trolled by its end product (*15*). Our speculation would imply several heme-synthesizing systems each, perhaps under separate genetic control, geared to α-, β-, or γ-chains.

MARTHA KREIMER-BIRNBAUM
ROBIN M. BANNERMAN
Department of Medicine, State University of New York at Buffalo, and *Buffalo General Hospital, 100 High Street, Buffalo 14203*

References and Notes

1. D. J. Weatherall, *The Thalassaemia Syndromes*. (Blackwell, Oxford, 1965).
2. R. M. Bannerman, G. Keusch, M. Kreimer-Birnbaum, V. K. Vance, S. L. Vaughan, *Amer. J. Med.*, in press; M. Kreimer-Birnbaum and R. M. Bannerman, in preparation.
3. A. M. M. Zade-Oppen, *Scand. J. Clin. Lab. Invest.* **15**, 491 (1963).
4. R. F. Labbe and G. Nishida, *Biochem. Biophys. Acta.* **26**, 437 (1957); A. Kassenaar. H. Morell, I. M. London, *J. Biol. Chem.* **229**, 423 (1957).
5. E. R. Huehns and E. M. Shooter, *J. Med. Genet.* **2**, 48 (1965).
6. M. Grinstein, R. M. Bannerman, J. D. Vavra, C. V. Moore, *Amer. J. Med.* **29**, 18 (1960); D. G. Nathan, S. Piomelli, F. H. Gardner, *J. Clin. Invest.* **40**, 940 (1961).
7. G. P. Bruns and I. M. London, *Biochem. Biophys. Res. Commun.* **18**, 236 (1965); A. I. Grayzel, P. Horchner, I. M. London, *Fed. Proc.* **25**, 648 (1966).
8. E. R. Burka and P. A. Marks, *Nature* **199**, 706 (1963); P. A. Marks and E. R. Burka, *Science* **144**, 552 (1964); A. Bank and P. A. Marks, *J. Clin. Invest.* **45**, 330 (1966).
9. J. D. Heywood, M. Karon, S. Weissman, *Science* **146**, 530 (1964); J. D. Heywood, M. Karon, S. Weissman, *J. Lab. Clin. Med.* **66**, 476 (1965); D. S. Weatherall, J. B. Clegg and M. Naughton, *Nature* **208**, 1061 (1965); J. D. Heywood, M. Karon, S. Weissman, *J. Lab. Clin. Med.* **67**, 246 (1966); B. Colombo and C. Baglioni, *J. Mol. Biol.* **16**, 51 (1966).
10. E. R. Huehns and C. B. McLoughlin, in *Proceedings International Symposium on Comparative Hemoglobin Structure*, Thessaloniki, Greece, 1966, in press.
11. E. R. Huehns and C. B. McLoughlin, in preparation.
12. T. G. Gabuzda, D. G. Nathan, F. H. Gardner, *J. Clin. Invest.* **42**, 1678 (1963).
13. P. Fessas and D. Loukopoulos, *Science* **143**, 590 (1964); P. Fessas, D. Loukopoulos, A. Kaltsoya, *Biochim. Biophys. Acta* **124**, 430 (1966).
14. H. S. Marver, D. P. Tschudy, M. G. Perlroth, A. Collins, *Science* **154**, 501 (1966).
15. J. Lascelles, *Biochem. J.* **100**, 184 (1966).
16. Supported by PHS grants AM-05581 and AM-10485 and grants from the United Health Foundation of Western New York. We thank Drs. M. Reichlin and P. H. Pinkerton for assistance in the separation of hemoglobins, and Dr. E. R. Huehns for criticism of the manuscript.

7 November 1966

Purified Staphylococcal Alpha Toxin: Effect on Epithelial Ion Transport

Abstract. *Purified staphylococcal alpha toxin, when added to the serosal bathing medium of the isolated toad bladder, causes a rapid fall in short-circuit current and transepithelial potential difference. It has no effect when added to the mucosal bathing medium. Oxygen consumption by suspensions of minced bladder tissue is stimulated by the toxin. These effects are neutralized by staphylococcal antitoxin.*

Purified staphylococcal α-toxin (hereafter referred to as toxin) is a heat-labile protein (molecular weight, 44,000) that produces hemolysis, dermatonecrosis, and death in animals (*1*, *2*). Studies of the action of the toxin on rabbit erythrocytes and cultures of kidney cells have indicated that the primary site of action may be the cell membrane. The very rapid release of potassium from erythrocytes prior to lysis, followed by loss of hemoglobin during lysis, suggests that a membrane "leak" occurs. Small molecules escape initially; large ones are lost as the leak increases in size (*3*). Tissue culture cells incubated with toxin release amino acids first and proteins later (*4*). This toxin appears to be one of several substances capable of causing lysis in a variety of membrane-bounded structures such as erythrocytes, platelets, lysosomes, bacterial protoplasts, and *Mycoplasma* (*5*). The purpose of our study was to determine the effect of purified staphylococcal α-toxin on transport phenomena and oxygen consumption in the isolated toad bladder.

