A Bactericidin for *Bacillus subtilis* in Mammalian Saliva.* (27154)

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Among the several apparently non-specific antibacterial systems, Jacox(1) described a bactericidin for *Bacillus subtilis* present in human serum which became enhanced during the acute phase of many illnesses. He found this serum bactericidal factor required calcium ions for its potentiation. More recently, Myrvik and Weiser(2), investigating a similar or identical bactericidin for *Bacillus subtilis*, reported relatively high concentrations in the sera of rabbits and rats, whereas sera from guinea pigs, dogs, cows and humans possessed low or undetectable levels of activity. Marked elevations in titer were noted in the human during acute phase of many illnesses. They found heating at 60°C for 2 hours at pH 7.4 inactivated the bactericidin. Evidence was presented indicating that this bactericidin is distinct from lysozyme, properdin or heat labile components of complement. Myrvik *et al.*(3) later demonstrated that both calcium and bicarbonate ions are required for the human system, whereas only bicarbonate is required in rabbit and rat serum. At least 2 nondialyzable fractions participate in this system(4).

It has been postulated that the innate resistance of the oral cavity is in part due to antibacterial systems of the saliva. The knowledge regarding the presence of such systems in the saliva is mainly confined to lysozyme(5,6) and a bactericidin for lactobacilli (7,8). The present communication is concerned with the levels and components of the *Bacillus subtilis* bactericidin in sera and saliva of humans and selected animal species.

Method. Pilocarpine-stimulated whole saliva was collected from New Zealand rabbits, Chase strain guinea pigs(9) and Sprague-Dawley rats(10). Paraffin was used to stimulate the flow of saliva in the human. All specimens of whole saliva were centrifuged at 3,000 rpm for 5 minutes at 4°C and sterilized by passage through a Swinney filter. The first 0.25 ml was discarded and the remaining sample was stored at –30°C until used.

The strain of *Bacillus subtilis* employed for titration of bactericidin activity was kindly supplied by Dr. Quentin Myrvik. It was maintained on either nutrient agar slants or nutrient broth.

The method for assaying sera and saliva are modifications of the method described by Myrvik and Weiser(2). Prior to titration, the serum and/or saliva was adjusted to pH 7.0-7.2 with 0.1 N HCl. The bactericidal activity of the material to be tested was assayed by 2-fold serial dilutions in physiological saline to give a final volume of 0.5 ml. Each tube was inoculated with 0.1 ml of a standardized suspension of *Bacillus subtilis*. In the case of serum, this was prepared by centrifuging a 3-hour nutrient broth culture and resuspending the organisms in sterile saline to obtain an optical density reading of 0.028 at 540 mμ with a 6A Coleman Jr. spectrophotometer. Since saliva does not contain sufficient nutrient to support the growth of the organism, the centrifuged 3-hour nutrient broth culture was resuspended in 10% Trypticase Soy broth also to obtain an optical density reading of 0.028 at 540 mμ.† After thorough mixing, the inoculated tubes were incubated in a 37°C water bath for 3 hours. The endpoint was expressed as the highest dilution of serum or saliva in which no visible growth of *Bacillus subtilis* was evident.

For quantitative comparison, bactericidal activity is expressed in units representing double the reciprocal of the maximum number of times 0.5 ml of saliva could be diluted and still inhibit growth of the assay organism.

To separate the cofactor(3), one volume of rat serum was dialyzed against 15 volumes of deionized water at 4°C for 48 hours in a

* Supported by grant from N.I.H., U.S.P.H.S.

† Utilizing both methods with specimens of sera identical results were obtained.

TABLE I. Bactericidin Levels in Some Mammalian Sera and Saliva.

Source	No. of subjects	Bactericidin, units/ml Serum	Saliva
Guinea pig	10	<2–2	<2
Rabbit	10	16–32	4–8
Human	10	<2–2	<2
Rat	10	16–64	<2

rotating dialyzer; the dialyzed serum was stored at 4°C. The dialysate containing the cofactor was concentrated by lyophilization and reconstituted with deionized water to the original volume.

Results. Effect of filtration. Since it was desirable to filter and sterilize the small quantities of saliva collected, the effect of filtration on the bactericidin was investigated. Rabbit serum which had been adjusted to pH 7.0-7.2 with 0.1 N HCl was passed through a Swinney filter. The first 0.25 ml of the serum which passed through the filter and the remaining portion were collected separately and assayed for the *B. subtilis* bactericidin. A sample of the same unfiltered rabbit serum served as a control. It was noted that the first aliquot yielded a filtrate with approximately 50% of the original activity, whereas the level of activity of the remaining aliquot was the same as the unfiltered control.

