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THE DEVELOPMENT OF TOLERANCE AND CROSS-TOLERANCE TO METHONIUM COMPOUNDS IN LABORATORY ANIMALS

BY

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Since the discovery of the ganglion-blocking action of hexamethonium and pentamethonium salts by Paton and Zaimis (1949), and a study of their clinical pharmacology by Arnold and Rosenheim (1949), these drugs have been used extensively in clinical practice, particularly in the treatment of hypertension (Restall and Smirk, 1950; Turner, 1950; Saville, 1950; Campbell and Robertson, 1950; Smirk and Alstad, 1951; Freis, 1951; McMichael, 1952; Morrison, 1953).

Smirk (1950) and Smirk and Alstad (1951) drew attention to the development of tolerance to pentamethonium and hexamethonium with their continued administration. In later communications Smirk (1952, 1953) reported cross-tolerance between these substances and between certain of their chemical relatives. As the development of such tolerance is a distinct disadvantage clinically —in that it is not possible to arrive at a stable dose level for several months, and hence necessitates continuous and close observation of the patient—the phenomenon has been studied experimentally. Some of the results have been mentioned in a preliminary communication (Mohanty, 1954).

METHODS

Toxicity Studies.—White mice aged 6–8 weeks and of homogeneous stock were used. The animals were divided into two groups by random sampling. One group received daily subcutaneous injections of hexamethonium bromide (C6) for a period of 10 days, the daily dose being increased gradually over this period. The other group received daily subcutaneous injections of 0.9% NaCl in equal volume. Next, 48 hr. after the last injection for the 10-day period, the mice of each group were subdivided by random sampling into 4 dose groups. Graded doses of C6 were given to mice of each of the 8 groups, suitable doses having been found previously by pilot experiments. The dose was proportional to the body weight and was given subcutaneously. Death or survival was recorded at the end of 6 hr.

Measurement of Mydriatic Effects.—The method used was essentially the same as that described by Ing, Dawes, and Wajda (1945). White mice aged 6–8 weeks and of homogeneous strain were kept under standard illumination for 30 min. before the measurement of the initial pupillary size and the injection of the drug. Pupillary diameters were again measured, 10 to 20 min. after the injection, by a microscope with an arbitrary scale in the eye-piece. In the first set of experiments, the dose-response curves were determined for the following compounds : C6, M&B 2050 [pentamethylene 1:5-bis-*N*-(*N*-methyl-pyrrolidinium) di-iodide], M&B 1863 [hexamethylene 1:6-bis-(ethyldimethylammonium) bromide], M&B 1950 [*N*, *N'*-tetramethyl-β-*p*-aminophenylethylamine) di-iodide], TEAB (tetraethylammonium bromide), SC-2159 and SC-1718. The last two compounds were kindly supplied by G. D. Searle & Co., and the remaining compounds by Messrs. May & Baker.

Fresh mice were assigned by random sampling into groups of 15 animals, one such group being used to test each compound. A test dose lying in the middle portion of the log dose-response curve for that substance was selected. Daily subcutaneous injections were given for 6–8 days. Changes in pupillary size were measured after each injection. A "cross-over" test was performed at the end of this period. Cross-tolerance was investigated mostly with C6 as reference standard drug.

Experiments with Isolated Intestine.—Trendelenburg's (1917) preparation was employed. Peristalsis was induced by raising the intraluminal pressure by 2.5 cm. of water. Intestinal strips from normal guinea-pigs and rabbits were used to determine the doses of the various methonium compounds which would produce paralysis of the peristaltic movement. To study tolerance and cross-tolerance, similar preparations were made with intestinal strips from guinea-pigs and rabbits which had been made tolerant by gradually increased daily subcutaneous injections of C6 or

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TEAB. Animals were normally sacrificed 48–72 hr. after the last injection for the 14-day period; but preparations from 3 tolerant guinea-pigs were examined at 1 week, 2 weeks, and 2 months (respectively) after the final injection in order to discover whether tolerance persisted. The preparations were taken from the lower part of the ileum and suspended in Ringer-Tyrode solution at 35° C. In all, 77 experiments were performed with this preparation.

Experiments with the Cat's Superior Cervical Ganglion.—Animals were anaesthetized with intraperitoneal injections of pentobarbitone-urethane. The nictitating membrane was prepared for recording in the usual way (Burn, 1952). Usually the experiment was elaborated by performing such operations as the following :

(1) It was often desirable to mount both nictitating membranes for simultaneous recording of their contractions, in order to demonstrate differences between the effects of C6 on a treated ganglion and on a control.

