TABLE II. Influence of β-alanine Upon Antibody Response and Growth of Pantothenic Acid-Deficient Rats.

Group	No. of rats	Body wt*		Antibody titers	
		Initial	Final†	Range	Avg
I. Normal control	5	42	223		1:2560‡
II. Pantothenic acid-deficient	10	44	89	1:5–1:320	1:148
III. Pantothenic acid-deficient + β-alanine	10	45	94	1:5–1:320	1:143

* Group avg in g.
† At the time of bleeding.
‡ All 5 rats had a titer of 1:2560.

sparing action of DL-methionine to become apparent have not been presented by these authors(5). Our failure to observe a significant improvement in growth and survival may be attributed to the shorter experimental period employed in the present experiments.

B. *Effect of β-alanine upon antibody response of pantothenic acid-deficient rats.* This experiment was conducted to determine whether β-alanine could substitute for pantothenic acid in the processes of antibody production. Twenty-five weanling rats were distributed into 3 groups as indicated in Table II. Each animal of Group III received 0.5 mg of β-alanine daily. The experimental diets were fed *ad libitum* to all rats and immunization was instituted during the 5th week.

It is evident from the results summarized in Table II that β-alanine cannot replace pantothenic acid for either antibody production or growth in the rat. The latter observation confirms the opinion of El-Sadr *et al.*(7) that the slight growth stimulation observed by Hoffer and Reichstein(8) was not significant.

Summary. 1. In albino rats DL-methionine possesses a significant sparing action upon the pantothenic acid requirement for antibody production. 2. β-alanine cannot replace pantothenic acid in the processes of antibody synthesis.

7. El-Sadr, M. M., Hind, H. G., Macrae, T. F., Work, C. E., Lythgoe, B., and Todd, A. R., *Nature,* 1939, v144, 73.

8. Hoffer, M., and Reichstein, T., *Ibid.,* 1939, v144, 72.

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Studies on β-Glucuronidase from *E. coli.* (18591)

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Although the preparation of β-glucuronidase from mammalian tissues has been studied by Masamune(1), Oshima(2), Fishman(3-6) and others(7-11), little attention has been

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1. Masamune, H., *J. Biochem. Japan,* 1934, v19, 353.

2. Oshima, G., *J. Biochem. Japan,* 1936, v23, 305.

3. Fishman, W. H., *J. Biol. Chem.,* 1939, v127, 367.

4. Fishman, W. H., *J. Biol. Chem.,* 1940, v136, 229.

5. Fishman, W. H., *J. Biol. Chem.,* 1947, v169, 7.

6. Bernfeld, P., and Fishman, W. H., *Arch. Biochem.,* 1950, v27, 475.

7. Graham, A. F., *Biochem. J.,* 1946, v40, 603.

8. Mills, G. T., *Biochem. J.,* 1948, v43, 125.

9. Sarkar, N. K. and Sumner, J. B., *Arch. Biochem.,* 1950, v27, 453.

10. Talbot, N. B., Ryan, J., and Wolfe, J. K., *J. Biol. Chem.,* 1943, v151, 607.

11. Levy, G. A., *Nature,* 1947, v160, 54.

devoted to microorganisms as a source of this important enzyme. That bacteria may possess β-glucuronidase activity has been suggested by the reports of Cohen and Marrian (12) and Patterson(13) that the estrogen of pregnancy urine was liberated by putrefaction. Barker, Brooksbank, and Haslewood(14) found that pregnanediol glucuronide and in some experiments glucuronic acid which was presumably liberated from the conjugate, were destroyed by some strains of *Staphylococcus albus*. Earlier, Bucher and Geschickter(15) had presented evidence which indicated that the liberation of pregnanediol in urine from pregnanediol glucuronide was brought about by the action of a bacterial enzyme. Our interest in β-glucuronidase lay in its use as a means of hydrolyzing steroid glucuronides. In an earlier paper, we reported the production of this enzyme(16) by *E. coli* and the hydrolysis of urinary estrogen and 17-ketosteroid conjugates by the bacterial culture fluid(17). The preparation of potent solutions of β-glucuronidase by the method described in this paper has been carried out by 3 other workers in this laboratory and one (18) of them has reported on the hydrolysis of conjugates of reducing lipids of urine by such solutions.

