periments were killed within a period ranging from the sixth to the 50th day after injection. The organs of the infected animals were cultured on agar plates as well as in broth tubes. In many cases the agar cultures yielded negative results, while after incubation of the broth cultures for 7 days positive results were obtained. The microorganisms isolated from these cultures were identified as *Brucellae* by agglutination with specific antisera. The following organs were examined: heart, lungs, liver, spleen, kidneys and genital glands. Seventy-six mice which were dissected up to the 28th day after injection proved to harbor *Brucellae* in all or in several organs. Fifteen out of 20 mice which were dissected between the 30th and the 50th day after the onset of the infection proved to harbor *Brucellae*, while only 5 yielded sterile cultures. The organs of 145 mice were cultured after death took place or when the mice were sacrificed. The positive results obtained from the different organs were as follows: Heart 131, lungs 126, liver 133, spleen 136, kidneys 136, genital organs 127. These figures prove that significant differences in the infection rate of the different organs do not exist, but only that foci of infection are equally distributed over the different organ systems of the infected animal.

Summary. *B. melitensis* and *B. abortus* did not produce lethal infections in mice even when 0.5 cc of broth cultures were injected intraabdominally. On the other hand lethal infections were produced if the broth cultures were administered together with 0.5 cc of a 10% mucin suspension. The LD_{50} for *B. melitensis* was 0.2 cc, for 2 strains of *B. abortus* 0.35 cc, and for a third strain of *B. abortus* 0.2 cc. In most of the surviving animals chronic infections were noted, if the dissections were performed up to the 50th day after the onset of the infection.

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17305. Absorption of Subtilin in the Rabbit.

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Subtilin,[1,2] an antibiotic active *in vitro* against Gram-positive bacteria[3] and *M. tuberculosis*,[3-6] has also been found active *in vivo* against the more sensitive pathogenic bacteria. For example, Salle and Jann injected subtilin intraperitoneally into mice infected with pneumococcus Type III,[7] *Streptococcus pyogenes*,[8] or *Staphylococcus aureus*,[9] and into guinea pigs subjected to experimental anthrax infections.[10] Prophylactic and therapeutic effects, depending on the relations of infection and dosage times, were striking.

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1 Garibaldi, J. A., and Feeney, R. E., *Ind. Eng. Chem.*, 1949, **41**, 432.

2 Fevold, H. L., Dimick, K. P., and Klose, A. A., *Arch. Biochem.*, 1948, **18**, 27.

3 Salle, A. J., and Jann, G. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 60.

4 Wong, S. C., Hambly, A. S., Jr., and Anderson, H. H., *J. Lab. Clin. Med.*, 1947, **32**, 837.

5 Knight, V., Shultz, S., and DuBois, R., Proc. 48th General Meeting Soc. Am. Bact., Minneapolis, Minn., May, 1948, p. 84.

6 Knight, V., and Tompsett, R., *J. Clin. Invest.*, 1948, **27**, 544.

7 Salle, A. J., and Jann, G. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 40.

8 Salle, A. J., and Jann, G. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 519.

9 Salle, A. J., Presentation at the Conference on Antibiotic Research, Washington, D. C., January 31 and February 1, 1947, under the auspices of the Antibiotics Study Section of the National Institute of Health.

10 Salle, A. J., and Jann, G. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 41.

These findings have been confirmed with respect to pneumococcal and streptococcal infections by Knight and colleagues[5,6] and with respect to protection against *Streptococcus hemolyticus* by Hobby.[11] On the other hand, in spite of *in vitro* potency, subtilin has been found ineffective against syphilitic infections in rabbits[12] and against *M. tuberculosis* infections in hamsters[13] and in mice.[5,6,14] Steenken and Wolinsky[15] found subtilin without effect on tuberculosis infections in guinea pigs, whereas Salle and Jann[16] have reported both positive and negative results with different lots of subtilin. Farber, Eagle, Anderson, and Gorman[17] found suggestive evidence of therapeutic activity from the topical application of subtilin on tuberculous laryngeal lesions.

