The subcutaneous injection of cholera toxin into adult mice resulted in a sustained increase in cyclic AMP levels in mouse epidermis after a lag period of about 2 hr. An increase in ornithine decarboxylase activity occurred between 7 and 10 hr, which was maintained for at least 10 hr. The increase in decarboxylase activity was localized to the area of epidermis visually affected by cholera toxin and was unaffected by hypophysectomy, suggesting a direct effect of the toxin on the epidermal cells.

The subcutaneous injection of cholera toxin also led to an increase in cyclic AMP levels in newborn mouse skin. In contrast to adult mice, newborn mouse skin contained high basal activities of ornithine decarboxylase in both the epidermal and dermal fractions. The activity in both fractions was markedly decreased following cholera toxin injection. The ability of cholera toxin to induce both epidermal and dermal ornithine decarboxylase activity developed between 10 and 21 days after birth.

Treatment of mouse skin with the tumour promoter 12-0-tetradecanoylphorbol-13-acetate (TPA) leads to a rapid, transient induction of the enzyme ornithine decarboxylase in the epidermis [1]. Enzyme activity reaches a maximum 4-6 hr after TPA application and has returned to the control value after 12 hr. An understanding of the mechanism of ornithine decarboxylase induction has intrinsic interest as the enzyme is believed to be rate-limiting for polyamine biosynthesis in mammalian cells and has an extremely rapid turnover (t_{1/2} approx. 17 min in mouse epidermis [1]). More specifically, induction of the decarboxylase has been implicated in the process of 2-stage carcinogenesis in mouse skin. Thus the ability of compounds to induce epidermal ornithine decarboxylase activity correlates well with their tumour promoting activity, and it has been proposed that induction is required for promotion to occur (1, 2, 3).

Little progress has been made in elucidating the mechanism of ornithine decarboxylase induction in mouse epidermis by TPA. In a number of other mammalian systems adenosine 3',5'-monophosphate (cyclic AMP) has been implicated in the induction of the decarboxylase [4-7], and some experiments have been carried out to determine if this is also the case for promoter-treated epidermis [8]. Mufson et al [8] were unable to confirm a report [9] that TPA causes an early rise in epidermal cyclic AMP levels. In addition, injection of isoproterenol, which caused a rapid accumulation of cyclic AMP in the epidermis, did not induce ornithine decarboxylase activity. It was concluded that an early rise in cyclic AMP concentration is not involved in the induction of the decarboxylase by TPA.

In the present communication we provide evidence that elevated concentrations of cyclic AMP can lead to increased epidermal ornithine decarboxylase activity. Thus the subcutaneous injection of cholera toxin into adult mice, which caused a sustained increase in epidermal cyclic AMP concentration, resulted in a marked induction of decarboxylase activity in the epidermis.

**MATERIALS AND METHODS**

**Materials**

Swiss albino mice were used in all experiments; the animals were housed as described previously [10]. The dorsal skin of adult animals was shaved, and only those animals which did not show a regrowth of hair over 7 days were used for experimentation. Female mice were used in all experiments with adult animals.

Purified cholera enterotoxin was obtained from Schwarz-Mann, New York, and as a gift from Dr. C. E. Miller, National Institute of Health, Maryland. DL-[1-14C]Ornithine (59 mCi/m mole) and L-[4,5-3H]-leucine (55 Ci/m mole) were purchased from The Radiochemical Centre, Amer­ sham, England. Pyridoxal 5'-phosphate was obtained from Calbiochem (Melbourne, Australia).

**Methods**

_Treatment with cholera toxin:_ Cholera toxin was injected subcutaneously into the lower dorsal skin in a volume of 0.1 ml of phosphate-buffered saline; control animals were injected with 0.1 ml of phosphate-buffered saline.

