increase of intake on the diluted diet *was not* prevented by the capacity of the opossum's stomach.

These findings are in sharp contrast to the results obtained with the albino rat. Several investigators have observed that caloric values are of greater consequence to this animal than are factors of palatability(2,5-7). Also, rats have been shown to adjust their food intake in accord with their base caloric intake within 3-5 days on dilutions as high as 75% and enrichment of 50%(2,11).

It is conceivable that this feral animal would want to overeat when food is abundant. Rigid caloric regulation would be of limited value to an animal with an irregular and varied food supply. A sensitivity to palatability would be of greater consequence to a wild animal than to a domesticated animal where rapid detection and discrimination among nutrients has survival value. The difference observed in caloric regulation between laboratory rats and opossum may be a species difference independent of domestication. That there may be species differences in caloric regulation is also suggested by the work of Janowitz and Hollander on food regulation in the dog(12).

Summary. The opossum did not precisely adjust its caloric intake over all the experimental conditions. Rather, as levels of dilution and enrichment increased, the precision of caloric adjustment decreased. There was no increase in grams of food intake on diluted diets, but there was a decrease in grams of food intake with calorically enriched diets. However, this reduction in gram intake constituted an increase in caloric intake. The physical capacity of the digestive tract was not a limiting factor.

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Some Observations on Staphylococcal Penicillinase. (29819)

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The resistance of many strains of staphylococci to penicillin has been shown by Kirby (1), Bondi and Deitz(2,3), Geronimus and Cohen(4) and others to be due to production of a penicillin degrading enzyme, penicillinase. Previous studies concerned with the isolation and properties of this enzyme have been conducted primarily on the extracellular penicillinase produced by *Bacillus cereus* (Pollock *et al*(5)).

Though there are scattered reports in the literature of certain properties of the staphylococcal penicillinase *in vitro*, the enzyme has not been purified to any extent nor crystallized. One possible reason for this is that the staphylococcal enzyme is usually intra-, rather than extracellular, and hence is more difficult to remove from the cells. Since the work on the crystallization of the enzyme from *B. cereus* is quite comprehensive we thought it of interest to attempt a purification of the staphylococcal enzyme using as a basis for

fractionation the procedures described by Pollock and his group. The staphylococcal enzyme, in the experiments reported here, was purified about 20 times, based upon specific activity. Some preliminary comparative immunologic data between this enzyme and that produced by the bacillus are also presented.

Materials and methods. Organism. The organism used throughout this study was a strain of *Staphylococcus aureus*, isolated from a scalp abscess, obtained from Dr. Cornelia Eddy, Dept. of Microbiology, Tulane University. The organism, when grown in trypticase soy broth (Baltimore Biological Laboratory), was found to be resistant to 500 units of penicillin per ml. Because of this resistance it was tested for penicillinase activity and found to be a good source of the enzyme.

Culture methods. Stock cultures were maintained in either trypticase soy broth at 37°C or on egg slants at 4°C. The trypticase soy broth cultures were transferred on alternate days, the egg slants monthly. For induction studies 400 ml of a stationary culture in trypticase soy broth was grown for 48 hours at 37°C. To this culture 2.5 mg (4175 units) of penicillin per ml was added, and the culture shaken for an additional 3 hours at 25°C on a rotary shaker. For isolation of penicillinase from the staphylococci, 300 ml of a culture were grown in trypticase soy broth for 18 hours at 37°C. This was then used to inoculate 10 liters of the same medium, which was then incubated for 18 to 20 hours at 37°C. The cells were collected by centrifugation, washed once with 0.9% sodium chloride and suspended in approximately 100 ml of isotonic saline.

Disruption of cells. The cells, after induction, were disintegrated by the method of Lamanna and Mallette(6) employing glass beads (Catphote Corp., size 12, 0.1 mm in diameter) in a Waring blendor. The concentration of the suspended organisms was not critical but the viscosity of the suspension was kept low enough to permit flow. Two hundred grams of the glass beads were placed in the blendor and cooled to 10°C for one hour. The suspension was then poured into the blendor and disrupted for 5 minutes at 4°C. The container and contents were then cooled for approximately 10 to 15 minutes at —10°C to prevent overheating of the solution. The process was repeated 6 times to give an overall grinding period of 30 minutes.

For comparison of cell disintegration methods a Raytheon 10KC magnetostrictive sonic oscillator was also used. The oscillation period for this method was 40 minutes (Geronimus and Cohen(7)).