The present work was undertaken to determine the absorption of subtilin and of methyl esters of subtilin[18] when administered by various routes and to note any pharmacological effects, as an indication of the value and the feasibility of more extensive clinical trials. The activity of these esters against *M. tuberculosis in vitro* is at least as high as that of subtilin.[19]

Methods. Thirty-one rabbits were used in 44 experiments. The animals used more than once had shown little or no absorption at the time of the original experiment and had not been used for at least a month. Except for treatments by slow infusion or injection into the ligated colon, the animals were not anesthetized. Anesthesia, when used, was with sodium pentobarbital. Initially, 40 mg/kg intravenously was used, with 10 mg/kg supplements as needed to maintain a light anesthesia. Intravenous injections of subtilin were made into the marginal ear vein, subcutaneous injections were divided between the two sides of the animal so as to insure a larger surface for absorption. Intramuscular injections were divided likewise and were made into the muscles of the thigh.

The subtilin was in nearly all instances from one composited batch of highly potent material prepared by methods described elsewhere.[1,2] Although electrophoretic analysis, fractional dialysis and salt fractionation[2] indicated that our subtilin was homogeneous, investigations at the research laboratories of Merck & Co., Rahway, N. J., by countercurrent distribution showed that our samples were probably about 90% pure. The methyl esters were prepared[18] from another batch of equally pure subtilin. Two lots were used: the first (No. 17ME-242) contained 5.9 equivalents of methoxyl per 10^4 g and was characterized by markedly enhanced bacteristatic potency and 4-fold increase in solubility under physiological conditions. The second (No. 37ME-242) contained 11.9 equivalents of methoxyl per 10^4 g and was characterized by substantially unchanged bacteristatic potency but 30-fold increase in solubility under physiological conditions. For intravenous administration, the solvent was 5% glucose; when 10% subtilin solutions were used for administration by other routes, the subtilin was dissolved in distilled water. Blood samples for subtilin assay were obtained by cardiac puncture. Urine, when desired, was obtained by catheter with washing of the bladder so as to assure complete collection.

Subtilin and the methyl esters of subtilin were determined by the cup-plate method. The test organisms used previously[20] for the

11 Hobby, G., personal communication.

12 Eagle, H., Musselman, A. D., and Fleischman, R., *J. Bact.*, 1948, **55**, 347.

13 Anderson, H. H., and Wong, S. C., *Tuberculology*, 1946, **8**, 77.

14 Rake, G. W., personal communication.

15 Steenken, W., Jr., and Wolinsky, E., *J. Bact.*, 1949, **57**, 453.

16 Salle, A. J., and Jann, G. J., Presentation at the Second National Symposium on Recent Advances in Antibiotics Research, Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section of the National Institute of Health.

17 Farber, S. M., Eagle, H. R., Anderson, H. H., and Gorman, R. D., *J. Lab. and Clin. Med.*, 1948, **33**, 799.

18 Carson, J. F., Jansen, E. F., and Lewis, J. C., *J. Am. Chem. Soc.*, in press.

19 Chin, Y. C., *Fed. Proc.*, 1948, **7**, 211, and unpublished data.

20 Lewis, J. C., Humphreys, E. M., Thompson, P. A., Dimick, K. P., Benedict, R. G., Langlykke, A. F., and Lightbody, H. D., *Arch. Biochem.*, 1947, **14**, 437.

turbidimetric assay of subtilin were much too insensitive by the cup-plate technic. This insensitivity proved particularly troublesome for the methyl esters of subtilin, which had much lower potencies than subtilin by cup-plate or agar-streak methods in contrast to their behavior in broth. The cause of this anomalous behavior is not known. A bacterium tentatively identified as *Sarcina lutea* was used as the test organism for subtilin. The test medium was that previously used[20] for the turbidimetric assay of subtilin with *Micrococcus conglomeratus*. Incubation for 20 hours at 35° gave an approximately 20-mm zone of inhibition when a solution containing 1 ppm of subtilin was placed in the cup. An unidentified Gram-negative diplococcus (C-7) isolated from chicken feces was chosen as the most sensitive organism available for the assay of methyl esters of subtilin. Two ppm of either ester gave an approximately 20-mm zone on nutrient agar containing 5% NaCl after 22 to 48 hours' incubation at 35°C.

In all assays the antibiotic under test was dissolved in an aliquot of whole citrated rabbit blood to provide a standard response. Control blood samples taken from animals never treated previously gave no inhibition of the test organisms. Citrated blood samples from treated animals and aqueous dilutions of this blood were pipetted directly into assay cups. Dilutions were not necessary for blood samples from animals treated with the subtilin esters. All assay results are expressed in terms of the weight of the particular type of subtilin under test, since the relative potencies of the three materials varied markedly with the experimental conditions.