_Preparation of extracts for assay of ornithine decarboxylase activity:_ In some experiments extracts were prepared from both the dermal and epidermal fractions of skin. The epidermis and dermis were separated by brief heat treatment [11]. Epidermis, dermis or whole skin from individual mice was homogenized with a glass-Teflon homogenizer in 1.2 ml of 50 mM Tris-HCl (pH 7.4) containing 0.1 mM pyridoxal 5'-phosphate and 0.1 mM EDTA. Homogenates were filtered through 2 layers of cheesecloth and centrifuged at 100 000 xg (20 min; 4°C). The supernatants were assayed for ornithine decarboxylase activity [12].

_Hypophysectomy:_ Animals were hypophysectomized as described before [13], and allowed access to 5% (w/v) glucose in 0.9% NaCl for 1 week before experimentation. At the end of each experiment, the successful removal of the pituitary gland was confirmed by dissection.

_Inflammatory index:_ The index was determined as described [14] on pieces of skin of area 201 mm² from individual mice. The inflammatory index is defined as:

\[
\text{Inflammatory index} = \frac{\text{Wet weight} - \text{dry weight}}{\text{dry weight}} \times 100
\]

**Other methods:** Protein [15] and cyclic AMP [16] were determined as previously described.

**RESULTS**

_Effect of Cholera Toxin on Ornithine Decarboxylase Activity and Cyclic AMP Levels in Adult Mouse Epidermis_

As shown in Fig 1, the subcutaneous injection of 10 μg of cholera toxin led to a sustained increase in epidermal cyclic AMP levels after a lag period of about 2 hr. In these experiments, the injection of cholera toxin resulted in a localized swelling extending for a radius of about 1 cm around the injection site. Samples of skin were only taken from this visually...
affected area for the determination of cyclic AMP levels and ornithine decarboxylase activities.

As also shown in Fig 1, cholera toxin induced an increase in epidermal ornithine decarboxylase activity. The increase began between 7 and 10 hr, and was maintained for at least 10 hr.

The increase in decarboxylase activity was restricted to the area of skin showing visual swelling (Table I). This localized effect implies that cholera toxin has a direct effect on the epidermal cells, and argues against the release of some indirect humoral factor. The experiment was necessary as it has previously been concluded that the induction of hepatic ornithine decarboxylase activity after the injection of dibutyryl cyclic AMP into rats may be an indirect consequence of the release of pituitary hormones [17]. The increase in liver decarboxylase activity (Table I) indicates that subcutaneously injected cholera toxin does have effects on tissues other than skin. However, these effects do not induce a generalized increase in epidermal ornithine decarboxylase activity. It was also demonstrated that the cholera toxin-induced increase in epidermal cyclic AMP levels was restricted to the epidermis from visually swollen areas of skin (data not shown).

The injection of cholera toxin caused a marked localized edema which could be quantified by measuring the inflammatory index (Table II, see reference 14). It was possible that the changes in ornithine decarboxylase activity were due to altered capillary permeability allowing the “leakage” of some stimulatory factor into the environment of the epidermal cells. For example, 2 pituitary hormones, ACTH and growth hormone, have been shown to induce ornithine decarboxylase activity [18]. However, prior hypophysectomy did not modify the response of the epidermal decarboxylase to cholera toxin (Table III). In addition, low doses of cholera toxin (2 µg/animal) enhanced the inflammatory index (Table II) but did not elevate epidermal cyclic AMP levels or ornithine decarboxylase activity (data not shown).

**Effect of Cholera Toxin on Ornithine Decarboxylase Activity in Newborn Mouse Skin**

The subcutaneous injection of 5 µg of cholera toxin into newborn (3 days old) mice resulted in a marked increase in skin cyclic AMP levels. Thus the cyclic AMP levels 5 hr after injection of cholera toxin was 0.57 ± 0.04 pmoles/µg DNA.

TABLE I. Localized induction of epidermal ornithine decarboxylase activity by the subcutaneous injection of cholera toxin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ornithine decarboxylase activity (pmoles/min/µg protein)</th>
<th>Uninvolved skin</th>
<th>Involved skin</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-buffered</td>
<td>2.54 ± 0.55</td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>1.17 ± 0.39</td>
<td>11.47 ± 1.46</td>
<td>4.66 ± 1.37</td>
<td></td>
</tr>
</tbody>
</table>

* Adult mice were injected subcutaneously with 10 µg cholera toxin. After 16 hr extracts were prepared from epidermis and liver and assayed for ornithine decarboxylase activity as described in “Materials and Methods.” Epidermis was prepared from the swollen area of skin around the injection site (involved skin), and from areas of dorsal skin visually free of swelling (uninvolved skin). Values are the mean ± SE of determinations done on 5 separate animals.

