of C^{14}, or even all of it, may appear in the positions 3 and 4. To test this, plants were at first starved for 72 hours and then permitted to carry on photosynthesis for various periods of time. The results are represented by Experiments 2, 3 and 4, Table I.

While the bulk of C^{14} is now in the 3 and 4 positions, considerable amounts still are present in the 2 and 5 and the least in the 1 and 6. The results are essentially the same in all 3 experiments in spite of the fact that the duration of photosynthesis in them varied from 3½ to 48 hours. One can conclude, therefore, that it was the length of the preceding starvation period and not the length of the illumination, which was responsible for the observed distribution of C^{14}.

One may also expect that if a period of photosynthesis in $C^{14}O_2$ is followed by that in $C^{12}O_2$, the final labelling in starch will be reversed. The greatest activity should be in 1 and 6, followed by that in 2 and 5, and the least in 3 and 4. To test this, 2 detached leaves were permitted to carry on photosynthesis for 3 hours in an atmosphere of $C^{14}O_2$. At the end of this period one leaf was killed and the distribution of C^{14} was determined. The other leaf was transferred to 5% $C^{12}O_2$

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2. Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, 1945, v159, 474.

3. Benson, A. A., Calvin, M., Haas, V. A., Aronoff, S., Hall, A. G., Bassham, J. A., and Weigl, J. W., *Photosynthesis in Plants*, 1949, Chap. 19, pp. 381-401, a monograph of the American Society of Plant Physiologists, edited by J. Franck and W. E. Loomis.