(2) Many experiments were performed with a vascularly isolated perfused ganglion preparation. In such experiments the superior cervical ganglion was dissected out and isolated from the rest of the body but for its nervous and vascular connexions; then the homolateral carotid artery was cannulated and perfused at a constant rate with a blood substitute. A rotary pump—the F.31 model of Messrs. C. F. Palmer (London), Ltd.—driven at slow speed was used for this purpose. Perfusion pressure and the contractions of the nictitating membrane were recorded. The perfusion medium was that described by Gallagher (1954). It was made by suspending washed sheep or human red cells in Krebs-Henseleit solution to which had been added 6% dextran and 0.1% glucose. The *p*H of the solution was kept at 7.2–7.4. The dose of C6 injected into the perfusion cannula was calculated to give a concentration in the perfusion fluid as great as, or greater than, that which would be given by the intravenous test dose. The injection was given at the height of contraction of the nictitating membrane. In most experiments the intravenous dose of C6 was 5 mg./kg. of the bromide and the intra-arterial dose 0.1–0.5 mg. Blood pressure was recorded from a carotid artery. Intravenous injections were made into a femoral vein.

(3) In some experiments, the ganglion was perfused for a time with heparinized blood obtained from untreated cats or patients in place of the simple blood substitute. In others, the perfusion medium was changed for 15–60 min. from the simple blood substitute to blood or plasma taken from cats or patients treated previously with C6.

The patients were some of the hypertensives under treatment with C6 at Dunedin Public Hospital. Details of their treatment have been published elsewhere (Smirk, 1953). All of those from whom blood was taken had become tolerant to C6 and were receiving at least 50 mg. of the bromide daily by subcutaneous injection, but no C6 was administered during the 24 hr. period before the removal of the blood. Immediately after collection the blood was heparinized. It was then centrifuged and the plasma collected. This plasma was mixed with the plain blood substitute in the proportion of 1:3.

The "C6-tolerant cat's blood" was obtained from cats that had been given daily subcutaneous injections of C6 in gradually increasing doses (5–20 mg./kg.) for a period of 14 days. Two to three days after the last injection they were anaesthetized, their carotid arteries were cannulated with polythene tubing, and the blood from them was collected in a paraffined beaker. Heparin was added to it.

Blood from normal cats, and blood and plasma from normal patients, were obtained in a similar fashion for control experiments.

(4) The procedures for applying C6 locally to a superior cervical ganglion were : (*a*) Both ganglia were exposed and isolated but for their nervous and vascular connexions. One ganglion was then surrounded by pledgets of cotton-wool soaked in 0.9% NaCl to which C6 (1%) had been added. The other ganglion was treated similarly, but with 0.9% NaCl alone. During the next 24 hr. C6 was applied continuously to the one ganglion and the plain physiological saline to the other. Lastly, both ganglia were washed with Ringer-Locke solution for some 30–60 min. before the pre-ganglionic nerves were again stimulated and the effects of an intravenous injection of C6 given at the height of contraction of the nictitating membrane recorded. (*b*) Alternatively, one ganglion was perfused for 1 hr. with blood substitute containing 5 μg./ml. of C6 and for a further 30–60 min. with the simple blood substitute before the test doses of C6 were repeated.

Results

Acute Toxicity of Hexamethonium for Normal and for C6-treated Mice

In both the C6-treated and untreated mice the test injection of C6 was followed within a few minutes by asthenia and flaccidity, due apparently to loss of muscular tone. Respiration became shallow and rapid, and the pupils widely dilated. Death occurred as the result of respiratory paralysis and was preceded in some mice by a short period of convulsions, probably due to asphyxia.

The results given in Table I were analysed graphically by the method of Litchfield and Fertig (1941). The LD50 for the untreated mice was found to be 148.0 mg./kg. with 95% fiducial limits of 144.3 and 151.6 mg./kg.; the LD50 for the C6-treated mice was 160.0 mg./kg. with 95% fiducial limits of 147.2 and 173.9 mg./kg. The difference between these LD50's is therefore not significant at the 95% level. The observed difference in slope of the regression lines was also not significant—for the untreated mice "b" was 23.6 with S.E. of 8.81 and for the treated mice it was 8.20 with S.E. of 5.65 (significance of difference,

TABLE I

ACUTE TOXICITY OF HEXAMETHONIUM IN NORMAL AND IN HEXAMETHONIUM-TREATED MICE

	Normal Mice				Tolerant Mice			
Dose of C6 (mg./kg.) ..	140	145	150	155	140	150	160	170
Log. dose	2·146	2·162	2·176	2·190	2·146	2·176	2·204	2·230
Mortality	9/30	12/30	17/30	21/30	7/20	8/20	10/20	12/20
% mortality ..	30	40	56·6	70	35	40	50	60
Probits	4·48	4·75	5·17	5·52	4·61	4·75	5·00	5·25

P=0.14). It therefore seems that little tolerance develops to the lethal action (or actions) of hexamethonium.