Results. Subtilin. Intravenous injection gave a satisfactory blood level, but the level was transitory and the procedure was accompanied by some danger. In a single experiment, 50 mg/kg caused death in 2 minutes, and 10 mg/kg did likewise in 1 of 5 trials. The concentration of subtilin in the blood of the other 4 animals was 100-200 ppm 5 minutes after the injection, falling to 10-30 ppm in 2 hours and to zero in 24 hours. Up to 7% of the injected subtilin was found in the 2-hour urine.

Subcutaneous and intramuscular injection of 100 mg/kg can be considered together. Blood levels ranging from a trace up to 1.4 ppm were found. A persistent lump was present at the injection site after subcutaneous injections, and a sterile abscess was present in muscles 2 months after subtilin administration. There was no antibiotic activity in an extract of this abscess, although an assay of a comparable muscle site 24 hours after injection indicated that the granular material still had considerable activity.

Intraperitoneal injection of 10 to 100 mg/kg led to blood levels of 0 to 1 ppm of subtilin. After 10 mg/kg there was no visible precipitate in the abdomen, but 50 mg/kg was sufficient to produce such a deposit.

Treatment of subtilin by crystalline pepsin or trypsin reduces its antibiotic activity.[21,22] This fact, along with the known low solubility of subtilin in body fluids, would suggest that administration by mouth would be ineffective. Nevertheless, 1 g/kg was given to one rabbit *per os*. The highest blood level noted was 0.1 ppm. After 5 hours, of the 4 grams originally given, only 2 μg were found in the urine, 15 mg in the intestine, and 0.5 g in the stomach. Two anesthetized animals were given 100 mg of subtilin/kg per rectum, with ligation of the gut near the anus to prevent loss. The solution was 1% of subtilin in water, and the volume was great enough to spread throughout the colon. From 2 to 4 ppm of subtilin were found in the blood stream, and autopsy 2 hours after administration showed subtilin deposits in the colon.

It is conceivable that a slow subcutaneous infusion of a dilute solution would allow the subtilin to be absorbed rather than precipitated. Concentrations of 0.05 to 0.5% in 5% glucose were infused in 3 unanesthetized animals under the skin of the back at rates of 1.7 to 27 mg/kg/hr for 6 hr. The volume of solution was approximately 5 cc/kg/hr. Some absorption did occur, as 27 mg of subtilin was found in the 24-hour urine sample in one

21 Stansly, P. G., and Ananenko, N. H., *Arch. Biochem.*, 1947, **15**, 473.

22 Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., *Arch. Biochem.*, 1947, **15**, 1.

instance and 4 mg in another; however, absorption was so slow that 6 ppm of subtilin was the maximum found in the blood.

The preceding experiments have indicated that little subtilin gets into the blood stream unless it is placed there directly. In the case of a single injection, there is some danger and activity is short-lived. A slow, intravenous infusion might give satisfactory blood levels without seriously embarrassing the animal. Three anesthetized rabbits were used, with infusion into the jugular or femoral veins of 0.25% subtilin in 5% glucose. One animal received 20 mg/kg/hr for 2 hours, 60 mg/kg/hr for 1 hour and 150 mg/kg/hr for 30 minutes until death. At the end of the third hour the blood level was 450 ppm and 6% of the injected subtilin was found in the urine. No symptoms were observed until sometime after the rate was raised to 60 mg, when tremors in the extremities were seen. Death was apparently of cardiac origin. The second animal was given 20 mg/kg/hr for 4 hours. No symptoms were noted, the animal appearing in good condition at the end of this time. The blood level had risen to 700 ppm.

In these 2 rabbits, the blood was observed for agglutination. We had found previously that there was an *in vitro* clumping of red cells at concentrations of subtilin lower than found in these two animals. No clumping was noted, indicating that *in vivo* actions differed from *in vitro,* or that agglutinated cells had been filtered out in the capillary systems of the body.

The third rabbit receiving intravenous infusion was prepared for records of blood pressure and respiration. Approximately 20 mg/kg/hr for 1½ hours did not modify blood pressure, heart rate or respiratory rate. Raising the infusion rate to 70 mg/kg/hr caused a gradual fall in pulse pressure (as measured with a membrane manometer) and a gradual increase in respiratory rate.

