received L-ascorbic acid (15 mg *per os*)(18).

Summary. D-Araboascorbic acid administered to guinea pigs in daily oral doses of 10 to 200 mg apparently replaced the antiscorbutic activity of L-ascorbic acid. The animals survived normally and the D-araboascorbic acid prevented them from developing any sign of scurvy discernible at autopsy.

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Immunity Against Experimental Cholera. (32121)

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Although cholera vaccines have been used since before the turn of the century, scientifically controlled field trials have only recently proven that they have any value in prophylaxis against cholera(1,2). The results of the field trials have been disappointing in that the vaccines which were sufficiently innocuous for wide scale usage in man conferred only a low degree of immunity of limited duration. However, one important fact emerged from these efforts: it is feasible, by means of parenteral immunization, to confer at least some degree of resistance against this bacterial infection which is restricted to the lumen of the intestine.

In previous studies on pathogenesis and immunity in experimental cholera(3,4,5), we have described a product, choleragen, which is elaborated *in vitro* under certain defined conditions by certain strains of cholera vibrios. This antigenic protein moiety, administered orally in microgram amounts with buffer, causes choleraic diarrhea in infant rabbits; it causes marked outpouring of fluid (enterosorption(6)) into the lumen of isolated loops of adult rabbit small bowel *in vivo;* in submicrogram amounts, it is an extremely potent permeability factor eliciting a delayed-sustained reaction in rabbit skin(7); and, it has been shown to cause a cholera-like syndrome in man(8). Our previous studies have shown that choleragen elicits the production of specific antibody which neutralizes and precipitates it(9). However, in our attempts to protect infant rabbits passively by parenteral administration of choleragen antiserum prior to challenge, little, if any, evidence of protection was obtained. The present communication presents evidence that parenteral administration of choleragen in adult rabbits gives rise to a highly effective degree of active immunity against intestinal challenge with either choleragen or a massive dose of living cholera vibrios. Some resistance can also be passively transferred to infant rabbits by means of serum from the immunized animals.

Materials and methods. A modification of the intestinal loop technique(10) was used for titration of the effect of choleragen or infection with *Vibrio cholerae* 569 B Inaba on enterosorption in control and immunized adult rabbits. Rabbits of either sex weighing 1.1-1.7 kg were allowed water but no food for one day preceding the test. Laparotomies were performed with aseptic precautions under local xylocaine anesthesia.* The vermiform appendix was identified and the first ligature was made on the ileum 2-3 inches above the blind end of the appendix. Usually the terminal ileum was found to be empty, but in occasional animals the intestinal contents had to be emptied into the proximal gut by elevating the caudal ligature and allowing the contents to move by gravity, thus avoiding unnecessary trauma to the gut. Five loops, each approximately 4 inches long, were made in each animal with 1 inch segments intervening. Thus, only the terminal 36 inches of ileum were used. Four loops were inoculated with 0.1 ml amounts of dilutions of purified choleragen(4) in saline and one loop received 1 ml of 1.0% peptone saline containing 10^8 viable *V. cholerae* harvested from 6-hour growth on meat extract agar plates. The experiments were designed so that the site receiving each inoculum was rotated among the animals: thus, the vibrios were inoculated into loops 1, 2, 3, 4 and 5 in separate animals and the choleragen doses were rotated similarly. The results were recorded 16-18 hours after challenge at which time survivors were sacrificed by air embolism. After noting the gross appearance of the loops, the loops were punctured with scissors and the fluid collected in graduated cylinders. The length of the straightened loops was measured to the nearest half inch. Control loops inoculated with diluent alone were not included in these experiments because in our previous experience there have been no false positive reactions.

Skin reactivity to choleragen was assayed in the same groups of rabbits. Two-fold serial dilutions of choleragen in sterile physiological saline, with saline as a control, were inoculated intracutaneously in 0.1 ml amounts on the shaven backs of the rabbits. The delayed, sustained, erythematous, edematous indurations(7) were observed and graded at 18-24 hours. The skin test was performed on the day preceding immunization in 2 of the immunized rabbits (no. 9 and 10) and again on the day preceding the laparotomies in all of the animals.

Immunization was accomplished by parenteral injection of choleragen in Freund's complete adjuvant (Difco Laboratories). Purified choleragen(4) dissolved in sterile physiological saline was mixed with an equal amount of adjuvant such that each 5 ml contained 5 mg of choleragen. The mixture, in an ice bath, was homogenized briefly with a sonifier (Model S110, Branson Instruments). The adult rabbits, after pre-bleeding, received multiple injections totalling 5 ml on a single occasion. The routes used for injection included: subcutaneous, 2 sites on the back; intramuscular, both rear limbs; intraperitoneal, 2 sites; and all 4 footpads. No untoward effects were observed although at sacrifice some evidence of persistence of adjuvant was noted in occasional animals. Three weeks later, the animals were bled from an ear vein and used in the procedure described.