Assay method. The penicillinase activity was followed manometrically by the method of Henry and Housewright(8). One unit of penicillinase activity is defined as that quantity of enzyme capable of hydrolyzing one μmole of penicillin in one hour at 37°C and pH 7.0. The sodium salt of benzylpenicillin (Penicillin G) was used as a substrate throughout. The results of the assay were reproducible within 5%.

Nitrogen determination. The protein concentration of the various fractions separated during the purification procedures was determined by the micro Kjeldahl method with nesslerization. Optical density was read photometrically at 570 mμ on a Bausch and Lomb Spectronic 20 spectrophotometer.

Electrophoresis studies. Electrophoresis of the purified preparation was carried out at 4° C in a Spinco paper electrophoresis cell, Durrum type, Model R, Series D, using Whatman "3 MM" strips, 30 mm wide. Two buffer systems were used: 0.01 M acetate at pH 5.0 and 0.05 M glycine-sodium hydroxide at pH 8.6. Duplicate strips were subjected to a potential difference of 370 volts for various periods. One strip was dyed according to the procedure of Block *et al*(9), the other kept moist for enzyme assay. For assay the bands were eluted with 5.9×10^{-3} M sodium bicarbonate.

Immunological techniques. The enzymes used for immunization and also for the *in vitro* experiments with the antiserum were a purified crystalline staphylococcal penicillinase showing a single band on electrophoresis, prepared as described here and a dry, amorphous *B. cereus* penicillinase concentrate purchased from Nutritional Biochemicals Corp. which also showed a single component by electrophoresis.

Immunization. To obtain antisera the same schedule of injections was followed with both enzymes. One ml of a saline solution contain-

ing 1.3 mg of each enzyme was injected into the subscapular region of a rabbit on 3 consecutive days. After 4 days the rabbits again received 3 more daily injections of the same concentration. The dosage was then increased to 3 mg and the triple injection schedule repeated twice with 4-day intervals. Each rabbit received a total of 12 injections or 25.8 mg of the enzyme.

Ten days after the last injection each rabbit was given one intravenous injection of one ml of a sterile saline solution containing 3 mg of the enzyme. Twenty days after the intravenous injection 30 ml of blood were withdrawn from the heart, allowed to clot, and antibody titer determined as described below. The serum was separated under sterile conditions and kept frozen in 5 ml quantities for use in neutralization tests or for fractionation.

Fractionation of antiserum. The γ-globulin fraction was obtained by the method of Howe (10). To each 0.5 ml of serum was added 15 ml of 22.2% sodium sulfate giving a final concentration of 21.4%. This solution was shaken and allowed to stand for one hour at 37°C to precipitate the γ-globulins. The precipitate settled to the bottom of the tube and most of the supernatant fluid was decanted. The precipitate was collected by filtration and suspended in 20 ml of distilled water. The suspension was dialyzed against 0.01 M potassium phosphate buffer (pH 7.0) at 4°C for 18 hours with 5 changes. The nondialyzable material, now free of sodium sulfate, was freeze-dried and stored at 4°C under vacuum.

Neutralization inhibition studies. Solutions of penicillinase were prepared in 0.9% sodium chloride and contained 5 mg of material per ml. The γ-globulin solutions were prepared so that one ml of the solution contained the equivalent of the amount of γ-globulin in one ml of serum. The tests were carried out in 2 ways. One ml of penicillinase solution and 0.5 ml of antiserum were mixed in a test tube and incubated for one hour. One ml of the solution was then transferred into each of 2 Warburg flasks and assayed for penicillinase activity. The second procedure was carried out directly in the Warburg flask. To 0.5 ml of the penicillinase solution was added 0.5 ml of serum or γ-globulin solution. This was then incubated for one hour. The buffer and penicillin were then added to the flasks and assayed.

TABLE I. Penicillinase Induction.

	Penicillinase activity	
	Units/ml	Total activity
Before induction	4.6	1800
After "	11.8	4720

One ml of the culture was assayed for penicillinase activity.

Precipitin studies. Solutions for these reactions were prepared as described under neutralization studies. Dilutions of 1:10, 1:50, 1:100 and 1:200 were prepared from the original γ-globulin solutions. Both microscopic and macroscopic tests were run. For the microscopic precipitin reaction, 0.01 ml of the dilutions was placed onto hanging drop slides and 0.01 ml of the penicillinase solution was added to each. These were incubated for one hour at 37°C and then observed. The macroscopic reactions were carried out in small test tubes. One-tenth ml of the antigen was placed with 0.1 ml of the globulin dilutions. These were also incubated for one hour and read visually. Capillary tube tests were set up with a 1:1 ratio of the antigen and dilutions of γ-globulin; those were incubated for 18 hours at 37°C.