Bactericidin activity in some mammalian saliva and sera. In Table I the bactericidin activity of serum and saliva of animals tested is summarized. Sera of rabbits contained 16-32 units of bactericidin per ml and saliva from the same animals yielded 4-8 units per ml. Rat sera demonstrated bactericidin activity in the range of 16-64 units per ml, whereas, in marked contrast, no activity was found in the saliva from the same animals. In the case of the guinea pigs and healthy humans, the serum contained <2 - 2 units of bactericidin activity per ml and the salivas similarly displayed no inhibition.

Components in rat saliva. It is notable that although both the rat and rabbit possessed high serum levels, the rat did not demonstrate any salivary bactericidin activity. To determine which component(s) were lacking in rat saliva, a series of experiments were carried out.

One volume of untreated rat saliva was added to one volume of heat inactivated rat serum (60°C/2 hr pH 7.4). The results of the bactericidin test indicated that addition of the heat stable (cofactor) portion of the system present in the serum did not supply bactericidin activity to the rat saliva. Moreover, the addition of bicarbonate ions, the previously demonstrated cofactor in the rat serum system(3), in varying amounts from 125 to 500 μg per ml did not provide activity to the rat saliva.

By dialysis, the bactericidin system of serum was separated into 2 components; namely, the dialyzable portion which contains the cofactors and the non-dialyzable portion. In order to provide additional information on the components in rat saliva, rat serum was dialyzed and reconstituted as described. Equal volumes of untreated rat saliva and rat serum cofactor were added as well as equal volumes of non-dialyzable component of rat serum and untreated rat saliva. Each combination and controls were assayed for bactericidin activity.

A typical result is illustrated in Table II. Addition of rat saliva to the dialyzed-inactivated rat serum reestablished the bactericidin activity, whereas addition of cofactor to the rat saliva did not potentiate the system.

Discussion. The bactericidin for *Bacillus subtilis* studied appears to be identical with the system described by Jacox(1) and Myrvik and Weiser(2). The appearance of the bactericidin in the saliva, although in decreased titer, parallels those previously reported in sera of the various animal species with the exception of the rat. None was

TABLE II. Effect of Dialysis and Recombination on the *Bacillus subtilis* Bactericidin of the Rat.

Substance	Bactericidin, units/ml
Serum	16
Dialyzed serum	<2
Cofactor*	<2
Cofactor + dialyzed serum (1:1)	8
Cofactor + saliva (1:1)	<2
Saliva + dialyzed serum (1:1)	16

* Cofactor is contained in the dialyzable portion of the sera. It is lyophilized and reconstituted to original volume.

found in either the sera or the saliva of the human and guinea pig; on the other hand, saliva of the rabbit contained about ¼-½ the bactericidin activity found in the sera. In the case of the rat, however, an animal which consistently demonstrated a high serum titer, none was detected in the saliva. The basis for this unexpected dichotomy was sought.

The results with the saliva of the rat indicate that the cofactor is present, whereas the non-dialyzable heat labile portion is lacking. This is confirmed by the inability of heat-inactivated rat serum to be reactivated by untreated rat saliva. Also, addition of cofactor did not potentiate the sysem, whereas addition of rat saliva to the dialyzed rat serum, from which the cofactor is missing, reestablished the bactericidal system.

The components of the saliva are suspected to be derived mainly from the plasma. As in the case of the kidney, the process is selective and, of course, variation between species is expected. For example, lysozyme, an antimicrobial enzyme found in high concentration in the polymorphonuclear leucocyte, was found to be present in both saliva and serum of the rat(11). Intravenously administered penicillin passed into the salivary secretions of the rat in high concentration(11). The present findings serve to emphasize the selective capacity of the salivary glands and the variability in this selectivity among the mammals studied.

Summary. 1. Sera and saliva from humans and guinea pigs possessed very low or undetectable amounts of the bactericidin for *B. subtilis*. 2. Rabbit saliva possessed ¼-½ the rabbit serum level. 3. The serum of the rat contained high levels of this bactericidin, whereas rat saliva demonstrated no activity. The non-dialyzable portion of the bactericidal system was lacking in the saliva; the cofactor was present.

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Received October 23, 1961. P.S.E.B.M., 1962, v109.

Glycogen in Adrenal Cortex of Sodium Depleted Rat.* (27155)

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The glycogen content and distribution in the adrenal cortex of the white rat has been studied by a number of investigators(1,2). As demonstrated histochemcially by the periodic acid Schiff (PAS) reaction, glycogen in this species concentrates in granular form in the cytoplasm of the cells of the fascicular and reticular zones, and is either absent or present in very small quantities in the glomerulosa. Haynes *et al.*(3,4) suggest that adrenocorticotropin (ACTH) acts to stimulate adrenal phosphorylase activity *via* an effect on adenosine-3′-5′-monophosphate. In the light of this theory the presence of considerable stored glycogen in the inner zones would seem reasonable since this is the area which responds most acutely to stress or ACTH and, therefore, should require a read-

* This research was supported by U. S. Public Health Service Grants.