as demonstrated by the approximate 98% recovery of added carotene to various controls.

The examination of radioactivity of the non-saponifiable fraction after 6 hours of incubating carotene with cell-free liver homogenate reveals considerable activity in a fraction which does not contain carotene or digitonin-precipitable sterols. It was not possible under these conditions to recover C-14 labeled Vit. A. Work is now in progress to isolate and characterize the various radioactive products present in this non-saponifiable fraction.

The liberation of $C^{14}O_2$ early in the incubation periods indicates that some cleavage of the carotene molecule takes place early in the degradation phase.

Addition of ATP, DPN, nicotinamide and $MgCl_2$ accelerated the destruction of carotene. Although there was considerable variation between one rat liver and another, the addition of these cofactors did accelerate the destruction of carotene by any one liver preparation. The amount of carotene destroyed by cell-free rat liver homogenates appeared to be approximately the same under aerobic and anaerobic conditions.

*Summary.* Ten to 20% of β-carotene was destroyed by incubating C-14 labeled β-carotene with various tissues from the rat. All tissues studied had the ability to transfer radioactivity from β-carotene to sterols, saponifiable material and steam-distillable compound or compounds. ATP, DPN, nicotinamide and $MgCl_2$ were cofactors that increased β-carotene destruction by cell-free homogenates. A negligible amount of $CO_2$ was liberated during incubation.

The author thanks Mrs. Phyllis Spiker for technical assistance.

1. Krause, R. F., Sanders, P. L., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 549.

2. Willmer, J. S., Laughlaud, D. H., *Can. J. Biochem. Physiol.*, 1957, v35, 819.

3. Fishwick, M. J., Glover, J., *Biochem. J.*, 1957, v66, 36.

4. Lilly, V. G., Barnett, H. L., Krause, R. F., Lotspeich, F. J., *Mycologia*, 1958, v50, 862.

5. Booth, V. H., *Carotene—Its Determination in Biological Material*. W. Heffer & Sons, Ltd., Cambridge, 1957.

6. Siekevitz, P., *J. Biol. Chem.*, 1952, v195, 549.

7. Schneider, W. C., Hogeboom, G. H., *Cancer Res.*, 1951, v11, 1.

8. Kelsey, F. E., *J. Biol. Chem.*, 1939, v127, 15.

9. Srere, P. A., Chaikoff, I. L., Daube, W. G., *ibid.*, 1948, v176, 829.

10. Twitchell, E., *Ind. Eng. Chem.*, 1921, v13, 806.

Received January 18, 1961. P.S.E.B.M., 1961, v107.

## Action of Synthetic Lysine Polypeptides on Isolated Guinea Pig Ileum.* (26627)

T. RICHARDSON† AND M. A. STAHMANN
*Department of Biochemistry, University of Wisconsin, Madison*

Synthetic lysine polypeptides or polylysines are polycations which readily interact with many polyanionic substances. *In vitro,* they agglutinate bacteria(1,2), viruses and virus deoxyribosenucleic acid(3), and red blood cells(4,5). Lysine polypeptides inhibit pepsin(6) and fibrinolysis(7), but enhance conversion of fibrinogen to fibrin(8), and are hydrolyzed by trypsin(9). *In vivo,* polylysine preparations possess antiviral(10) and

* This report is based on a portion of a Ph.D. thesis by T. Richardson and was supported in part by research grant from Nat. Microbiol. Inst., N.I.H., U.S.P.H.S., and the Research Committee, Graduate School, Univ. of Wisconsin, from funds supplied by Wisconsin Alumni Research Foundation. Approved for publication by Director of Wisconsin Agr. Exp. Station.

† Predoctoral Research Fellow of Nat. Cancer Inst. Present address: Dept. of Food Science and Technology, Univ. of California, Davis.

antitumor(11) activities and are weakly antigenic in rabbits(12,13). Subcutaneous injections of polylysine into rats increases capillary permeability, presumably as a direct action on the vascular endothelial cement (14). A preliminary abstract(15) suggested that polylysine and protamine released histamine when large doses were injected intravenously into dogs or cats. Histamine was also released from washed cell-free liver homogenates. In the work reported here, we have continued studies on the biological properties of polylysine and will describe the release of a contractile substance, probably histamine, by lysine polypeptides from the isolated guinea pig ileum, preparation of polylysyl histamine, and the inability of trypsin to hydrolyze a terminal lysyl-histamine bond.