Results. We first demonstrated, confirming the work of other investigators, that a marked enhancement of penicillinase was produced after induction by addition of penicillin to the culture medium (Table I).

Experiments were then carried out to determine the relative efficiency of the disruption of cells by use of a Waring blendor and sonic oscillator. The comparison of a typical set of the results obtained is given in Table II. Since it is obvious that the Waring blendor technique was most satisfactory in terms of total activity recovered, this procedure was used throughout the remaining experiments.

Enzyme isolation. Pollock *et al*(5) obtained from the culture medium of *B. cereus* 5/B the exopenicillinase in crystalline form. Various modifications of their technique were tested before the procedure described below was adopted.

The cells, grown as previously described,

TABLE II. Comparison of Disruption Methods.

Fraction	Waring blendor		Sonic oscillator	
	Amt (ml)	Total activity (units)	Amt (ml)	Total activity (units)
Cell suspension (initial)	42.5	6800	42.5	6800
Clarified supernate after disruption	237	2773	67	678
Cell fragments	47	634	25	483
Freeze-dried powder	798 mg	1554	72 mg	205

were collected by centrifugation at 2,600 rpm and washed once with 0.9% sodium chloride. They were then disrupted in the Waring blendor. At the end of the disintegration period the contents of the blendor were transferred to a large, empty chromatography column and allowed to drain. The beads were washed free of the cell suspension with 0.9% sodium chloride. The suspension and the bead washings were combined, and the solution was freed of the cell fragments by centrifugation at 12,000 rpm for 30 minutes at 0°C. Penicillinase was then precipitated by slowly adding 1.6 volumes of acetone (previously cooled to −10°C) with constant shaking. The solution was kept at −10°C for 18 hours. Most of the supernate could then be decanted, and the remainder removed by filtration at 4°C. The enyzme was brought into solution by dissolving the precipitate in 70 ml of ice water. The solution was kept at 4°C for 16 hours. The solubilized enzyme was removed from the undissolved salts by filtration at 4°C and the filtrate was then freeze-dried.

One gram of the freeze-dried powder was taken for further purification. This was dissolved in 50 ml of 0.6% saturated ammonium sulfate at 4°C and allowed to stand at that temperature for 2 hours. The enzyme is soluble at this salt concentration and the portion which remained insoluble was removed by centrifugation at 12,000 rpm for 15 minutes at 0°C and discarded. The penicillinase was precipitated from the supernatant fluid by addition of solid ammonium sulfate to saturation. The solution was allowed to stand for 3 hours at 4°C and the precipitated enzyme removed by centrifugation.

The enzyme was dissolved in cold distilled water and subjected to increasing concentrations of ammonium sulfate. The fractions soluble at 0.6 saturation and insoluble at 0.8 saturation were combined and dialyzed against 5 changes of 0.1 M potassium phosphate buffer (pH 7.0) for 6 hours to remove the ammonium sulfate which interfered with the later precipitation and assay of the enzyme. The contents of the dialysis bag were then transferred to a beaker and cooled until ice crystals began to form. Ninety-five per cent ethanol was then added dropwise with shaking to a concentration of 40% (w/v), at which time the enzyme began to precipitate. It was left at 4°C overnight. The precipitate was removed by centrifugation at 0°C and dissolved in cold water containing enough 0.1 M potassium phosphate buffer (pH 7.0) to bring the enzyme into solution.

On gradual cooling of the above solution to 2°C brownish colored crystals of indefinite shape began to form. The solution was left undisturbed for one week. The crystals which formed were collected by centrifugation at 0°C, suspended in distilled water, and freeze-dried.

The penicillinase activity of the individual fractions was followed by manometric assay and is given in Table II.

It was found that the freeze-dried powder was stable for several weeks if kept at 4°C in a desiccator under vacuum.

Electrophoretic studies of the crystalline material showed only one band at pH 5.0 in 0.01 M acetate buffer and at pH 8.6 in 0.05 M glycine-sodium hydroxide buffer. On comparison of the purified staphylococcal penicillinase with the commercial concentrate of *B. cereus* penicillinase, only one stainable migrating band was observed with each preparation. In each case the band moved the same distance from the origin. There also appeared to be a non-migrating, stainable, artifact observable in both buffers. This has not as yet been identified. On elution of both bands the activity was present only in the band which migrated.