Tolerance and Cross-tolerance to the Mydriatic Action of Methonium Compounds in Mice

The dose-response curves obtained with various onium compounds are illustrated in Fig. 1. They are non-linear, with the exception of the dose-response curve for SC-1718. The linearity of the dose-response curve was not improved by plotting the dose against the increase in pupil diameter instead of against the final diameter (Fig. 1). Four separate determinations of the dose-response curve for C6 indicated that results were closely reproducible. The dose-response curves for all the other drugs except TEAB are almost parallel to that for C6 with the dose ranges tried.

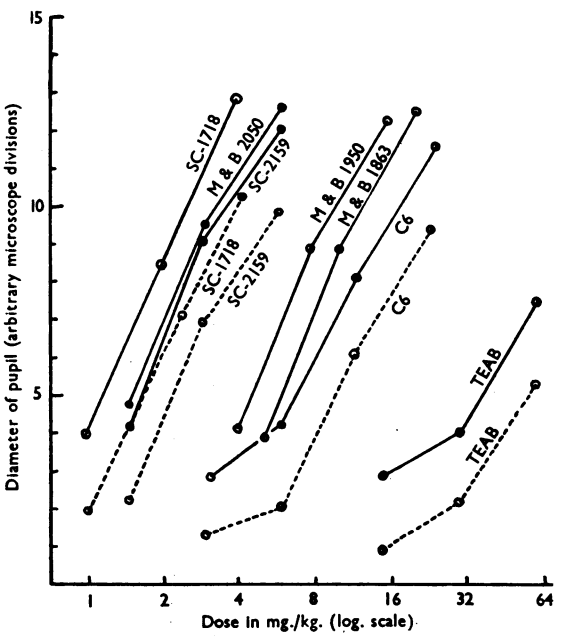

FIG. 1.—Log dose-response curves for the mydriatic action of various methonium compounds in mice. A broken line indicates that allowance has been made for the initial size of the pupil. See "Methods" for structures of drugs.

Tolerance was observed with all the compounds except SC-1718 (Figs. 2 and 3). The greatest degree was observed with C6. With the remaining drugs the degree of tolerance under comparable conditions decreased in the order: M&B 1950, SC-2159, M&B 1863, M&B 2050, TEAB (Fig. 3). Some tolerance could be detected after the first injection and, with continued daily injections, tolerance developed progressively until the completion of the experiment. Cross-tolerance between C6 and M&B 1950, M&B 2050, M&B 1863, and SC-2159 was observed. M&B 1863 gave rise to most tolerance to C6, followed (in descending order of potency) by SC-2159, M&B 2050, and M&B 1950. Mice first treated with C6 showed most tolerance to TEAB, followed by M&B 1863, M&B 2050, SC-2159, and M&B 1950. There was no cross-tolerance between SC-1718 and C6. Although mice treated with C6 developed a considerable degree of tolerance to TEAB, mice treated with daily injections of TEAB did not develop tolerance to C6.

Tolerance to the Action of Methonium Compounds on Isolated Intestinal Preparations

The smallest dose which would abolish the contractions of the circular muscle of isolated strips of ileum taken from normal guinea-pigs and rabbits was determined for various drugs. For a 100 ml. organ-bath, the doses were as follows: C6 (0.3–2.0 mg.), M&B 2050 (0.2–0.5 mg.), M&B 1863 (0.3–2.0 mg.), M&B 1950 (0.5–2.0 mg.), M&B 2024B (0.5–2.0 mg.), TEAB (2–5 mg.), and TMAB (4–6 mg.). TMAB is tetramethylammonium bromide, and M&B 2024B is hexamethylene 1:6-bis-*N*-(*N*-methyl-pyrrolidinium) dibromide. A typical result is illustrated in Fig. 4a. This shows that, although a small dose of C6 is sufficient to abolish the contractions of circular muscle, a period of washing is followed by complete recovery.

By contrast, intestinal strips obtained from animals pre-treated with C6 consistently showed a very high degree of tolerance to C6 (Fig. 4b). The pre-treatment consisted in giving daily subcutaneous injections increased gradually from 20 mg. to 50 mg. for rabbits and from 5 mg. to 15 mg. for guinea-pigs over a 2-week period. Under these conditions, such high concentrations of C6 as 100 mg./100 ml. produced neither abolition of the contractions of the circular muscle nor the appearance of any latent period. Furthermore, there was a very high degree of tolerance to M&B 1863, M&B

