interactions are available, the first being the recombinants between anurans and urodeles (*11*) but examples in which tissues from widely different species were employed are rare. There are virtually no previous studies in which an unspecialized responding epithelium has been used: perhaps the closest to the present combination is that of Coulombre and Coulombre (*12*), who reported that replacement of the lens of a 5-day chick embryo with dermis from mouse flank led to feather formation in the relatively unspecialized host-chick cornea.

This investigation demonstrates that human embryonic skin dermis specifically directs the transformation of unspecialized chick chorionic epithelium into a typical keratinized epidermis, histologically indistinguishable from chick skin. That the differentiated epidermis may exert an influence on human dermis in its turn is suggested by the additional modifications observed in the human dermis after recombination culture. This study clearly distinguishes the roles of both the inducing dermis and the transforming epithelium in a tissue interaction.

ROGER H. SAWYER
URSULA K. ABBOTT
Department of Avian Sciences, University of California, Davis 95616
JOHN D. TRELFORD
Department of Obstetrics and Gynecology, University of California Medical School, Davis

References and Notes

1. C. B. McLoughlin, *J. Embryol. Exp. Morphol.* **9**, 385 (1961); M. E. Rawles, *ibid.* **11**, 765 (1963); P. F. Goetinck and U. K. Abbott, *J. Exp. Zool.* **154**, 7 (1963); P. Sengel and U. K. Abbott, *J. Hered.* **54**, 255 (1963); J. W. Dodson, *J. Embryol. Exp. Morphol.* **17**, 83 (1967); N. K. Wessells, *N. Engl. J. Med.* **277**, 21 (1967).
2. Y. Kato, *J. Exp. Zool.* **170**, 229 (1969).
3. T. Mizuno, *C. R. Acad. Sci.* **271**, 2027 (1970).
4. R. E. Billingham and W. K. Silvers, in *Epithelial-Mesenchymal Interactions*, R. Fleischmajer and R. E. Billingham, Eds. (Williams & Wilkins, Baltimore, 1968), pp. 252–266.
5. R. A. Briggaman and C. E. Wheeler, Jr., *J. Invest. Dermatol.* **51**, 454 (1968).
6. Y. Kato and Y. Hayashi, *Exp. Cell Res.* **31**, 599 (1963).
7. ———, *Transplantation* **10**, 354 (1970).
8. E. W. Goodpasture, B. Douglas, K. Anderson, *J. Exp. Med.* **68**, 891 (1938).
9. J. M. Cairns, *Dev. Biol.* **12**, 36 (1965); P. Sengel and M. P. Pautou, *Nature* **222**, 693 (1969).
10. B. Garber, E. J. Kollar, A. A. Moscona, *J. Exp. Zool.* **168**, 455 (1968).
11. H. Spemann and O. Schotté, *Naturwissenschaften* **20**, 463 (1932).
12. J. L. Coulombre and A. J. Coulombre, *Dev. Biol.* **25**, 464 (1971).
13. We are indebted to Rae Lester for technical assistance. Work supported in part by NIH training grant 5T01-GM-00701.

16 August 1971

Crystalline Cholera Toxin and Toxoid

Abstract. *The exo-enterotoxin of* Vibrio cholerae *has been obtained in crystalline form. A solution of the crystalline protein was equal in potency to the parent pure toxin in both choleragenicity and skin reactivity. Crystals of the natural toxoid, choleragenoid, resemble those of the toxin in appearance. A solution of crystalline choleragenoid was equivalent to the parent preparation in the flocculation test.*

We have reported the isolation and purification of the cholera exo-enterotoxin, which was designated choleragen, and indicated that the pure protein was both choleragenic and skin reactive (*1*, *2*). Other investigators using cruder preparations have reported some dissociation of skin reactivity and choleragenicity and suggested that the two activities may not be present on the same molecule (*3*). We have also reported the purification and some of the properties of a natural toxoid—designated choleragenoid, also found in fermenter-grown cultures of the highly toxigenic strain of *Vibrio cholerae*, 569B Inaba (*1*, *2*)—and presented some details of how the toxoid is formed from the toxin (*4*, *5*). We now report the application of an additional criterion of purity, the solubility test, and the crystallization of these two proteins. Redissolved crystalline cholera toxin was found to be equally as potent as the parent purified toxin in choleragenicity, skin reactivity and flocculation (Lf) tests.

Choleragen and choleragenoid, purified by either of the methods described (*2*, *5*), were each subjected to an additional chromatographic passage on Bio-Gel P-150 (Bio-Rad Laboratories) to remove residual traces of the other protein and traces of aggregated protein which may have been formed. As an additional test of purity, the phase rule solubility test was applied. Increasing increments of choleragen solution (25 mg/ml) in tris (hydroxymethyl) aminomethane (tris) buffer (*1*) were added to test tubes, and tris buffer was added to a total volume of 1 ml. To each tube 1 ml of 4*M* $(NH_4)_2SO_4$ was added and the tubes were incubated overnight at 22° to 23°C with gentle shaking. The tubes were centrifuged, and the optical density of the supernatant was determined at 280 nm. The results, Fig. 1A, were consistent with those expected from a pure protein. The solubility curve showed a sharp change in slope at the inflection point (at approximately 1.7 mg of protein added), and there was no further increase in absorbance with added increments of protein.