Purified staphylococcal α-toxin was prepared by a modification of the method of Madoff and Weinstein (*1*). The final preparation was frozen in borate buffer (0.3*M*, *p*H 8.6) and stored in 0.5-ml portions at −60°C. The specific activity of the toxin was 30 to 60 hemolytic units (HU) per microgram of protein. Analysis by the Ouchterlony double-diffusion technique with Burroughs-Wellcome antitoxin to staphylococcus (1 ml neutralized 100,000 HU of purified α-toxin) revealed a single band with toxin containing 35,000 HU/ml; concentrations of 70,000 HU/ml, and less potent preparations stored at −60°C for 2 to 3 months, produced a faint second band.

Short-circuit current (SCC) in microamperes (μa), and spontaneous transepithelial potential difference (PD) in millivolts (mv) across the isolated toad bladder (*Bufo marinus*) were determined by the method of Leaf, Anderson, and Page (*6*). Each half of a single bladder was mounted between two sections of a lucite chamber with cross-sectional area of 7.07 cm^2. The bladder tissue was bathed on each side by 20 ml of toad Ringer solution, *p*H 7.35 (*6*). Only bladders with which the current fluctuated over a range of no more than ± 10 μa for 20 minutes or showed a continuous rise during this period were used. Bladders exhibiting a potential difference of less than 15 mv or an electrical resistance of less than 150 ohm (calculated as the quotient of PD and SCC) were discarded.

In initial studies, 500 μl of purified toxin (40,000 HU/ml) was added to the serosal bathing medium of one hemibladder. The same quantity of toxin, inactivated by heating at 56°C for 30 minutes, was similarly applied to the control. A rapid decline in both SCC and PD was observed after the addition of toxin in all of ten experiments. The heated material produced no significant change. These experiments were then repeated, with toxin neutralized by antitoxin to exclude the possibility of a nonspecific effect. Six studies were carried out with 150 μl of α-toxin (6000 HU). One set of bladders was treated with toxin alone and another with the same quantity previously incubated with a fully neutralizing dose of staphylococcal antitoxin (75 μl). In order to maintain equal volumes in all the preparations, 75 μl of Ringer solution was added to the unneutralized toxin in three experiments, and 75 μl of normal horse serum in three others. An abrupt but small rise in short-circuit current occurred within 1 to 2 minutes after treatment with toxin in half the experiments; this was followed by a rapid fall in all experiments to less than 10 μa in 30 to 45 minutes. During the same time interval, PD declined steadily. The control hemibladders remained unchanged (Fig. 1). The difference in

Table 1. Effect of staphylococcal alpha toxin and a toxin-antitoxin mixture on oxygen consumption (microliters per minute) by minced toad-bladder tissue. Numbers in parentheses indicate the number of samples.

Test substance	Oxygen consumption (Mean ± S.E.M.) *	*P* †
Toxin (17)	6.5 ± .19	<.001
Toxin + antitoxin (7)	4.5 ± .28	>.9
Antitoxin (8)	4.5 ± .20	>.9
Borate buffer (11)	4.4 ± .20	>.8
Tris buffer (15)	4.8 ± .15	

* S.E.M. = Standard error of the mean. † Each *P* value represents comparison by *t*-test to the control (tris buffer alone).