This report describes the more important points which must be taken into account in preparing potent solutions of β-glucuronidase from cultures of *E. coli*.

Experimental. The basal medium employed in our survey of bacteria as a source of β-glucuronidase consisted of 1.0% Difco bacto-peptone, 0.3% Difco bacto-beef extract, 0.5% sodium chloride, and 3.0% sodium glycerol phosphate. β-Glucuronidase activity was determined by the hydrolysis of sodium phenolphthalein glucuronidate, a chromogenic substrate introduced by Talalay, Fishman, and Huggins(19). Of twenty-three bacteria tested, only *C. xerose, C. hoffmani,* and *E. coli* demonstrated β-glucuronidase activity. *E. coli* was selected for further study for the production of the enzyme, because of the ease with which this organism can be cultured. However, not all strains of *E. coli* possess the capacity to produce β-glucuronidase. The strain (available on request) employed in our studies was isolated from a case of cystitis.

Culture pH. The optimum pH for the production of β-glucuronidase was determined by growing *E. coli* in the basal medium at various pH levels for 5 days at 25°C.† Using the Beckman pH meter, the pH of the medium was adjusted by the addition of HCl or NaOH. Autoclaving and subsequent incubation did not alter the original pH of the medium. After incubation, the cultures were centrifuged for 1 hour at 3000 r.p.m. (International centrifuge, Size 1) to remove the bacterial debris, and the clear culture fluids were adjusted to pH 7.0‡ and analyzed for their β-glucuronidase activity. Aliquots of the various culture fluids were incubated with sodium phenolphthalein glucuronidate at 37°C for 24 hours. Chloroform, which does not inhibit bacterial β-glucuronidase, was added as a preservative during the incubation. The optimum pH for β-glucuronidase production was found to be approximately 7.3. At pH 6.5 and 8.0, the enzyme production was respectively 26% and 38% less than at pH 7.3.

Stimulation by substrate. Because of the well known influence of substrate upon the

12. Cohen, S. L., and Marrian, G. F., *Biochem. J.*, 1934, v28, 1603.

13. Patterson, J., *Brit. Med. J.*, 1937, v2, 522.

14. Barker, M., Brooksbank, B. W. L., and Haslewood, G. A. D., *Nature*, 1948, v162, 701.

15. Bucher, N. L. A., and Geschickter, C. F., *Endocrinology*, 1940, v27, 727.

16. Buehler, H. J., Katzman, P. A., and Doisy, E. A., *Fed. Proc.*, 1949, v8, 189.

17. Buehler, H. J., Katzman, P. A., Doisy, P. P., and Doisy, E. A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 297.

18. Kinsella, Jr., R. A., Doisy, R. J., and Glick, Jr., J. H., *Fed. Proc.*, 1950, v9, 190.

19. Talalay, P., Fishman, W. H., and Huggins, C., *J. Biol. Chem.*, 1946, v166, 757.

† This strain of *E. coli* produced much less β-glucuronidase when grown at 37°C.

‡ In the course of developing the procedure for potent preparations of β-glucuronidase, many assays were conducted before the pH of optimum activity was established. Some were carried out at pH 7; some at pH 7.3, but in each case the pH was kept constant for the series of each experiment.