TABLE III. Fractionation of Staphylococcal Penicillinase.

Fraction	Activity	Amount	Total activity (units)	Specific actvity (units/mg N)
	First trial			
Cell suspension	102.0 u/ml	95 ml	9690	9.0
Waring disruption clarified	17.8 u/ml	200 ml	3460	15.0
Freeze-dried powder	2.7 u/mg	1000 mg	2700	22.0
1st crystallization after ethanol ppt	2.5 u/mg	25 ml	62.5	20
Final freeze-dried crystals	2.7 u/mg	52 mg	140	130.0
	Second trial			
Cell suspension	78.0 u/ml	100 ml	7800	7.8
Waring disruption clarified	4.4 u/ml	300 ml	1320	6.3
Freeze-dried powder	.8 u/mg	860 mg	688	10.0
Final freeze-dried crystals	3.0 u/mg	57 mg	171	150.0

Immunological experiments. It was found that the *B. cereus* penicillinase antiserum would neutralize both its homologous antigen and the staphylococcal penicillinase. *S. aureus* penicillinase antiserum was not as active with respect to the bacillus penicillinase (Table IV) but did show cross reactivity in increased concentration in one experiment (Exp. II, Table IV) out of a series of 6, and requires further investigation.

A cross reaction was observed in the precipitin tests. The reactions were carried out as previously described and the results are shown in Table VI.

Discussion. Because of the lack of information available on the properties of staphylococcal penicillinase, it was felt that the purification of the enzyme should be attempted. By obtaining a purified crystalline preparation of the staphylococcal penicillinase, a comparison could be made with some of the known properties of a similar enzyme from *B. cereus* which has been obtained in purified form.

TABLE IV. Neutralization Studies with Antiserum.

Antigen source	Exp No.	% Inhibition after treatment with antiserum: *S. aureus* antiserum	% Inhibition after treatment with antiserum: *B. cereus* antiserum
Staphylococcal penicillinase	I	15	15
	II	33	36
B. cereus penicillinase	I	0	39
	II	34	47

Exp I: 1 ml of penicillinase solution (5 mg/ml) and 0.5 ml of antiserum were combined and incubated for 1 hr. One ml was then assayed.

Exp II: 0.5 ml of penicillinase solution (5 mg/ml) and 0.5 ml of serum were combined and incubated for 1 hr before assaying. One ml was assayed.

Staphylococcus aureus penicillinase is intracellular. For this reason a method of liberation of the enzyme from the cell was required before an attempt could be made to crystallize the enzyme.

While the two methods of disintegration tested gave essentially the same units on a milliliter basis the much greater total activity recovered by the use of the Waring blendor made this the method of choice.

TABLE V. Neutralization Studies with γ-Globulin Fraction of Immune Sera.

Antigen source	% Inhibition after treatment with γ-globulin fraction: *S. aureus* γ-globulin	% Inhibition after treatment with γ-globulin fraction: *B. cereus* γ-globulin
Staphylococcal penicillinase	43	24
B. cereus penicillinase	7	40

0.5 ml of penicillinase solution (5 mg/ml) and 0.5 ml of γ-globulin solution were combined, and incubated 1 hr before assaying. One ml was assayed.

Pollock *et al*(5) reported a method of purification of the penicillinase from a strain of *B. cereus* whereby the enzyme was adsorbed on glass surfaces and eluted by ammonium sulfate. If other proteins were present at a concentration exceeding one per cent the adsorption did not occur. Since other proteins were freed during the disruption of the staphylococcal cells the clarified supernate obtained in our preparations contained a high protein concentration. This additional pro-

TABLE VI. Precipitin Tests.

	Exp No.	None	Dilutions 1:10	1:50	1:100	1:200
			Staphylococcal γ-globulin			
S. aureus penicillinase	I	3	3	3	1	trace
	II	3	trace	trace	1	—
	III	2	1	1	—	—
B. cereus penicillinase	I	2	3	3	3	1
	II	trace	trace	—	—	—
	III	1	1	1	—	—
			B. cereus γ-globulin			
S. aureus penicillinase	I	1	1	1	1	
	II	4	3	trace	trace	—
	III	2	1	1	±	—
B. cereus penicillinase	I	4	2	2	3	
	II	4	3	2	trace	trace
	III	2	2	2	1	1

I, microscopic; II, macroscopic; III, capillary ring test. 4 = maximum precipitation observed.

tein apparently facilitated their recovery of the enzyme from the glass beads which were used to disintegrate the cells. Since the staphylococcal penicillinase could not be adsorbed on glass under our conditions, their method was modified as described to obtain the enzyme.