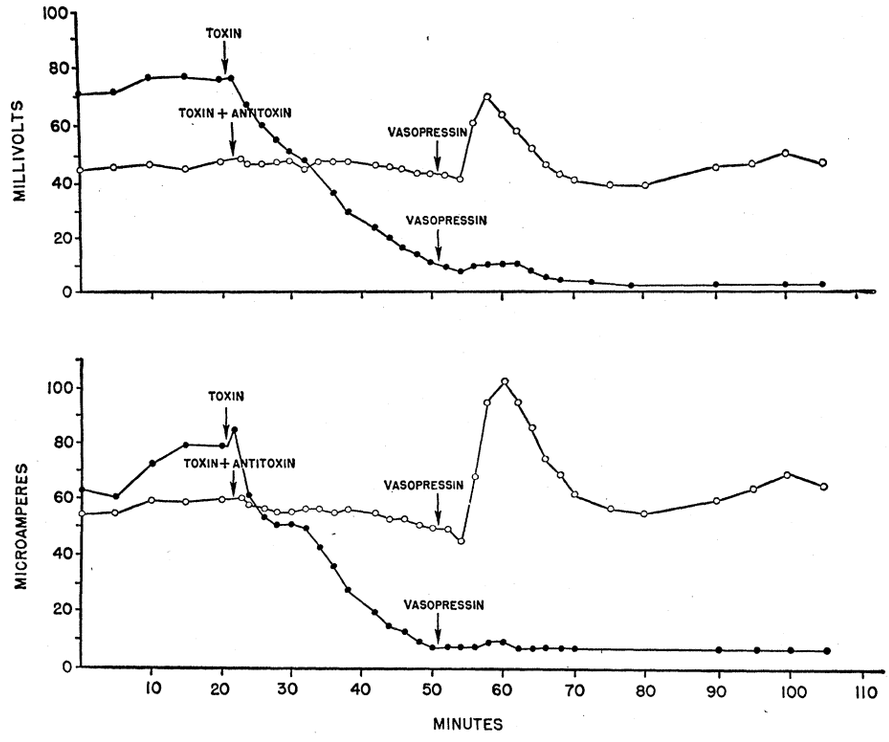

Fig. 1 (right). Addition of staphylococcal α-toxin to the serosal bathing medium on transmembrane potential and short-circuit current; 6000 HU (0.150 ml) of toxin was used. The effect of toxin combined with antitoxin is representative of that obtained in experiments with antitoxin alone, heated toxin, or Ringer solution as control. Vasopressin was added to the serosal bathing medium.

current and potential difference between treated and control preparations after 30 minutes was significant by Student's t-test analysis ($P < .01$). No consistent difference was observed when Ringer solution or normal horse serum was employed for volume equilibration; however, in some instances, the serum appeared to retard the activity of the toxin.

Vasopressin (final concentration, 0.1 unit/ml) was applied to the serosal medium of both the experimental and control preparations 45 minutes after exposure to toxin or at the time when the SCC of either hemibladder fell below 10 μa. The addition of vasopressin to the toxin-treated hemibladders did not significantly stimulate either current or potential difference. The controls responded to the hormone with a characteristic rise in both SCC and PD and a fall in electrical resistance (Fig. 1).

Six experiments were carried out to determine the effect of toxin added to the mucosal bathing medium. The quantity used (18,000 HU) was three times greater than that employed in the previous studies. No effects were noted when either toxin or Ringer solution was applied to the mucosa of the bladder. Analysis of the difference in SCC and PD between the experimental and control hemibladders at 30 minutes revealed $P > .4$. The change in PD paralleled that of SCC in each experiment. The addition of vasopressin to the serosal surface produced a characteristic stimulation of short-circuit current and potential difference in both the control and toxin-treated preparations (Fig. 2).

We made studies to determine whether the inhibition of SCC and PD by staphylococcal α-toxin was secondary to inhibition of aerobic respiration. Suspensions of minced toad bladder (400 mg, wet weight) were made in the following buffered solution at 4°C (*p*H 7.1, 795 milliosmoles): tris (0.1*M*), sucrose (0.25*M*), succinate (0.08*M*), NaCl (0.012*M*), and $MgCl_2$ (0.004*M*). Two groups of experiments were carried out in a Warburg respirometer at 37.2°C. In the first, 0.4 ml of toxin (16,000 HU) was added to bladder suspensions. The controls were treated with (i) borate buffer, (ii) toxin and antitoxin, (iii) antitoxin alone, or (iv) tris buffer. The final volume in each flask was 3.0 ml. Oxygen consumption was determined for a period of 45 minutes. In the second group of experiments, bladder suspensions were allowed to respire for 45 minutes, and either toxin or buffer was added to duplicate flasks. Oxygen consumption in each flask during a