Choleragen was crystallized by adjusting the *p*H of a solution containing approximately 20 mg/ml to the isoelectric point, *p*H 6.6 (*2*), and adding 4*M* $(NH_4)_2SO_4$ (adjusted to the same *p*H) until slight opalescence was observed at room temperature. Sufficient water to clarify the solution was added, and the mixture was placed at 4°C. On subsequent days, a white precipitate was observed in the bottom of the conical test tube which, on swirling, exhibited a refractile sheen characteristic of dispersed crystals. Examination by dark-field microscopy revealed the crystalline nature of the sediment (Fig. 1B). A variety of crystalline forms was observed. Some were needle-like structures and some shield-like forms, but,

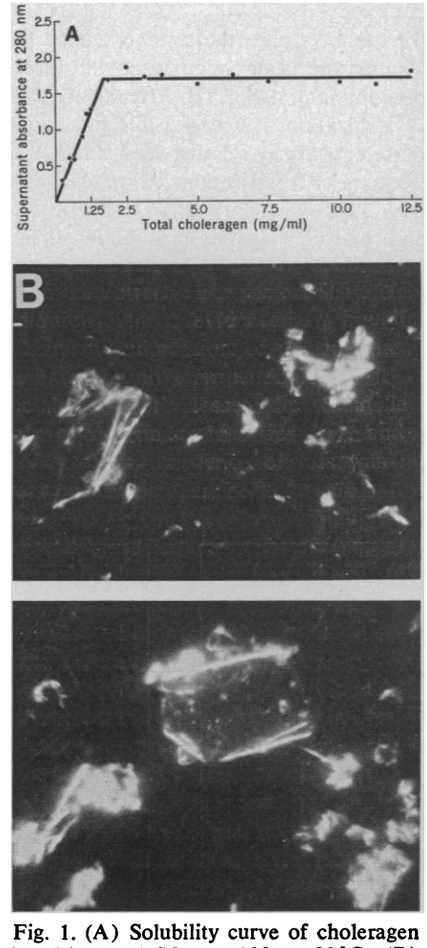

Fig. 1. (A) Solubility curve of choleragen in 2*M* $(NH_4)_2SO_4$, at 22° to 23°C. (B) Two views of crystals of choleragen, dark-field microscopy; original magnification approximately 600 ×.

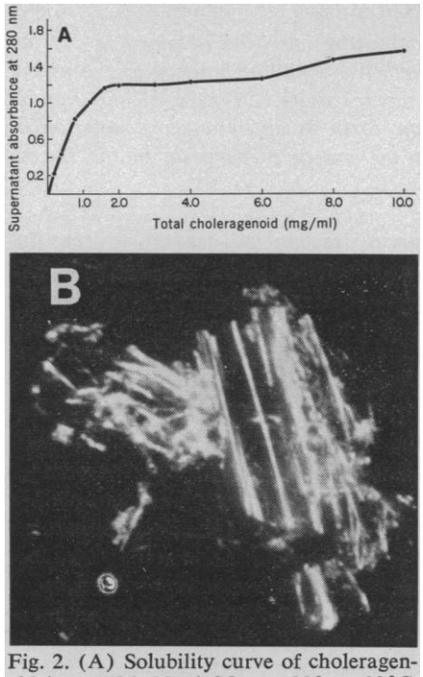

Fig. 2. (A) Solubility curve of choleragenoid in 2.67*M* $(NH_4)_2SO_4$, at 22° to 23°C. (B) Crystals of choleragenoid, same conditions as Fig. 1B.

for the most part, the crystals tended to assume the plate or rhomboidal form predominant in Fig. 1B. After the initial crystallization, it was possible to obtain more crystals by seeding fresh $(NH_4)_2SO_4$-treated preparations. Crystals were harvested by centrifugation, redissolved, and dialyzed against tris buffer. The potency of the redissolved choleragen was tested, in parallel with the parent toxin, for choleragenicity in infant rabbits (*6*), for skin reactivity in adult rabbits (*7*), and by the Lf test (*8*). The results in each case were identical with the parent toxin. The dose causing a mean choleragenic score (*1, 6*) of 5 was estimated from titrations in groups of five infant rabbits each, to be 0.4 μg. The skin reactive dose was between 0.0003 and 0.001 μg, determined in duplicate titrations in each of two rabbits. [This dose was somewhat higher than that which we reported previously (*2*), but we have found that results of this test vary depending on the sensitivity of the rabbits used on a given occasion.] The Lf dose, as already reported (*8*), was 1.0 μg. Disc electrophoresis revealed a single protein band in the separating gel with a trace of aggregated material which failed to penetrate the polyacrylamide gel.