Serial dilutions of sera were tested for agglutinins against live vibrios(11). Choleragen neutralization tests were performed by diluting serum 1:10 in choleragen-buffer solution (K_2HPO_4, 2%; Na_2HPO_4, 2%) such that the final mixture contained 25 μg of choleragen per ml. After the mixtures had stood at room temperature for 1 hour, they were fed in 1 ml amounts to previously gastrically lavaged infant rabbits by means of a catheter inserted *per os* into the stomach (3,4). At least 3 animals were used for each test. Precipitating antibody against choleragen was detected by a modified Ouchterlony technique(4). Passive protection tests were performed by administering 1 ml of pooled antisera intraperitoneally in infant rabbits 18 hours prior to oral challenge with 25 μg of choleragen in buffer. After feeding

* We are indebted to Mrs. Usha Bhargava for demonstrating the technique she uses for laparotomies in rabbits under local anesthesia.

TABLE I. Effect of Parenteral Immunization* with Choleragen on Resistance to Intestinal and Intradermal Challenge.

Challenge	Control rabbits				Immunized rabbits					
	1	2	3	4	5	6	7	8	9	10
Intra-intestinal										
10^8 *V. cholerae*	3.3†	7.0	5.8	6.7	2.8	0	2.8	0	0	0
Choleragen										
50 μg	—	—	—	—	2.6	0	1.0	5.3	0	0
10 μg	5.1	7.0	5.6	6.6	0	0	2.3	0	0	0
2 μg	0	.8	4.0	5.0	0	0	0	0	0	0
0.4 μg	0	0	0	4.0	2.5	0	0	0	0	0
0.08 μg	0	0	0	0	—	—	—	—	—	—
Intradermal choleragen	.005‡	.01	.02	.005	1.25	2.5	>10.0	2.5	1.25	5.0

* Immunized rabbits were inoculated with 5 mg of choleragen in Freund's complete adjuvant in multiple sites 3 weeks prior to challenge.

† ml of fluid per inch of intestinal loop, 18 hr after challenge.

‡ Smallest dose of choleragen producing + reaction, 18 hr after inoculation. Rabbits 9 and 10 were pretested, prior to immunization. The minimal skin reactive doses at that time were 0.01 and 0.15 μg, respectively.

of choleragen, the animals were observed for evidence of diarrhea and survivors were sacrificed at 18 hours to determine the presence of excessive fluid in the intestines. Normal rabbit sera had no activity in any of the tests described.

Results. Table I summarizes the results of titrations of choleragen and infection with *V. cholerae* in immunized animals and in a parallel group of controls. In the immunized group, only 2 out of 6 animals responded to the massive live vibrio challenge, and then to a lesser degree than the control group all of which responded with marked outpouring of fluid into the loops. Immunization also conferred a marked, although not absolute, degree of resistance to intra-intestinal challenge with choleragen. Similarly, the skin reactivity to intradermal choleragen was markedly decreased in the immunized group in comparison with the simultaneous controls.

Neutralization tests revealed that each of the post-immunization sera neutralized choleragen, with the possible exception of serum from rabbit 5. In the latter case, 2 out of 3 infant rabbits had excessive fluid in the intestine at sacrifice.

Each of the post-immunization sera had antibody capable of precipitating choleragen in Ouchterlony tests (Fig. 1). The serum of rabbit 5, however, contained less anti-choleragen antibody than did the others as evidenced by the nearness of the precipitin band to the serum well. On the other hand, serum from rabbits 9 and 10 appeared to have a somewhat higher content of anti-choleragen antibody as the specific precipitin band was further from the serum well. Some sera, notably 5, 9 and 10, gave two bands of precipitate; these rabbits apparently responded to additional antigens in "purified choleragen."

Passive protection tests employing a pool of antisera from animals 6, 7 and 8, and another of 9 and 10 gave some evidence of protection in terms of either survival during the experimental period or reduction in the diarrheal rate and enterosorption (Table II). The pool of sera from rabbits 9 and 10 was somewhat more effective.

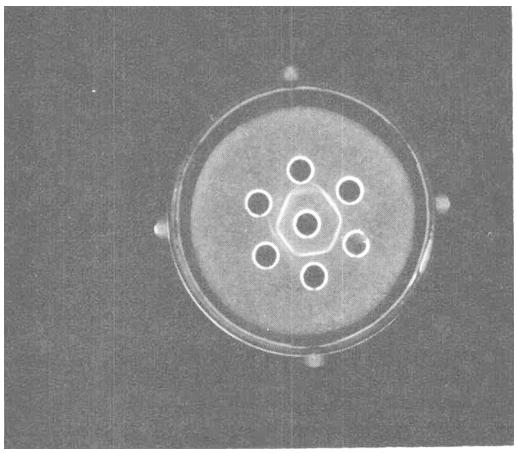

FIG. 1. Agar gel double diffusion precipitation patterns of sera from rabbits immunized with choleragen. Starting from the top, clockwise, sera from rabbits 5, 6, 7, 8, 9, and 10. Center well: 25 μg of purified choleragen.