*Materials and methods.* Materials were obtained as follows: L-lysine from Mann Biochemicals, protamine sulfate from Eli Lilly Co., histamine from Distillation Products, and salt-free crystalline trypsin from Pentex Laboratories, Inc. Benadryl (2-diphenylmethoxy-N, N-dimethylethylamine hydrochloride) was purchased locally as a 1:100 solution for injection. Young adult female guinea pigs were obtained from Zeimet Farms, Madison.

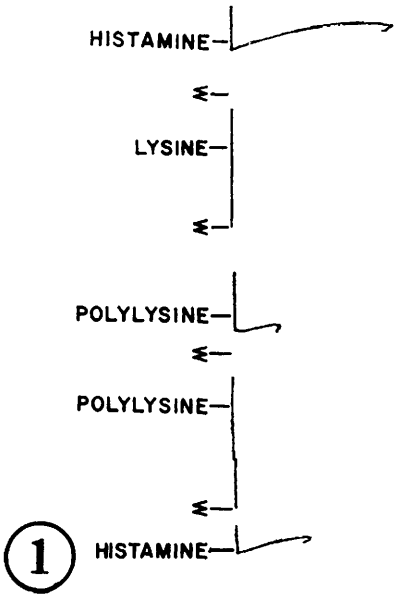
ε-Carbobenzoxy L-lysine polypeptide was prepared by ammonia-initiated polymerization of ε-carbobenzoxy-N-carboxy-L-lysine anhydride in dry dioxane(16). Degree of polymerization was determined by α-amino nitrogen analysis. Carbobenzoxy protective groups were removed by treatment with anhydrous hydrochloric acid in glacial acetic acid(17) and the product isolated as polylysine hydrochloride.

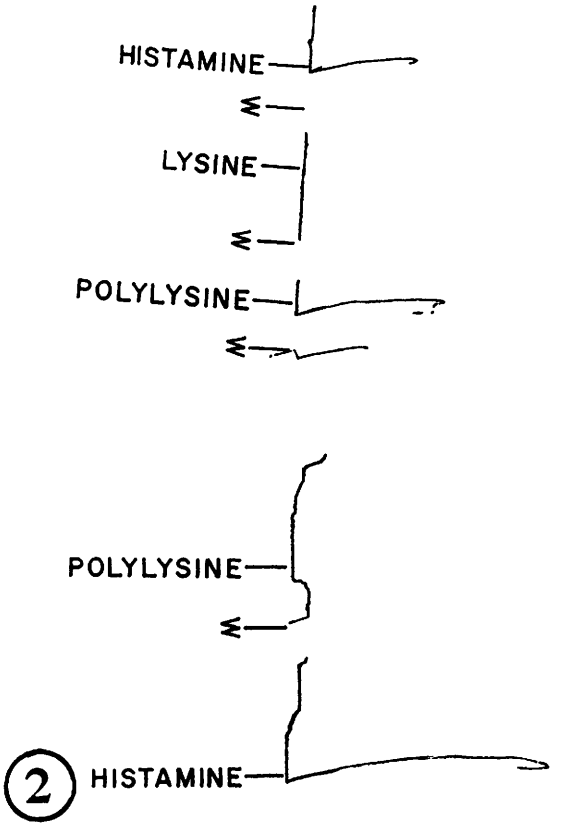
Poly-L-lysyl histamine was prepared by initiating polymerization of ε-carbobenzoxy-N-carboxy-L-lysine anhydride with histamine in dry dimethyl formamide. In the polymerization procedure, 15.3 g (50mM moles) of the anhydride were dissolved in 275 ml of anhydrous dimethyl formamide. To this solution were added 556 mg (5mM moles) of histamine base representing an anhydride to initiator ratio of 10. The clear yellowish solution was stirred for 36 hours, protected with a calcium chloride drying tube. Subsequently, the polymerization mixture was poured into excess water to precipitate the polymer. The product was washed thoroughly with water and lyophilized. Carbobenzoxy groups were removed with anhydrous hydrochloric acid(17) to yield poly-L-lysyl histamine hydrochloride in a yield of 84% of theory. The product gave strong positive Ehrlich diazo and biuret reactions.