TABLE II. Effect of cholera toxin on the inflammatory index of adult mouse skin

<table>
<thead>
<tr>
<th>Time after treatment (hr)</th>
<th>0.9% NaCl</th>
<th>Cholera toxin (10 µg)</th>
<th>0.9% NaCl</th>
<th>Cholera toxin (2 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.99 ± 0.17</td>
<td>2.86 ± 0.38</td>
<td>2.50 ± 0.26</td>
<td>2.72 ± 0.50</td>
</tr>
<tr>
<td>5</td>
<td>1.55 ± 0.17</td>
<td>3.65 ± 0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1.16 ± 0.15</td>
<td>2.00 ± 0.29</td>
<td>1.58 ± 0.39</td>
<td>3.79 ± 0.57</td>
</tr>
<tr>
<td>8</td>
<td>1.36 ± 0.17</td>
<td>3.94 ± 0.32</td>
<td>1.38 ± 0.23</td>
<td>4.57 ± 0.22</td>
</tr>
<tr>
<td>24</td>
<td>1.10 ± 0.24</td>
<td>10.92 ± 1.45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The index was measured at varying times after the subcutaneous injection of cholera toxin or 0.9% NaCl into adult mice as described in the “Materials and Methods.” Each value is the mean ± SE of determinations carried out on 4 animals.

TABLE III. Effect of cholera toxin on the epidermal ornithine decarboxylase activity of hypophysectomized mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ornithine decarboxylase activity (pmoles/min/µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operated</td>
<td>1.36 ± 0.79</td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>7.13 ± 2.27</td>
</tr>
<tr>
<td>Hypophysectomized</td>
<td></td>
</tr>
<tr>
<td>0.9% NaCl</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>5.07 ± 0.60</td>
</tr>
</tbody>
</table>

* Epidermal extracts were prepared 16 hr after the subcutaneous injection of 0.9% NaCl or 10 µg of cholera toxin into adult mice as described in the Materials and Methods section. Each value is the mean ± S.E. of determinations carried out on 4 separate animals.
compared with 0.12 ± 0.01 pmoles/μg DNA in control animals. However in contrast to the results obtained with adult mice, the ornithine decarboxylase activity in newborn mouse skin was decreased by the injection of cholera toxin. The results obtained with extracts prepared from whole mouse skin are shown in Fig 2. After an initial transient increase in ornithine decarboxylase activity (reproducible in 3 experiments), there was a sustained decrease in enzyme activity. The decrease was not due to the presence of an inhibitor of the decarboxylase in extracts from cholera toxin-treated skin. Thus assays carried out with a mixture of extracts prepared from control and cholera toxin-treated animals showed strictly additive ornithine decarboxylase activities (data not shown).

Histological examination indicated that doses of cholera toxin up to 10 μg in adult mice did not cause obvious damage to the epidermal cells. The cholera toxin-induced decrease in ornithine decarboxylase activity was observed in extracts from both the epidermis and dermis of newborn mice (Fig 3). In addition, the basal activity of the decarboxylase decreased with increasing age of the animal. The decrease was most pronounced in the dermis. Histological examination demonstrated that the decreased basal activity could not be accounted for by a decrease in the cellularity of the dermal layer. As indicated in Fig 3, the ability of cholera toxin to induce both epidermal and dermal ornithine decarboxylase activity developed between 10 and 21 days after birth.

