A second conclusion is that Ertron and calciferol are indistinguishable in their effects on the serum calcium of the rat when corresponding U.S.P. or international antirachitic vitamin D dosages are administered. In both experiments the curves for the two products are not separated by a statistically significant amount at any point; the probable error calculated for each point is from 0.2 to 0.5 mg %.

The effects of the larger dosage of A.T.10 very closely resemble those of the 100,000 I.U. dosage of vitamin D_3. In terms of antirachitic activity, *i. e.*, vitamin D, the dose of this solution given represents only 22 to 25 I.U. per mg or a total dose of 110 to 125 units. Such a dosage is thus approximately 1/850 of the potency of the vitamin D_3 so that the effects of A.T.10 on serum calcium cannot be attributed primarily to the presence of contaminating antirachitic sterols. Finally, the marked difference between the effects of the two doses of A.T.10 given suggest that the determination of serum calcium might prove to be a satisfactory basis for the bioassay of the product to replace the original "toxic borderline dose" method.

Summary. The effects of vitamins D_2, D_3, and of Ertron and A.T.10 on serum calcium have been compared. Crystalline vitamin D_2 and Ertron are statistically indistinguishable in their effects. Vitamin D_3 produces a more prolonged hypercalcemia than does vitamin D_2. On the basis of responses to the above preparations, A.T.10 produces an effect far greater than would be predicted from its antirachitic value.

13071

Inhibition of Bacteriostatic Action of Sulfanilamide by Yeast Extracts.

T. A. LOOMIS, ROGER S. HUBBARD AND ERWIN NETER.

From the Departments of Pharmacology and Bacteriology, University of Buffalo, School of Medicine, and The Children's Hospital, Buffalo, N.Y.

Substances capable of preventing the bacteriostatic action of sulfanilamide have been found widely distributed in nature.[1] Woods[2] reported that a substance was present in an extract prepared

[1] MacLeod, C. M., *J. Exp. Med.*, 1940, **72**, 217.

[2] Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

from Baker's yeast which lessened the inhibitory effect which sulfanilamide has upon the growth of *Streptococcus hemolyticus.* Further experiments upon the properties of this compound led him to the conclusion that it resembled p-aminobenzoic acid, and investigations of preparations of that substance in pure solution showed that it markedly inhibited the anti-bacterial action of sulfanilamide. Since this paper was published, Rubbo and Gillespie[3] have isolated p-aminobenzoic acid as the benzoyl derivative from a Brewer's yeast extract. *In vivo,* p-aminobenzoic acid prevents the favorable action of sulfanilamide upon experimental infections with streptococci and pneumococci as shown by Selbie[4] and McCarty.[5]

Woods[2] found that his extracts contained, in addition to the anti-sulfanilamide factor, a compound which promoted the growth of the organisms studied. This substance was present in small amounts because after dilution of these extracts its presence could no longer be demonstrated, although the anti-sulfanilamide action could still be shown. It is generally agreed that the bacteriostatic action of sulfanilamide is influenced by the composition of the culture medium, particularly by peptone and other growth-promoting factors.[6, 7] Therefore, at the present time anti-sulfanilamide activity appears to be due to (a) factors that are favorable to the growth of the organisms and (b) compounds that specifically interfere with the action of sulfanilamide.

In this paper experiments designed to extend further the study of anti-sulfanilamide activity of yeast extracts are reported. Attempts were made to prepare extracts from Baker's yeast in which substances favoring the growth of organisms would be absent.

A fat-free, protein and peptone-free, extract from Baker's yeast was made by a technic similar to that developed by Stamp[8] and Woods.[2] Eight different samples of yeast were used, each weighing approximately 250 g. The procedure involved extraction with ammonia, precipitation with phosphotungstic acid, removal of excess phosphotungstic acid with barium hydroxide, and concentration at reduced pressure at a pH of approximately 7.0. The extract was then treated with 10 volumes of acetone because it was thought that the growth-promoting property of the extracts may be due to the presence of complex carbohydrates. A precipitate was obtained

[3] Rubbo, S. D., and Gillespie, J. M., *Nature,* 1940, **146**, 838.

[4] Selbie, F. R., *Brit. J. Exp. Path.,* 1940, **21**, 90.

[5] McCarty, M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 61.

[6] Long, P. H., and Bliss, E. A., *So. Med. J.,* 1938, **31**, 308.

[7] Lockwood, J. S., and Lynch, H. M., *J. Am. Med. Assn.,* 1940, **114**, 835.

[8] Stamp, T. C., *Lancet,* 1939, **2**, 10.

which was removed by centrifuging the solution, and the treatment with acetone was repeated twice. Acetone was removed from the supernatant fluid by evaporation to dryness. This dry residue was dissolved in distilled water, the pH adjusted to 7.0, and dilution was made so that 1 cc was equivalent to 1 g of the original yeast cells. This solution will be referred to hereafter as extract A. This preparation gave a positive reaction for reducing compounds (3 to 5 mg % as dextrose) by the method of Klendshoj and Hubbard,[9] but no fermentable sugar was present. No significant increase in reducing action was obtained after acid hydrolysis. When treated by the diazo reaction of Bratton and Marshall,[10] its content of p-aminobenzoic acid appeared to be 0.06 mg %. The precipitate obtained from the action of acetone was dried, dissolved in distilled water to give 1 cc equivalent to 1 g of the yeast cells, and the solution adjusted to a pH of 7.0. This solution, which is referred to as extract B, gave a negative reaction for starch with iodine. It contained a small amount of reducing material (15 to 20 mg %) the larger proportion of which was fermentable by treatment with yeast. Acid hydrolysis yielded small additional amounts of reducing compounds (5 to 10 mg %). No reaction was obtained by the diazo technic of Bratton and Marshall.[10]