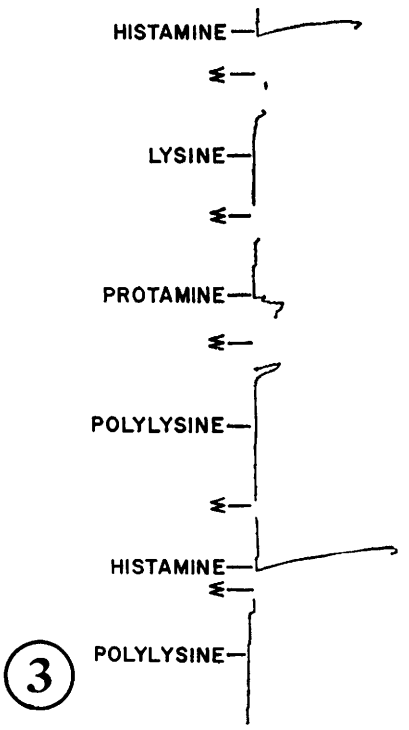
Polylysyl histamine, its hydrolysate, lysine, and histamine were spotted on Whatman 4 paper, and paper chromatograms were developed 16 hours in secondary butanol-98% formic acid-water (70:15:15). Papers were sprayed with 0.5% ninhydrin in water-saturated n-butanol or with the imidazole reagent of Block *et al.*(18). Polylysyl histamine did not migrate while histamine, lysine, and hydrolytic products migrated as expected. Furthermore, histamine when mixed with polylysyl histamine migrated freely. Although the solvent system did not effect good separation of L-lysine from histamine, it showed there was very little contamination of the polymer with lysine or histamine.

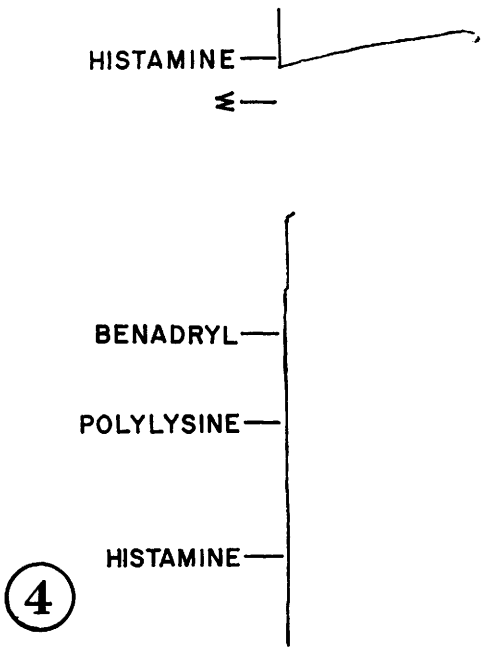
Polylysyl histamine was analyzed for histamine by adaptation of the colorimetric determination of histidine described by Jorpes (19). Diazotized sulfanilic acid and histamine formed a cherry-red color that reached maximum intensity in 10 minutes and had a maximum absorption at 500 mμ. Lysine did not interfere with the color measurement at this wavelength. Polylysyl histamine, hydrolyzed in 6N HCl, contained 4.5% histamine indicating an average of 15 lysyl residues per molecule.