Choleragenoid was processed similarly, except that a higher concentration of $(NH_4)_2SO_4$, 2.67*M*, was used in the solubility test. The results (Fig. 2A) indicated that choleragenoid was not homogeneous according to this criterion. A gradual curve was observed with increasing absorbance at high concentrations of protein. This is entirely consistent with our previous observations that there are multiple species of choleragenoid (*2*). Nevertheless, using $(NH_4)_2SO_4$ at the isoelectric point, *p*H 7.75, of the predominant species of choleragenoid (*2*), we were able to obtain crystals (Fig. 2B) which resembled those of choleragen. In this case, however, a slight amount of amorphous precipitate was removed after the first $(NH_4)_2SO_4$ addition, and crystallization was achieved after a subsequent increase of the $(NH_4)_2SO_4$ concentration to approximately 2.7*M*. Disc electrophoresis of the redissolved crystalline material suggested that it consisted primarily of the predominant choleragenoid species (*2*). The Lf dose of the redissolved crystals was identical with the parent preparation of choleragenoid, 0.6 μg (*8*). We believe this to be the first time that crystallization of a natural toxoid has been reported.

In addition to showing that the cholera toxin, like diphtheria, tetanus, and other classical bacterial toxins, is crystallizable, our results indicate that we were working with a pure protein. Our study reemphasizes the differences between our previous work and that of investigators working with cruder preparations (*3*) who reported dissociation of diarrheagenic activity and skin reactivity. We can conclude only that the discrepancies are attributable to the use of impure preparations or to the possibility that in the procedures used, which resulted in losses of most of the original toxic activity (*3*), fractions or subunits of the toxin molecule may have been separated perhaps in combination with other materials present. Although we have demonstrated the heterogeneity of the subunits of the choleragen molecule (*9, 10*), we have thus far been unable to separate the subunits and thereby evaluate this hypothesis. In contrast to previous reports on less pure materials (*11*), chemical analyses (*10*) indicate that the purified products are simple proteins essentially free of lipid and carbohydrate.

RICHARD A. FINKELSTEIN
JOSEPH J. LOSPALLUTO
Departments of Microbiology and *Biochemistry, University of Texas Southwestern Medical School, Dallas 75235*

References and Notes

1. R. A. Finkelstein and J. J. LoSpalluto, *J. Exp. Med.* **130**, 185 (1969).
2. ———, *J. Infec. Dis.* **121** (Suppl.), S63 (1970).
3. A. C. Lewis and B. A. Freeman, *Science* **165**, 808 (1969); G. F. Grady and M. C. Chang, *J. Infec. Dis.* **121** (Suppl.), S92 (1970).
4. R. A. Finkelstein, J. W. Peterson, J. J. LoSpalluto, *J. Immunol.* **106**, 868 (1971).
5. R. A. Finkelstein, K. Fujita, J. J. LoSpalluto, *ibid.* **107**, 1043 (1971).
6. R. A. Finkelstein, J. J. Jehl, A. Goth, *Proc. Soc. Exp. Biol. Med.* **132**, 835 (1969).
7. J. P. Craig, in *Microbial Toxins*, S. Kadis, T. C. Montie, S. J. Ajl, Eds. (Academic Press, New York, 1971), vol. 2A, p. 189.
8. R. A. Finkelstein, *Infec. Immun.* **2**, 691 (1970).
9. J. J. LoSpalluto and R. A. Finkelstein, *Fed. Proc.* **30**, 304a (1971).
10. ———, *Biochim, Biophys. Acta*, in press.
11. W. H. Coleman, J. Kaur, M. E. Iwert, G. J. Kasai, W. Burrows, *J. Bacteriol.* **96**, 1137 (1968); J. Kaur, H. C. König, W. R. Martin, W. Burrows, *J. Infec. Dis.* **121**, 78 (1970); S. H. Richardson and D. J. Evans, Jr., *J. Bacteriol.* **96**, 1443 (1968).
12. We thank Dr. Iwao Kato who set an example in his purification and crystallization of diphtheria toxin; see I. Kato, H. Nakamura, T. Uchida, J. Koyama, T. Katsura, *Japan. J. Exp. Med.* **30**, 129 (1960).
13. We thank Barbara Smith, Judith Head, and Katherine Grundman for technical assistance. Supported by PHS research grant AI-08877 under the United States–Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases.

9 July 1971

Microsomal Lipid Peroxidation: Morphological Characterization

Abstract. *Lipid peroxidation of liver and kidney microsomes induces a highly characteristic sequence of morphological changes typified by detachment of ribosomes and formation of large aggregates of vesicles bound together by dense amorphous material and myelin figure-like debris. The trilaminar structure of the membrane is, however, retained even after complete peroxidation, though its spacing may be increased. The aggregates resemble lysosomal lipofuscin pigment as well as the membranous aggregates of endoplasmic reticulum seen in the liver after carbon tetrachloride poisoning.*

Lipid peroxidation of cell membranes may play an important, although for the present poorly understood, role in many types of cell injury. It has been detected, by chemical means, in the liver after administration of toxic compounds; in the brain, muscle, and liver of vitamin E–deficient rats, and in the kidneys of choline-deficient rats (*1*). It may also be associated with radiation