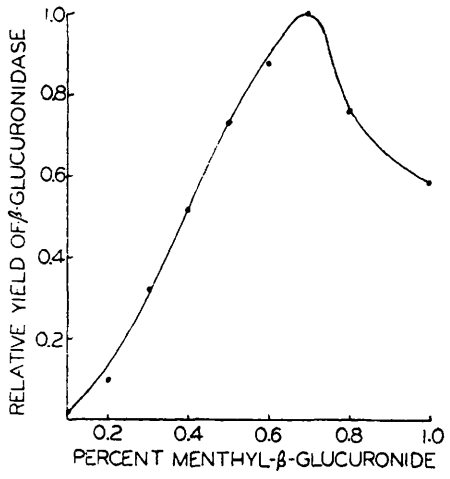

FIG. 1.

Influence of concentration of menthyl-β-glucuronide in the culture medium on production of β-glucuronidase by *E. coli*.

production of enzymes, a β-glucuronide was added to the growing cultures of *E. coli* to ascertain whether this organism would respond by producing larger amounts of β-glucuronidase. Varying amounts of menthyl-β-glucuronide, prepared according to Williams (20), were added to the basal medium at pH 7.3 and after inoculation the cultures were incubated for 5 days at 25°C. Since we had found that shaking the growing cultures increases the yield of the enzyme (see below), these cultures were kept on a shaking machine, operating at 75-100 r.p.m., during the incubation period. After filtration of the culture through paper to remove the liberated menthol and centrifugation to remove bacterial debris, the clear culture fluids were assayed for β-glucuronidase activity. It was found that the greatest yield of the enzyme (Fig. 1) was produced in a medium containing 0.7% of menthyl-β-glucuronide. The factors responsible for the apparent fall of activity in media containing higher concentrations of menthyl-β-glucuronide have not been studied.

Influence of surface area. The influence of the surface area on the production of β-glucuronidase was determined by incubating equal volumes of culture containing 0.7% of menthyl-β-glucuronide at pH 7.3 in flasks of different capacities. Thus the surface area per unit volume of culture varied with the size of the flask employed. These were cultured without shaking for 5 days, after which time assays were made for enzymatic activity. It was found that the production of enzyme was increased with increasing size of the flask in which the culture was grown. This experiment was repeated with the same series of flasks but employing constant agitation during the incubation period. A shaking machine operating at 75-100 r.p.m. was used for this purpose. This treatment completely overcame the effect of surface area, since all the cultures produced the same amount of β-glucuronidase, irrespective of the capacity of the flasks in which they were grown (Fig. 2). From these studies, it seems likely that an adequate amount of oxygen is necessary for the optimum production of β-glucuronidase by *E. coli*.

Incubation period. To determine the optimum period of incubation for the production of β-glucuronidase, cultures at pH 7.3 containing 0.7% of menthyl-β-glucuronide were grown with continuous shaking for varying lengths of time ranging from 2 to 11 days.

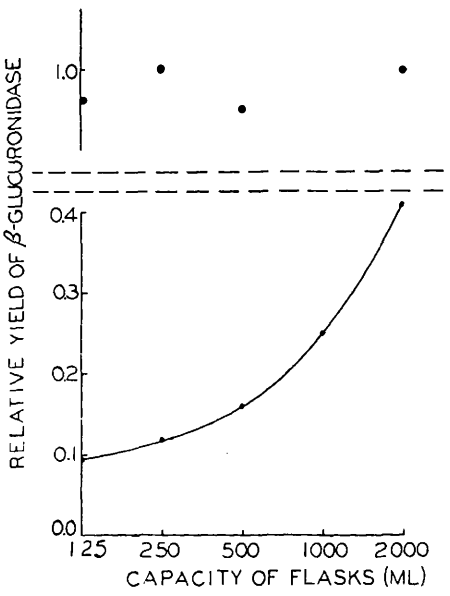

FIG. 2.

Influence of surface area of culture on production of β-glucuronidase by *E. coli*. Curve shows results when flasks were stationary, while effect of shaking them is indicated by points above broken lines.