The polypeptides were tested on the isolated guinea pig ileum using the technic as outlined by Code and McIntire(20). Sections of ileum 4 to 6 cm were mounted in Tyrode's solution gassed continuously with 95% oxygen and 5% carbon dioxide, U.S.P. The muscle bath, similar in design to Coulson's(21), had a 30 ml capacity and was immersed in a water bath at 35°C. Solutions to be assayed were incubated at 35°C, and test aliquots of never more than 1 ml were added to the muscle bath with a


HISTAMINE
LYSINE
POLYLYSINE
POLYLYSINE
1
HISTAMINE


HISTAMINE
LYSINE
POLYLYSINE
POLYLYSINE
2
HISTAMINE


HISTAMINE
LYSINE
PROTAMINE
POLYLYSINE
HISTAMINE
3
POLYLYSINE


HISTAMINE
BENADRYL
POLYLYSINE
4
HISTAMINE

syringe. After the muscle response was recorded on a Bird Horizontal Kymograph, the bath was drained and, unless otherwise indicated, the muscle was washed 3 times each with 30 ml of Tyrode's solution from a reservoir in the water bath. Stock test solutions were prepared in the required amount of Tyrode's solution, and if necessary, the pH was brought to 7.2 with sodium bicarbonate.

Tryptic hydrolyses of poly-L-lysyl histamine in 0.22 M collidine buffer at pH 7.6 and 26°C were patterned like those described by Waley and Watson(9). The reaction mixtures contained 48 μg trypsin and 20 mg poly-L-lysyl histamine hydrochloride in the collidine buffer. Ten μl aliquots of the reaction were spotted on Whatman 1 paper at zero time, and at 5, 10, 20, 40, 80, and 1,000 minutes after initiation of the reaction. Enzyme and substrate blanks were spotted at the end of each experiment. The enzymatic reactions were terminated on paper by applying 2N acetic acid to the spots. After addition of lysine and histamine markers, the papers were developed 15 hours in ascending fashion in n-butanol-pyridine-water (1:1:1), and the dried papers were sprayed with the imidazole reagent of Block *et al.*(18). In addition, distribution of lysine peptides with and without histamine residues was determined from identical chromatograms containing spots from a 1,000 minute reaction mixture. One chromatogram was sprayed with ninhydrin reagent and the other with imidazole reagent.

*Results.* The tracings in Fig. 1 indicate that L-lysine (1:2,000) failed to induce contraction of the freshly excised guinea pig ileum, whereas polylysine containing 28 lysyl residues (1:2,000) stimulated the ileum to contract. There was only a slight lag in the response of the muscle to the polymer compared to its immediate response to histamine (1:30,000,000) suggesting that the site of action of the polymer was not hindered. Other isolated segments of ileum contracted in the presence of synthetic L-lysine polypeptides prepared with anhydride to initiator ratios of 10, 40, and 320. The muscles were generally refractory to a second addition of polylysine to the bath but were always sensitive to subsequent additions of histamine. In exceptional cases it was possible to demonstrate (Fig. 2), a very weak response to a second challenge with polylysine. Protamine (1:2,000), although much less effective than polylysine, also stimulated the ileum to contract (Fig. 3). A subsequent addition of polylysine to the bath, however, had no effect on the ileum. Also, polylysine was ineffective after a second histamine response indicating the polymer was not displacing non-specifically bound exogenous histamine. These data indicate polylysine *per se* was not mediating the contraction but was stimulating the release of an active material. The first contact with polylysine presumably depleted the available contractant and subsequent additions of the polymer were ineffective.

Benadryl (1:10,000,000) completely prevented the intestinal response to polylysine, thus implicating endogenous histamine as the contractile agent (Fig. 4). Responses to the synthetic L-lysine polypeptides were obtained only with freshly excised ileum, and there was variation in the magnitude of the contraction. When the muscles were maintained in aerated Tyrode's solution for periods exceeding ½ hour the response to polylysine, but not to histamine, lessened and in most cases became almost nil.

Polylysyl histamine (1:6,000) also induced a contraction of the isolated guinea pig ileum which was blocked by Benadryl. The polylysine moiety alone at this concentration would not produce a contraction of the observed magnitude, and the same muscle responded to a second addition of the polylysyl histamine. These 2 factors preclude activity due to the polylysine portion of the polylysyl histamine and suggest that the response may

FIG. 1. Effect of polylysine on isolated guinea pig ileum.
FIG. 2. Effect of polylysine on isolated guinea pig ileum.
FIG. 3. Effect of protamine on isolated guinea pig ileum.
FIG. 4. Blocking of polylysine action on isolated guinea pig ileum with Benadryl.

be due to traces of free histamine in the preparation.