The bacteriological tests were carried out *in vitro* on strains of beta *hemolytic streptococci* (group A Lancefield) and *E. coli.* In tests with the former organisms the solution to be tested was added to 1% dextrose phenol red broth (Difco) containing sulfanilamide in varying concentrations. In tests with *E. coli* the synthetic medium of Sahyun, Beard, *et al.*,[11] which was free of demonstrable antisulfanilamide activity was prepared in a similar manner. The mediums were sterilized in test tubes by autoclaving at 15 lb for 12 minutes. Organisms freed from nutrient material by washing and resuspending in physiological saline were used for seeding purposes. Inoculated material was incubated at 37°C. Visible growth of the organisms in the material tested was taken as evidence of the presence of growth factors. Visible growth in the medium containing the test material and sulfanilamide, in contrast to the absence of visible growth in the sulfanilamide control indicated antisulfanilamide activity.

The results of these tests showed that both extract A and extract

[9] Klendshoj, N. C., and Hubbard, R. S., *J. Lab. Clin. Med.*, 1940, **25**, 1102.

[10] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

[11] Sahyun, M., Beard, P., Schultz, E. W., Snow, J., and Cross, E., *J. Inf. Dis.*, 1936, **58**, 28.

B inhibited the bacteriostatic action of sulfanilamide toward *hemolytic streptococci* as well as *E. coli.* Both preparations also promoted growth of *E. coli* under these experimental conditions. Because there is evidence that the action of these yeast extracts may be due to p-aminobenzoic acid, a comparative study of the anti-sulfanilamide activity of these extracts and p-aminobenzoic acid was carried out. p-aminobenzoic acid showed anti-sulfanilamide activity when tested in concentrations approximating those found in extract A. It did not promote growth of *E. coli,* although in higher concentrations (10 to 100 mg %) it prolonged the survival of this organism. p-aminobenzoic acid was extracted from acid aqueous solutions by ether, and the anti-sulfanilamide activity was destroyed by acetylation as carried out by the method of Woods[2] but was subsequently recovered on acid hydrolysis.

After extraction with ether from an acid medium or acetylation of extract A, the diazo reaction became negative, but only part of the anti-sulfanilamide activity was lost. On subsequent hydrolysis following acetylation the extract did not recover demonstrable anti-sulfanilamide activity, although the amino compound was regenerated as shown by the development of a positive diazo reaction. In the ether-soluble portion of extract A which was evaporated to dryness and taken up in water, anti-sulfanilamide activity could be demonstrated as well as a positive diazo reaction, and this ether-soluble portion was not growth-promoting to *E. coli.* Thus, the ether extracted and acetylated anti-sulfanilamide factor in extract A may be p-aminobenzoic acid. Extract A showed a much greater anti-sulfanilamide activity than did the presumably equivalent solution of p-aminobenzoic acid. In extract A, therefore, there was apparently not only p-aminobenzoic acid but also another factor which diminished the bacteriostatic action of sulfanilamide. The results of the study of extract B were in some ways similar to those obtained with extract A. As in extract A, acetylation procedures destroyed part of the anti-sulfanilamide activity and subsequent hydrolysis completely destroyed this property of extract B. However, unlike extract A, growth-promoting properties of extract B were partially recovered by acid hydrolysis. Extract B did not contain p-aminobenzoic acid as shown by a negative diazo reaction and negative anti-sulfanilamide activity of an ether extract prepared from it. The anti-sulfanilamide activity and the growth-promoting properties of the extract persisted apparently unchanged after ether extraction. The results of these experiments are summarized in Table I.

In conclusion, the anti-sulfanilamide activity of the yeast ex-

TABLE I.
Effect of Ether Extraction and Acetylation on Activity of Para-aminobenzoic Acid and Yeast Extracts.

	Anti-sulf. activity	Growth-promoting activity	Diazo test
1. p-aminobenzoic acid (100 mg%)			
natural	+	—	+
treated with ether	—		—
ether soluble fraction	+		+
acetylated	—	—	—
acetylated then hydrolysed	+	—	+
2. Extract A			
natural	+	+	+
treated with ether	+	+	—
ether soluble fraction	+	—	+
acetylated	+	—	—
" then hydrolysed	—	—	+
natural extract hydrolysed	+	+	+
3. Extract B			
natural	+	+	—
treated with ether	+	+	—
ether soluble fraction	—	—	—
acetylated	+	—	
" then hydrolysed	—	+	
natural extract hydrolysed	+	+	—

+ = Test is positive or activity is present.
— = " "negative " " "absent.

tracts was due to at least two factors. One has properties which closely resemble p-aminobenzoic acid and the other factor differs from p-aminobenzoic acid because anti-sulfanilamide activity persists after ether extraction and acetylation procedures which destroy the anti-sulfanilamide activity of p-aminobenzoic acid. The experiments suggest that this second factor is possibly made up of two different compounds, one soluble and the other insoluble in acetone. The experiments further showed no absolute relationship between growth-promoting properties and anti-sulfanilamide activity of these extracts, since fractions of yeast extracts were prepared which promoted the growth of *E. coli* without showing demonstrable anti-sulfanilamide activity, and conversely, fractions which had anti-sulfanilamide activity without promoting growth of the organisms.