TABLE II. Passive Protection of Infant Rabbits with Anti-choleragen Sera.*

Response	Serum pool: None	Rabbits 6–8	Rabbits 9 and 10
Excessive fluid	9/9†	7/8	5/7
Diarrhea	9/9	7/8	4/7
Dead or moribund	9/9	4/8	3/7

* Serum (1 ml) administered I.P. 18 hr prior to 25 μg of choleragen *per os*.
† No. responding/total.

All sera from the immunized rabbits contained high levels of agglutinins. Against an Inaba serotype agglutinating antigen, the titers were equal to or greater than 20,480; against Ogawa antigen, they ranged from 5,120 to a high of 20,480 (in rabbit 6).

Discussion. It is evident from the present study that parenteral immunization with choleragen actively protects adult rabbits against intestinal challenge with either live vibrios or with choleragen. A partial degree of immunity to the choleragen may be passively transferred to infant rabbits by means of serum administered parenterally prior to challenge.

The latter observation stands in contrast to our previous study(9) in which it was found that neutralizing antisera had little, if any, protective effect in the infant rabbit under similar conditions. The differences in methodology in the two studies which could account for the differences in results include the use of more potent and purified antigen for immunization in the present study and the use of adjuvant and a longer interval between vaccination and bleeding. In this regard, recent observations of Feeley(12) may have some relevance. He found that sera obtained later in the course of immunization were more protective against live vibrio challenge in the infant rabbit. The late sera did not contain higher titers of agglutinins or vibriocidins than the earlier sera, but they were less susceptible to inactivation by 2 mercapto-ethanol. These observations suggest a need for further elucidation of the origin, nature and molecular species of the antibody involved in the anti-choleragen protective effect.

Since agglutinating antibody was produced in high titer in all of the immunized rabbits in the present study, no conclusion may be drawn at the present time as to which antibody, the anti-choleragen, the anti-bacterial or both, has primary responsibility for the active protection observed against the live vibrio challenge. Other investigators(13-16), including ourselves(9), have shown that some degree of resistance against intestinal challenge with live vibrios can be passively transferred to infant rabbits by means of anti-vibrio antisera. Thus, the anti-choleragen antibody may only provide a second line of defense. Although it would be desirable to settle this point, the important consideration is that choleragen, at its present state of purity, simultaneously stimulates resistance to both the effect of infection and the choleragenic factor and thus offers promise as an effective immunogen against cholera. Obviously, additional study is needed before it could be tested in the field. The present and previous(4,17) observations on the lack of toxicity of parenterally administered choleragen suggest that detoxification may not be necessary. The choleragenic activity is inactivated by formaldehyde(17) should inactivation prove desirable although it remains to be shown that the resulting product is still antigenic.

Summary. Choleragen, administered parenterally, immunized adult rabbits against intestinal challenge with either living cholera vibrios or with choleragen. A degree of resistance against choleragen could be passively transferred by serum to infant rabbits. These results suggest that choleragen offers promise as an effective immunogen against cholera although additional study is needed before any field studies can be performed.

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Metabolism of 3-(p-Chlorophenoxy)-2-Methoxypropyl Carbamate in the Rat. (32122)

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Alkyl ethers are reported to be quite stable in animals and are usually excreted unchanged (1). Such ethers, in addition, do not serve as substrates for the liver microsomal enzyme responsible for cleavage of aromatic ethers (2) although glycerol ethers of long chain fatty alcohols can be split by a similar microsomal system(3).

Recently it has been shown that the muscle relaxant drug chlorphenesin carbamate† [3-(p-chlorophenoxy)-2-hydroxypropyl carbamate], a compound potentially capable of forming both O- and N-glucuronides(4) *in vivo,* was excreted only as the O-glucuronide from the rat and human(5). The methyl ether of chlorphenesin carbamate has now been investigated to determine if blockage of the hydroxyl group would result in N-glucuronide formation. The present report describes the fate of 3-(p-chlorophenoxy)-2-methoxypropyl carbamate‡ (MCC, Fig. 1) in the rat.

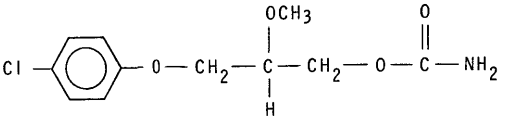

FIG. 1. 3-(p-Chlorophenoxy) - 2 - methoxypropyl carbamate.

Experimental. Four male Wistar rats weighing 260-290 g were each given an oral dose of 54 mg MCC in 2 ml homogeneous saline suspension (dose approximately 190 mg/kg). Administration was repeated every 24 hours for 5 days until the animals had received a total dose of 1.08 g of drug. Following initial dosing, the animals were placed in individual metabolism cages and urine was collected under toluene for a period of 7 days. Urine samples were removed every 24 hours, pooled and frozen.

The pooled 7-day urine was thawed, adjusted to pH 4 with glacial acetic acid and extracted 4 times with ½ volume of chloroform. The chloroform extract containing the nonconjugated metabolites then was fractionated into nonconjugated neutral and non-

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† Maolate is The Upjohn Co. trademark for chlorphenesin carbamate.

‡ Synthesized by G. Youngdale, Chemistry Research, The Upjohn Co.