Polylysyl histamine, as a model substrate, offered an opportunity to study the effects of trypsin on the C-terminal lysyl-histamine bond and further to study the proteolytic theory of histamine release(22). Chromatograms of the tryptic hydrolyzate sprayed with the imidazole reagent clearly showed the lysine polymer was readily hydrolyzed within 5 minutes after initiation of the enzymatic reaction. As the reaction proceeded, 3 imidazole-positive spots became the primary reaction products, and the spot at the origin decreased until it was completely gone at the end of 1,000 minutes. Only a very small trace of the bound histamine, however, was released during the reaction. The chromatograms of the 1,000 minute reaction mixture sprayed with imidazole reagent indicated 3 spots corresponding to a faint trace of histamine with an $R_f$ of 0.44 and 2 major components, probably dilysyl and trilysyl histamine(9) with $R_f$ values of 0.38 and 0.24, respectively. The ninhydrin-treated chromatograms showed the same pattern in addition to large quantities of lysine peptide fragments close to the origin. The results indicate that the lysyl-histamine bond was resistant to tryptic hydrolysis.

*Discussion.* The contractile response of freshly excised segments of guinea pig ileum to polylysine and protamine and the subsequent refractoriness of the muscle to the polypeptides indicate that the contraction is not due to a direct action of the polymers. The failure of an equivalent amount of L-lysine to induce a contraction suggests that polylysine activity may be due to its basic polyelectrolyte character. There was only a slight lag in response of the muscle to the polymers, as compared to the immediate response to histamine. This small delay and the observation that the muscle is refractory to a second stimulation by the polypeptide indicates that polylysine released some loosely bound substance which in turn induced the contraction. The first addition of polylysine depletes the available contractant, and subsequent additions of polymer are then ineffective. Blocking of the contractile response by Benadryl indicates that loosely bound histamine or similar substances mediates the contraction. Boreus and Chakravarty(23) have recently correlated release of histamine during the antigen-antibody reaction in sensitized guinea pig tissues with disappearance of mast cells. In the jejunum, however, the mast cells did not disrupt significantly, and only a maximum of 3% of total histamine was released. Perhaps only those mast cells near the surface of the intestine are available for histamine release. Assuming that the response to histamine was linear and that concentration of the histamine in the tissues was of the same order of magnitude as those found by Boreus and Chakravarty(23) [10.9 to 14.7 $\mu$g/g] an approximation can be made of the quantity of contractant released calculated as per cent of total histamine. Figures which give maximal calculated values indicate that only 4 to 5% of the histamine in the intestine was released in the present studies. The low release of histamine by the intestine may reflect the impermeability or great reactivity of the intestine toward polylysine. These observations are compatible with either the ion exchange(24) or the lecithinase activation (25) concepts of histamine release.

When the muscles were maintained in aerated Tyrode's solution for periods of time exceeding ½ hour the response to polylysine, but not to histamine, lessened considerably. Responses to the synthetic polypeptides were obtained only with freshly excised ileum. The decreased response with time might reflect a slow release of that portion of endogenous histamine which can be rapidly displaced by the polylysine.

Paton(26) has suggested that basic histamine liberators might release endogenous histamine in the gut to stimulate intestinal motility. Perhaps as proteins are hydrolyzed in the gastrointestinal tract, basic peptides similar in their action to polylysine mediate the release of small amounts of endogenous histamine which in turn increases intestinal motility.

Rocha e Silva(27) tested a series of acylated histamines for antihistaminic activity on the isolated guinea pig ileum and demon-

strated that blocking the primary amino group but leaving the imidazole ring free for binding reduced histamine activity and resulted in compounds with weak antihistaminic activity. The concentration of polylysyl histamine required for contraction showed that coupling the histamine to the peptide also greatly reduced the activity of the histamine.