Chin[23] has reported that the LD_{50} of intravenously injected subtilin to mice is 100 mg/kg. This is slightly higher than the approximately 70 mg/kg found by us in a small series of animals, but either value would give a body concentration well above the minimum *in vitro* effective tuberculostatic dose. Death from intravenously administered subtilin is presumably due to embolism since subtilin, which dissolves readily in water, is only slightly soluble in physiological saline[18] or serum.[24] The amount held in solution varies somewhat with the manner of manipulation, being around 0.05 to 0.1 g/100 cc under physiological conditions of temperature and salt concentration. The intraperitoneal LD_{50} was some 2 to 3 times the intravenous LD_{50}. Subcutaneously, mice were able to tolerate more than 3 g/kg of purified subtilin without demonstrable symptoms.[25] The lack of subcutaneous toxicity is likewise explained by the low solubility in body fluids, a deposit of precipitated material being formed at the site of injection.

Subtilin methyl esters. The increased solubility of some of the methylated subtilin preparations, and the increased antibiotic activity of others[18] led to the hope that satisfactory blood levels could be obtained. Twelve animals were used. On 4 of them blood assays were not made. There were no untoward reactions in these or any of the other rabbits receiving the esters. It was found that blood levels of the 8 other animals were slightly higher after intravenous, subcutaneous and intramuscular injection of the more soluble ester than the levels found after administration of unmodified subtilin. The levels (up to 2.4 ppm after intramuscular administration) were not great enough to warrant further study. The ester with increased activity was absorbed so poorly that it had an unimportant antibiotic effect in the blood.

Several lots of methyl esters of subtilin were tested for approximate intravenous toxicity in mice. The values obtained did not differ markedly from values for unchanged subtilin.

Subtilin-pectin complex. Subtilin, when dissolved with 5 to 8 times its weight of pectin gives a product which is soluble in physiological saline to the extent of 0.5% of subtilin. After some time the complex begins to

23 Chin, Y. C., *Fed. Proc.*, 1947, **6**, 317.

24 Klose, A. A., unpublished data.

25 Wilson, R. H., Lewis, J. C., and Humphreys, E. M., *Fed. Proc.*, 1948, **7**, 266.

break down and subtilin comes out of solution. Dr. Harry S. Owens of this laboratory prepared such a complex for us. It was given intravenously 3 times, intramuscularly and subcutaneously once each. After intravenous injection it disappeared from the blood as rapidly as did unmodified subtilin, and it was as poorly absorbed when given by the other routes. Since it offered no advantages over subtilin and, because of its great viscosity, was very difficult to inject or else required considerable dilution, it was not considered further.

Pectin-treated subtilin. At a recent symposium, Salle and Jann[16] stated that a described treatment of subtilin eliminated abscess formation and that subcutaneously injected material so treated was effective against tuberculosis in guinea pigs. The treatment consisted in dissolving 1.5 g of subtilin in 50 ml of 10% urea solution and mixing with an equal volume of 0.1 g of pectin in distilled water. After standing overnight, the supernatant liquid was used for injection. Following this report, we prepared a subtilin solution in the described manner and injected it subcutaneously into 2 rabbits. The dosage was 100 mg/kg, assuming that none of the subtilin was lost during the manipulations. Assays of periodic blood samples showed blood concentrations similar to those reported earlier in this paper.

The question of abscess formation was studied on mice and guinea pigs. We felt that lack of abscesses might be due to simple dilution. Four groups of mice were used, 6 animals to a group. Each mouse received 1 ml of solution under the skin of the back, as follows: (1) 1.5% subtilin, Lot No. 317, in 5% glucose, (2) 5% subtilin No. 317; (3) the solution prepared as described by Salle and Jann, using subtilin No. 317; (4) a 5% solution of subtilin recovered from (3) by precipitation with 10% NaCl.[2] All animals in groups 2 and 4 and a majority in groups 1 and 3 showed abscess formation, the abscesses being smaller and less apt to ulcerate with the more dilute solutions.