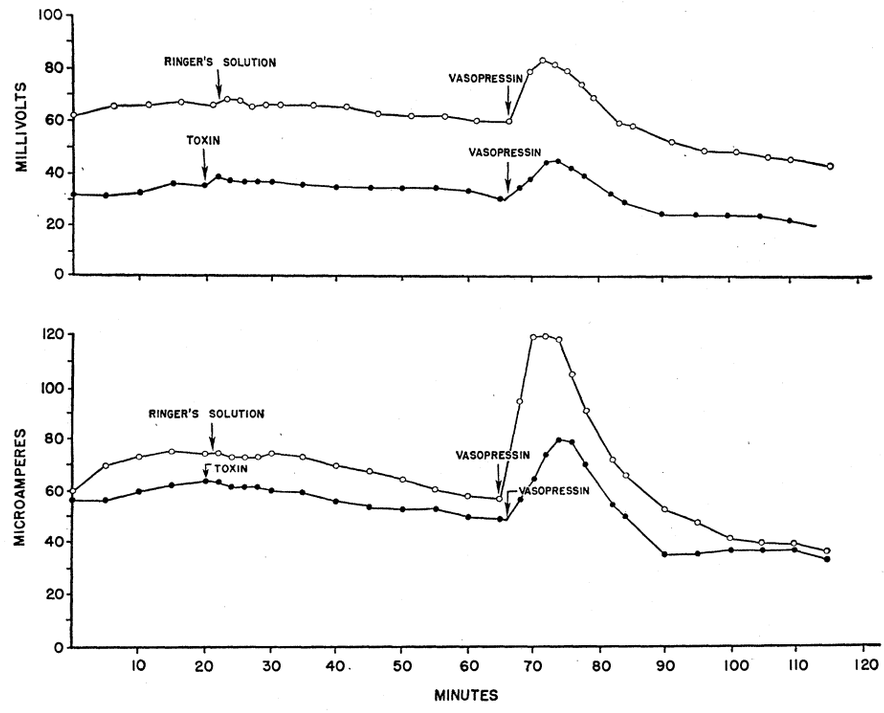

Fig. 2 (right). Addition of staphylococcal α-toxin (18,000 HU; 0.45 ml) to the mucosal bathing medium on transmembrane potential and short-circuit current. Vasopressin was added to the serosal bathing medium.

period of 60 to 90 minutes was compared with that during a period of 15 to 45 minutes. A dose of α-toxin (16,000 HU) that produced maximal suppression of SCC and PD was used. The addition of toxin prior to incubation produced an increase in mean oxygen consumption in paired tissue from 4.5 to 6.5 μl per minute ($P < .001$) (Table 1). When toxin was added to bladder suspensions which had been allowed to respire for 45 minutes, oxygen consumption observed at 60 to 90 minutes was increased by 15.4 μl over that observed at 15 to 45 minutes. Oxygen consumption by untreated tissue declined by 4.6 μl during the same interval ($P < .001$).

The inhibition of both the short-circuit current and potential difference of the isolated toad bladder by staphylococcal α-toxin suggests that an alteration in epithelial ion movement has occurred. This does not appear to be secondary to inhibition of oxygen consumption since this was stimulated by the toxin. Active sodium transport has been shown to be directly related to SCC across the isolated bladder in a variety of circumstances (*6*). Whether the inhibition of SCC and PD by this toxin reflects a specific effect on active transport mechanisms, a destructive action on cell membranes, or is mediated by other metabolic or structural changes remains to be determined.

JAMES J. RAHAL, JR., MARTIN E. PLAUT
HERMAN ROSEN, LOUIS WEINSTEIN
Infectious Disease Service and Renal Service, New England Medical Center Hospitals, and *Tufts University School of Medicine, Boston, Massachusetts 02111*

References and Notes

1. M. A. Madoff and L. Weinstein, *J. Bacteriol.* **83**, 914 (1962).
2. A. W. Bernheimer and L. L. Schwartz, *J. Gen. Microbiol.* **30**, 455 (1963); K. Goshi, L. E. Cluff, P. S. Norman, *Bull. Johns Hopkins Hosp.* **112**, 15 (1963); I. Lominski and J. P. Arbuthnott, *J. Pathol. Bacteriol.* **86**, 258 (1963); A. W. Jackson, *Can. J. Biochem.* **41**, 219 (1963); S. Kumar, K. I. Loken, A. J. Kenyon, R. K. Lindorfer, *J. Exp. Med.* **115**, 1107 (1962).
3. L. Z. Cooper, M. A. Madoff, L. Weinstein, *J. Bacteriol.* **87**, 136 (1964); M. A. Madoff, L. Z. Cooper, L. Weinstein, *ibid.*, p. 145.
4. M. S. Artenstein, M. A. Madoff, L. Weinstein, *Yale J. Biol. Med.* **35**, 373 (1963).
5. A. W. Bernheimer and L. L. Schwartz, *J. Pathol. Bacteriol.* **89**, 209 (1965); *J. Bacteriol.* **87**, 1100 (1964); *ibid.* **89**, 1387 (1965); A. W. Bernheimer and M. Davidson, *Science* **148**, 1229 (1965).
6. A. Leaf, J. Anderson, L. B. Page, *J. Clin. Invest.* **41**, 657 (1958).
7. Supported by USPHS grant AI-02564-06. J.J.R. and M.E.P. were supported by USPHS graduate training programs HE 5391 and 5TI-AI 276-03, respectively. We thank W. B. Schwartz, A. Leaf, and A. Essig for criticism and suggestions, and R. Workman for technical assistance.