Since trypsin was found to induce contraction of the isolated guinea pig ileum(28) and to release histamine from rabbit platelets (29), Rocha e Silva postulated that endogenous histamine was bound to the carboxyl group of a lysine or arginine residue forming an amide bond with the primary $\beta$-ethyl amino group of histamine. Histamine was thought to be released by some proteolytic enzyme with the specificity of trypsin which was activated during anaphylaxis(22). Although the proteolytic theory has generally been displaced in favor of other mechanisms (24,25), the action of trypsin on the lysyl-histamine bond has not been previously experimentally tested. Paper chromatographic studies of the action of trypsin on polylysyl histamine showed that after 16 hours only trace quantities of histamine with an $R_f$ of 0.44 were released from the polypeptide. The bulk of the imidazole residues remained in 2 spots with $R_f$ values of 0.38 and 0.24. Based on the work of Waley and Watson(9), these unknown spots should correspond to lysyl-histamine peptides. A ninhydrin spray revealed the same pattern and other lysine peptides close to the origin. These data indicate that very little of the covalently bound histamine is released by trypsin and are incompatible with the proteolytic theory of histamine release. Perhaps higher levels of trypsin would attack the lysyl-histamine bond as in the case with lysyl amide(30); however, higher levels of trypsin would not be consistent with the concentrations used in biological experiments(28,29,31). Furthermore, based on chemical manipulations of the polymer, there is no unique lability of the lysyl-histamine bond which was once suggested (32) as due to the amplitude of the peptide chain. These data furnish direct evidence that the lysyl-histamine bond is refractory to trypsin hydrolysis, and that adjacent lysyl-lysyl bonds are much more easily split by trypsin.

*Summary.* L-lysine polypeptides stimulated contraction of freshly excised guinea pig ileum. Ileal segments were refractory to a second challenge of polylysine, and the antihistamine, Benadryl, blocked the action of the polypeptide. Consequently, the contraction was attributed to release of endogenous histamine or a histamine-like substance which was displaced by the basic polypeptide. Polylysyl histamine was synthesized by initiation of polymerization of $\epsilon$-carbobenzoxy-N-carboxy-L-lysine anhydride with histamine. Although polylysyl histamine was readily hydrolyzed by trypsin, the primary products were lysyl histamine peptides which indicated that the histamine-lysine bond was not hydrolyzed by trypsin. The relationship of these data to the proteolytic theory of histamine release was discussed.

1. Burger, W. C., Stahmann, M. A., *Arch. Biochem. Biophys.,* 1952, v39, 27.

2. Katchalski, E., Bichowsky-Slomnicki, L., Volcani, B. E., *Biochem. J.,* 1953, v55, 671.

3. Burger, W. C., Stahmann, M. A., *J. Biol. Chem.,* 1951, v193, 13.

4. Rubini, J. R., Stahmann, M. A., Rasmussen, A. F., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 659.

5. Katchalsky, A., Danon, D., Nevo, A., *Biochim. et Biophys. Acta,* 1959, v33, 120.

6. Katchalski, E., Berger, A., Neumann, H., *Nature,* 1954, v173, 998.

7. Ginsburg, I., Vries, A. de, Katchalski, E., *Science,* 1952, v116, 15.

8. Bienzunski, N., Shafrir, E., Vries, A. de, Katchalski, E., *Biochem. J.,* 1955, v59, 55.

9. Waley, S. G., Watson, J., *ibid.,* 1953, v55, 328.

10. Tsuyuki, E., Tsuyuki, H., Stahmann, M. A., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 318.

11. Richardson, T., Hodgett, J., Linder, A., Stahmann, M. A., *ibid.,* 1959, v101, 382.

12. Buchanan-Davidson, D. J., Stahmann, M. A., Lapresle, C., Grabar, P., *J. Immunol.,* 1959, v83, 552.

13. Stahmann, M. A., Tsuyuki, H., Weinke, K., Lapresle, E., Grabar, P., *Compt. rend. Acad.,* 1955, v241, 1528.

14. Stein, O., Vries, A. de, Katchalski, E., *Arch. intern. pharmacodynamie,* 1956, v57, 243.

15. Garcia-Arocha, J., Ashwin, J. G., Grossberg, A. L., *XIXth Internat. Physiol. Congress,* Abst. Communications, Montreal, Canada, 1954, p 378.