20. Williams, R. T., *Biochem. J.*, 1938, v32, 1849.

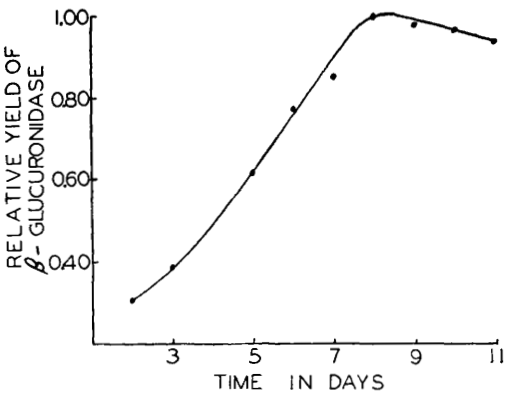

FIG. 3.
Effect of length of incubation period on production of β-glucuronidase by *E. coli*.

It was found that an incubation period of eight days gave the greatest amount of enzyme (Fig. 3).

Method of preparation. As a result of the foregoing studies, we have adopted the following procedure for the preparation of bacterial β-glucuronidase. Fifty ml of medium containing 1.0% Difco bacto-peptone, 0.3% Difco bacto-beef extract, 0.5% sodium chloride, 3.0% sodium glycerol phosphate (Mallinckrodt, U.S.P.), and 0.7% menthyl-β-glucuronide at pH 7.3 are placed in a 250 ml Erlenmeyer flask and autoclaved for 15 minutes at 15 pounds pressure. After cooling, the medium is inoculated with *E. coli* which had been carried on nutrient agar slants. The inoculated medium is incubated for 8-10 days at 25°C with continuous shaking by a rotary shaking machine operating at 75-100 r.p.m. The pH of the culture remains at 7.3 during the incubation period. The culture is then filtered through paper to remove the suspended menthol, and the filtrate is centrifuged in the cold room at 3500 r.p.m. to separate the bacterial cells from the enzyme solution. The resulting clear culture fluid without further purification can be used as the β-glucuronidase preparation. Such preparations have been stored in the refrigerator at 5°C for months without any detectable loss of activity. Stable powders can also be prepared by lyophilization. The culture fluid is rich in phosphatase activity as demonstrated by the hydrolysis of phenolphthalein phosphate. However, phenolic or alcoholic sulfatase could not be detected. Other enzymic activity was not studied.

Method of assay. The determination of the glucuronidase activity of our potent enzyme preparations is carried out by a modification of the Talalay, Fishman and Huggins procedure(19). The culture fluid is adjusted to pH 6.2 with HCl after it has been filtered and centrifuged. One ml is diluted to 10 ml with distilled H_2O; to 2 ml of this is added 1 ml of a sodium phenolphthalein glucuronidate solution containing the equivalent of 450 μg of phenolphthalein per ml, and the mixture is incubated for 4 hours at 37°C. Then to 1 ml of this incubated mixture are added 5 ml of 0.4 molar glycine buffer (pH 10.4) and the color is read in a colorimeter using a green filter (range 500-570 mμ) to determine the micrograms of phenolphthalein liberated.

$$\frac{\gamma\text{-Phenolphthalein} \times 3 \times 10}{2} = \text{micrograms of}$$

phenolphthalein liberated per ml of original culture fluid. One ml of enzyme preparations made and assayed according to the above procedures usually hydrolyzed phenolphthalein glucuronide equivalent to 1500-2500 μg of phenolphthalein.

Effect of pH on β-glucuronidase. To study the effect of pH on bacterial β-glucuronidase activity, thoroughly dialyzed preparations of the enzyme were incubated with sodium phenolphthalein glucuronidate and with sodium estriol glucuronidate.§ The incubation mixtures were buffered with 0.067 molar phosphate at pH levels ranging from 4.5 to 8.5. The optimum pH for the hydrolysis of sodium phenolphthalein glucuronidate was approximately 6.2. However, with sodium estriol glucuronidate no variation in rate of hydrolysis was found between pH 4.5 and 7.0, but above pH 7.0 the activity fell off very abruptly. Because the optimum pH range was so broad in this case, duplicate determinations were made with 3 different preparations of β-glucuronidase. The results of these experiments which are summarized in Fig. 4, were identical.