18 November 1966

Mammalian X-Chromosomes: Change in Patterns of DNA Replication during Embryogenesis

Abstract. *One arm of both X-chromosomes in female eight-cell embryos of the golden hamster replicates late in the period of DNA synthesis. Mid-gestation embryos and adult fibroblasts show an increase in late-replicating DNA. Here, one X-chromosome is labeled in one arm; the other is labeled throughout.*

In somatic cells of female mammals, one of the two X-chromosomes has the following characteristics: it is relatively inactive, both genetically and metabolically (*1*); it shows positive heteropycnosis (*2*); and its DNA is synthesized later in the DNA-synthetic or S period than is most of the other chromosomal DNA (*3*). According to the Lyon hypothesis (*4*), this differentiation is a random event involving either X-chromosome during early embryogenesis. However, once a given chromosome has been inactivated it will remain so in all subsequent cell generations.

Interphase and prophase heteropycnosis of the X-chromosome does not appear until the time of implantation (*5*). Likewise, estimates of the time of inactivation from genetic studies do not implicate the first few cell divisions (*6*). Therefore, if the previously mentioned relationships hold, the patterns of DNA replication in the X-chromosomes during the early stages of cleavage should differ from those found after implantation.

In this report, comparisons are made of autoradiographs of metaphase cells from 3-day-old, eight-cell embryos before implantation, and 7½- to 9½-day-old embryos after implantation, and of fibroblasts from adult skin. All cells have been continuously exposed to tritiated thymidine during the latter part of the period of DNA-synthesis, a time when few chromosome segments become labeled. The golden or Syrian hamster (*Mesocricetus auratus*) was chosen for this study because it can be easily hand-bred, it produces litters of 9 to 13 young, and its early developmental patterns are known (*7*). Even with these advantages, over 300 females have been sacrificed in the course of this study.

The eight-cell embryos were flushed from the uterine horns of pregnant females and placed in a medium, used by Brinster (*8*), containing 8 μc of tritiated thymidine per milliliter, (New England Nuclear Corporation; specific activity > 10.6 c/mmole) for 7 to 8½ hours. Colchicine (0.2 mg/ml) was added for the last hour. Mid-gestation embryos and adult fibroblasts were cultured in enriched Eagle's medium containing 15 percent human and 5 percent calf serum and 2 μc of tritiated thymidine per milliliter for 5½ to 6¼ and 6 hours, respectively. Colchicine was present during the last ½ hour in the embryo cultures and during the last 4 hours in the fibroblast cultures. All cells were treated with hypotonic citrate, fixed, and squashed in 1.5 percent aceto-orcein either by direct pressure or by heating the slide; autoradiographs were then made (*9*).

The X-chromosome is the largest of the golden-hamster complement: it is nearly metacentric and is morphologically distinguishable (*10*). The patterns of DNA replication of the sex chromosomes have been described for cultures of 15-day fetal cells and for adult cells (*11*). These published reports are confirmed by our findings in 35 cells from adult skin fibroblasts from females, which have been labeled during the latter part of the S period. Here, one of the X-chromosomes is labeled along its whole length, the other being labeled in only one arm. Credence is given to this by the finding of one and a half heteropycnotic X-chromosomes in prophase cells (*12*) and one and one-half X-chromosomes in metaphase cells showing an alteration in morphology after special fixation and spreading (*13*). Our examination of 29 cells from 7½-day-old and of 29 cells from 9½-day-old female embryos showed that the replication pattern just described is clearly established by these times in development.

The patterns of replication in X-chromosomes reported for the Syrian hamster differ in some respects from those found in most mammalian species (*3, 14*). Usually, pronounced asynchrony is found in the times at which the two X-chromosomes replicate; one chromosome is labeled at the end of the S period, the other at an earlier time. As in other mammals, asynchronous replication of X-chromosome DNA is present in the Syrian hamster, but only in one arm. The other arm of both X-chromosomes is late replicating. The presence of this late-replicating DNA in both chromosomes may be related to their larger size (*12*), since other mammals with large X-chromosomes show replication pat-