We have observed that this penicillinase is not stable to dialysis against ammonium sulfate solutions. The inactivation appears to be due to the ammonium ion and was first observed when assaying the various fractions during the purification. A few preliminary experiments using a *B. cereus* penicillinase concentrate showed that this enzyme is also inhibited in the presence of this ion. Further work needs to be done with both purified enzymes to arrive at a more definite indication of whether the degree of inhibition is the same for both.

Certain physical properties of both *B. cereus* and the staphylococcal penicillinase are markedly similar. The crystals obtained from *S. aureus,* when isolated, have a brownish color. This was also reported to be true of *B. cereus* penicillinase. Both enzymes migrated the same distance when subjected to electrophoresis at pH 5.0 and 8.6 and both possess similar solubility properties.

Precipitin test revealed a cross reaction between the staphylococcal penicillinase antibodies and the enzyme from *B. cereus.* A possible difference between the two enzymes was shown by the enzyme inhibition studies. Approximately the same amount of inhibition of the staphylococcal penicillinase was produced by both antisera. The *B. cereus* enzyme was inhibited to a marked degree only by its homologous antiserum. These results however may be due to non-specific reactions and should be carried out with more highly purified preparations.

The results of the inhibition studies may be of significance in that they indicate a combination of the penicillinase with the antiserum, possibly blocking specifically the active center of the enzyme. Further studies are necessary to clarify this assumption.

Summary. 1. A twenty-fold purification of staphylococcal penicillinase, based upon specific activity, has been achieved. The enzyme, from a penicillin resistant strain of *S. aureus,* was freed from the cells by grinding with glass beads and purified by ammonium sulfate fractionation and precipitation with ethanol. Brown crystals of indefinite shape have been obtained by the procedure outlined. 2. The purified, crystalline, penicillinase was subjected to electrophoresis at pH 5.0 and 8.6. Only one band was observed. At both pH's this band migrated the same distance from the origin as did a partially purified preparation of *B. cereus* penicillinase. 3. Precipitin tests showed an immunological cross reaction between the enzymes. Inhibition studies however indicated possible

differences. Staphylococcal penicillinase activity was inhibited by both homologous and heterologous antiserum. *B. cereus* penicillinase was neutralized to lesser degree by the heterologous antiserum.

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Malaria Induced Endotoxin Tolerance. (29820)

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Hyperreactivity of the reticuloendothelial system (RES) has been considered to be one of the major mechanisms of endotoxin tolerance, the state of resistance to the pyrogenic properties of endotoxin induced by a series of injections(1). Diminished febrile reactivity to endotoxin, noted in patients recovering from malaria, who are known to develop hepatosplenomegaly, the major organs of the RES, has been cited as supporting evidence for this theory(2). This evidence was based primarily on a report that the febrile response to injection of heat killed typhoid vaccine was less marked in neurosyphilis patients recently treated with malaria than in untreated controls(3). In addition, experimental malaria in mice has been reported to induce increased RES activity as measured by the rate of clearance of colloidal carbon particles(4).

We have recently had the opportunity to study febrile reactivity to a well characterized endotoxin in the same subjects before and after induction of experimental malaria. Volunteers, inoculated with malaria parasites, who developed hepatosplenomegaly but no parasitemia, were studied in addition to those who developed clinical malaria. Endotoxin tolerance was observed in those subjects who did develop malaria. In addition, a preliminary report on the functional capacity of the RES is presented.

Materials and methods. Eight normal white male federal prisoner volunteers, age 21 to 37, comprised the study group. Four of the subjects were inoculated with *Plasmodium cynomolgi* (M. strain) by mosquito bites; the remaining 4 were inoculated with *Plasmodium vivax* (Chesson strain), 2 by mosquito bites and 2 by intravenous administration of blood from infected patients. After inoculation Giemsa stained thick and thin blood films were examined daily for malaria parasites. A minimum of 750 oil immersion fields was inspected before concluding that a preparation was negative.

All patients were examined daily by the same physician and particular attention was paid to the possibility of hepatosplenomegaly. Rectal temperatures were taken at least 4 times daily.

Subjects with *P. vivax* malaria were treated with chloroquine and primaquine after 2-3 weeks of illness while treatment was not instituted in the patients inoculated with *P. cynomolgi* until all endotoxin studies were completed. Endotoxin was administered to the patients with *P. vivax* malaria before