16. Becker, R. R., Stahmann, M. A., *J. Am. Chem. Soc.,* 1952, v74, 38.

17. Ben-Ishai, D., Berger, A., *J. Org. Chem.,* 1952, v17, 1564.

18. Block, R. J., LeStrang, R., Zweig, G., *Paper Chromatography,* 1st edit., Academic Press Inc., New York, 1952, 62-64.

19. Jorpes, E., *Biochem. J.,* 1932, v26, 1507.

20. Code, C. F., McIntire, F. C., in *Methods of Biochemical Analysis,* 1956, vIII, 62.

21. Coulson, E. J., *J. Allergy,* 1953, v24, 458.

22. Rocha e Silva, M., *Histamine, Its Role in Anaphylaxis and Allergy,* 1955, C. C. Thomas Co., Springfield, Ill.

23. Boreus, L. O., Chakravarty, N., *Acta Physiol. Scand.,* 1960, v48, 315.

24. MacIntosh, F. C., Paton, W. D. M., *J. Physiol.,* 1949, v109, 190.

25. Hogberg, B., Uvnas, B., *Acta Physiol. Scand.,* 1957, v41, 345.

26. Paton, W. D. M., *Pharmacol. Rev.,* 1957, v9, 269.

27. Rocha e Silva, M., *J. Pharm. Exp. Ther.,* 1944, v80, 399.

28. ———, *Arquiv. inst. biol. (Sao Paulo),* 1939, v10, 93.

29. Dragstedt, C. A., Rocha e Silva, M., PROC. SOC. EXP. BIOL. AND MED., 1941, v47, 420.

30. Waley, S. G., Watson, J., *Biochem. J.,* 1954, v57, 529.

31. McIntire, R. C., Roth, L. W., Sproull, M., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 605.

32. Rocha e Silva, M., *J. Pharmacol. Exp. Ther.,* 1943, v77, 198.

Received February 7, 1961. P.S.E.B.M., 1961, v107.

## A Non-Competitive β-Glucuronidase Inhibitor in Rabbit Urine.* (26628)

GAYLORD M. CONZELMAN, JR., AND DANIEL W. CROUT
(Introduced by L. H. Schmidt)
*The Christ Hospital Institute of Medical Research, Cincinnati, Ohio*

It has been suggested by Boyland(1) that urinary glucuronidase may be an important factor in induction of bladder cancer by certain aromatic amines. According to this concept, non-carcinogenic glucuronic acid conjugates of 2-amino-1-naphthol might be hydrolyzed by glucuronidase in the bladder to yield the carcinogenic 2-amino-1-naphthol (2).

In an investigation of the relationship of 2-naphthylamine metabolism to bladder tumor induction, urinary β-glucuronidase has been assayed in various species by the method of Boyland *et al.*(3). These determinations revealed marked variations in β-glucuronidase levels of various species. An unexpected finding was the apparent absence of this enzyme in urine obtained from several kinds of rabbits(4). Subsequent experiments showed, however, that rabbit urine did contain β-glucuronidase and that the enzyme could be precipitated from urine with ammonium sulfate. The failure to demonstrate β-glucuronidase activity in whole urine was due to the presence of inhibitory materials. Similar investigations indicated that glucuronidase inhibitors were also present in urine of man, dog, rhesus monkey, mouse and rat(4). These observations confirm and extend the findings of Abul-Fadl(5) and Lewis and Plaice(6) who demonstrated β-glucuronidase inhibitors in human urines. The present report deals with the partial characterization of the β-glucuronidase inhibitor(s) in rabbit urine.

*Materials and methods.* Two male and 2 female rabbits, inbred animals from a tuberculosis-resistant race,† were used for this study. They were maintained on a diet of Purina Rabbit Pellets with water *ad libitum.*

During experimental periods the animals were housed in metabolism cages equipped with stainless steel funnels; urine was collected into iced vessels, without preservative,

* This project was supported in part by a gift from the Jacob G. Schmidlapp Memorial Fund.

† We are indebted to Dr. Max Lurie, Henry Phipps Inst. for the gift of these animals.