§ Our thanks are due to Professor G. F. Marrian for supplying estriol monoglucuronide.

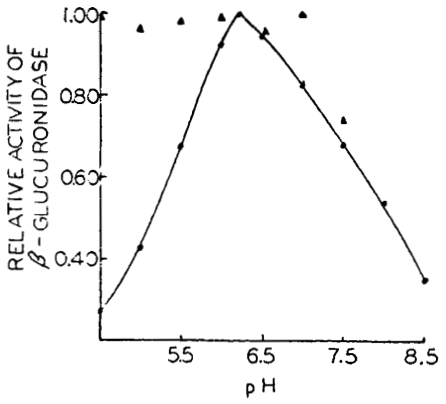

FIG. 4.

Influence of pH on activity of β-glucuronidase produced by *E. coli*.

●—Phenolphthalein-mono-β-glucuronide as substrate.

▲—Estriol-β-glucuronide as substrate.

Some properties of the β-glucuronidase. As the main object of this investigation was to prepare adequate quantities of β-glucuronidase for use in the determination of steroid glucuronides, properties of the enzyme have not been extensively studied. Dialysis of the culture fluid containing the enzyme caused the loss of at least one-half of the activity. This loss of activity was not prevented by controlling the temperature and the pH during dialysis. This bacterial β-glucuronidase is readily inhibited by ammonium chloride, a concentration of 0.1% of this salt causing a 20% reduction of activity. For this reason, the free acid and not the ammonium salt of menthyl-β-glucuronide is used in the culture medium for the preparation of the enzyme. Sodium chloride, in concentrations above 1.0%, decreases the activity of the enzyme appreciably.

Summary. Potent culture fluid preparations of β-glucuronidase are obtained when *E. coli* is grown at 25°C and pH 7.3 in a simple medium containing menthyl-β-glucuronide. The optimum incubation time is 8 days when continuous agitation is employed. The optimum pH for the hydrolysis of phenolphthalein mono-β-glucuronide is 6.2, while that for estriol-β-glucuronide is 4.5-7.0. Bacterial β-glucuronidase is readily inhibited by ammonium ion. Dialysis markedly decreases the enzymatic activity of our preparations. The culture fluid contains phosphatase but no sulfatase activity.

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Biological Activity of Alkali Treated Aureomycin. (18592)

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Recently, the growth promoting effect of aureomycin on the chick(1,2), the turkey(3), the rat(4,5), and the pig(6) have been reported. The general interpretation of these results has stressed the probable effect of the antibiotic on the intestinal flora of the test animal. The inactivity of intramuscularly administered aureomycin or penicillin(1,7) was in accord with this hypothesis.

Data presented by Stokstad and Jukes(2) suggested that aureomycin after microbiological inactivation with alkali was still active for the chick. As these data were not in agreement with the results we had obtained, it was decided to investigate more fully the biological

1. Whitehill, A. R., Oleson, J. J., and Hutchings, B. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 11.

2. Stokstad, E. L. R. and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 523.

3. Stokstad, E. L. R. and Jukes, T. H., *Poultry Science*, 1950, v29, 611.

4. Oleson, J. J., Hutchings, B. L., and Whitehill, A. R., *Poultry Science*, 1950, v29, 774.

5. Stern, J. R., and McGinnis, J., *Proc. A.C.S. Meetings*, Sept. 1950, p33C.

6. Carpenter, L. E., *Arch. Biochem.*, 1950, v27, 469.

7. McGinnis, J., Berg, L. R., Stern, J. R., Wilcox, R. A. and Bearse, G. E., *Poultry Science*, 1950, v29, 771.