Three guinea pigs were used, each animal receiving subcutaneously the following 4 solutions: (1) 1.5 ml of the solution prepared as described by Salle and Jann; (2) 1.5 ml of 1.5% subtilin No. 317 in 5% glucose; (3) the same *weight* (22 mg) of subtilin in a 10% solution; (4) the same *volume* (1.5 ml) of subtilin in a 10% solution. A large, hard, subcutaneous lump was formed in all 3 animals by solution 4. Solution 3 caused a prompt development of small lumps in 2 animals, and solutions 1 and 2 produced more slowly developing lumps in 1 and 2 animals, respectively. These results indicate that the proposed treatment does not modify the absorption of subtilin nor its tendency to produce abscesses. The decreased reaction was due to the dilution of the material. Since approximately 1/3 of the subtilin was precipitated by the pectin (as estimated by sulphur balance) the concentration of subtilin in the solution prepared according to the method of Salle and Jann was actually about 1%.

Discussion. Consideration of the foregoing results indicates that high systemic levels of subtilin are not obtained readily in the rabbit. The amount absorbed after administration in a variety of ways would not produce bactericidal or even bacteristatic levels in the blood stream, except possibly for very sensitive Gram-positive organisms. Furthermore, injection under the skin or into muscle is followed by a lasting deposit which is gradually transformed into an abscess. The poor absorption and the local deposition of precipitated subtilin are caused by the low solubility of subtilin in physiological fluids. It is possible to obtain substantial blood levels in the rabbit only by intravenous administration. If this is done by single injection there is some danger, due again to low solubility causing precipitation in the blood stream, and furthermore, the blood levels are not maintained. The one method of administration which, within the limits of the above experiments, would give a satisfactory blood level with no ill effects to the animal is a slow intravenous infusion.

It is conceivable that results reported here would differ with other species. In the introduction it was noted that certain infections in mice and guinea pigs could be controlled, and we find mice may be killed by intraperitoneal injections of subtilin. However, the

difference is probably only one of degree. In the mouse, as in the rabbit, a subcutaneous deposit is formed, and in the former animal an abscess is formed at the site of injection. Since the lack of absorption and the manifestations of toxicity are dependent on the physical properties of subtilin in physiological fluids, it seems unlikely that a species difference would be marked.

Such modifications of subtilin as have been tried to date have not greatly modified the physiological absorption or bacteristatic level, even though the products have had somewhat greater *in vitro* antibiotic activity or greatly increased solubility under physiological conditions.

Although the results in this paper indicate that high systemic levels of subtilin are not practical, it is quite possible that subtilin would be beneficial when topical application is indicated. We have observed no reactions which would contraindicate local application.

Conclusions. The antibiotic, subtilin, has been administered to rabbits in a variety of ways, with analyses of blood to determine absorption. The bioassay procedures are described.

Because of precipitation in physiological fluids, injection into subcutaneous tissue, muscle or peritoneal cavity is an ineffective way of reaching bacteristatic levels of subtilin in the blood stream. Administration by mouth or per rectum is likewise unsatisfactory.

A single injection intravenously is accompanied by some danger and gives a blood level of subtilin which is not maintained. A satisfactory level can be maintained, at least for 4 hours, by slow intravenous infusion, without apparent harm to the animal.

A subtilin-pectin complex, which temporarily allows more subtilin to be soluble in physiological saline, a methyl ester of subtilin which is likewise more soluble and which possesses greater antibiotic activity, and a second methyl ester which is much more soluble, are all absorbed in amounts similar to that of unmodified subtilin.

No observations were made which would contraindicate the topical application of subtilin.

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17306. The Antidiuretic Action of Relaxin-Containing Preparations.*

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The phenomenon of water retention in the female during pregnancy has received a great deal of attention from many investigators. It has been known for some time that both an extract of the posterior pituitary and desoxycorticosterone acetate have antidiuretic action. In addition, Thorn, Nelson, and Thorn[1] have shown that the sex steroids can also produce water retention in the dog. Nevertheless a suitable explanation for the shift in water balance during pregnancy is still lacking. In the present report data are presented to show that extracts from the ovaries of pregnant sows and blood serum of pregnant rabbits, prepared for relaxative activity on the symphysis pubis of the guinea pig, possess an antidiuretic action in the rabbit.

Several different extracts of relaxin were used in these experiments. Preparation J-46 was prepared from unselected ovaries† of the sow according to the method of Albert,

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1 Thorn, G. W., Nelson, K. R., and Thorn, D. W., *Endocrinol.*, 1938, **22**, 155.

† Unselected ovaries were obtained from both pregnant and non-pregnant sows.