The Specificity of Cholera Toxin Effects

Some experiments were carried out to determine whether the effects of cholera toxin on mouse skin were specific to ornithine decarboxylase or could be a consequence of generalized changes in the rates of protein synthesis. As discussed previously [17, 19], generalized changes would be expected to lead to rapid fluctuations in the activity of rapidly turning over enzymes such as ornithine decarboxylase. As shown in Table IV, the injection of cholera toxin did not lead to an increased rate of leucine incorporation into acid-soluble material. In newborn mice, chole-

Fig 2. Effect of cholera toxin on ornithine decarboxylase activity in newborn mouse skin. Determinations were done at various times after the subcutaneous injection of phosphate-buffered saline (△) or 5 μg cholera toxin (●) into 3 days old mice. Each point is the mean ± SE of determinations on the whole skin from 5 separate animals.

FIG 2. Effect of cholera toxin on ornithine decarboxylase activity in newborn mouse skin. Determinations were done at various times after the subcutaneous injection of phosphate-buffered saline (△) or 5 μg cholera toxin (●) into 3 days old mice. Each point is the mean ± SE of determinations on the whole skin from 5 separate animals.

Fig 3. Effect of cholera toxin on ornithine decarboxylase activity in the epidermis and dermis of mice of different ages. Determinations were done 16 hr after the subcutaneous injection of 5 μg cholera toxin or phosphate-buffered saline. Epidermal and dermal extracts were prepared after cholera toxin (● and △, respectively) or phosphate-buffered saline (○ and ▲, respectively) injection. Each point is the mean ± SE of determinations on 5 separate animals.

Fig 3. Effect of cholera toxin on ornithine decarboxylase activity in the epidermis and dermis of mice of different ages. Determinations were done 16 hr after the subcutaneous injection of 5 μg cholera toxin or phosphate-buffered saline. Epidermal and dermal extracts were prepared after cholera toxin (● and △, respectively) or phosphate-buffered saline (○ and ▲, respectively) injection. Each point is the mean ± SE of determinations on 5 separate animals.

The mechanism for the various effects induced by cholera toxin in mouse skin is unclear. In adult mice, an elevation of epidermal ornithine decarboxylase activity was observed and a number of observations suggest that the enhanced activity is a consequence of the direct interaction of cholera toxin with the epidermal cells. Thus the elevation was localized to the epidermis immediately around the injection site, was unaffected by hypophysectomy and could be uncoupled from the marked inflammatory response induced by the toxin. However, unequivocal establishment of this point will require work with cultured epidermal cells. If a direct interaction with epidermal cells does occur, the induction is presumably mediated via cyclic AMP which is thought to be solely responsible for cholera toxin effects in mammalian cells [20].

DISCUSSION

Interpretation of the effects of cholera toxin on skin ornithine
The activity of rapidly synthesized or inactivated. For example, as discussed by other will respond sharply to either increases or decreases in the overall rates of protein synthesis. Thus the data obtained in the changes could involve effects on the rates of either enzyme activity, it cannot necessarily be assumed that cyclic AMP synthesis occurring after isoproterenol stimulation is evenly distributed throughout the cell [22].

TABLE IV. Effect of choleragen on the incorporation of [3H]leucine into acid-insoluble epidermal material

<table>
<thead>
<tr>
<th>Treatment</th>
<th>6 hr</th>
<th>16 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-buffered saline</td>
<td>236 ± 34</td>
<td>148 ± 14</td>
</tr>
<tr>
<td>Cholera toxin (adult)</td>
<td>148 ± 15</td>
<td>139 ± 9</td>
</tr>
<tr>
<td>Cholera toxin (newborn)</td>
<td>1989 ± 270</td>
<td>1390 ± 207</td>
</tr>
<tr>
<td>Cholera toxin (newborn)</td>
<td>837 ± 130</td>
<td>525 ± 50</td>
</tr>
</tbody>
</table>

* Adult and newborn (4 days old) mice were injected subcutaneously with choleragen (10 and 5 μg respectively) or with phosphate-buffered saline. After 6 and 16 hr, animals were injected with [3H]leucine and the incorporation into acid-insoluble material was determined after a further 30 min (see "Materials and Methods"). Values are the mean ± SE of determinations done on 5 separate animals.

REFERENCES