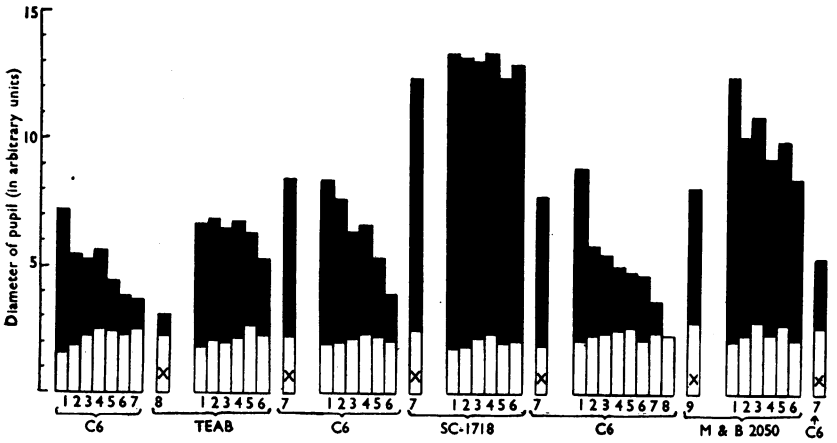

FIG. 2.—Histograms showing the progressive development of tolerance and cross-tolerance to C6 and related drugs. The numbers under each column refer to the day of injection. An X shows the result of a cross-over test, e.g., the effect of the standard dose of TEAB on mice already treated with C6. The whole column gives the mean diameter of the pupil after the injection of the drug (15 mice). The blank part gives the mean initial diameter and the solid part the mean increase in pupil diameter. These results are representative.

1950, M&B 2050 and M&B 2024B. Concentrations of the order of 100 mg./100 ml. of M&B 1950 or M&B 2050 were unable to prevent the contractions of the circular muscle obtained from C6-treated animals. Pre-treatment with C6 also established a high degree of tolerance to TEAB and a considerable degree of tolerance to TMAB. Thus with TMAB a dose of 30 mg. had no effect, though a 60 mg. dose in the 100 ml. bath abolished the contractions (compared with a 4–6 mg. dose for an intestinal strip from an untreated animal). Yet intestinal preparations taken from TEAB-treated animals showed no cross-tolerance to C6, the dose of C6 necessary to abolish the contractions in such preparations being of the same order as for untreated animals (about 2 mg./100 ml.). These preparations, however, exhibited a considerable degree of tolerance to TEAB. It should be added that small doses of TEAB produced immediate contraction of the longitudinal muscle of the TEAB-tolerant ileum, spontaneous contractions of this muscle not normally being observed with preparations from guinea-pigs. Subsequent elevation of the intraluminal pressure gave rise to considerable spasm of the circular muscle, but the addition of further quantities of TEAB (up to 30–45 mg./ml.) was followed by paralysis of the circular muscle.

In a further series of experiments, intestinal strips obtained from untreated guinea-pigs and

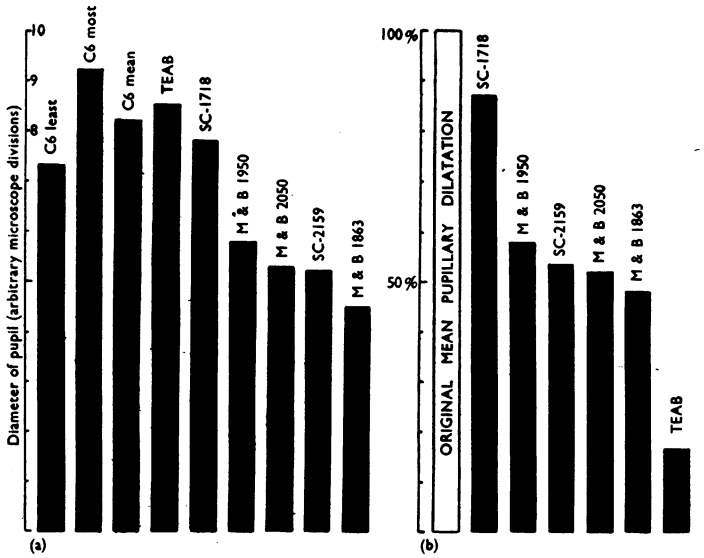

FIG. 3.—Histograms showing the degree of cross-tolerance obtained with the repeated daily injection of C6 and related compounds. The cross-over was performed after one of the pair of drugs had been injected daily for 6–8 days. Each column gives the mean effect for 15 mice. (*a*) The first three columns represent the mean pupillary dilatation in separate experiments with C6. The remaining columns show the mean pupillary dilatation following the test dose of C6 in groups of mice previously treated with the methonium compound named above each column. (*b*) The drugs named above each column were given after C6. The height of each column gives the degree of cross-tolerance as a percentage calculated by comparing the mean effect of the test dose after C6 with its effect in untreated mice.

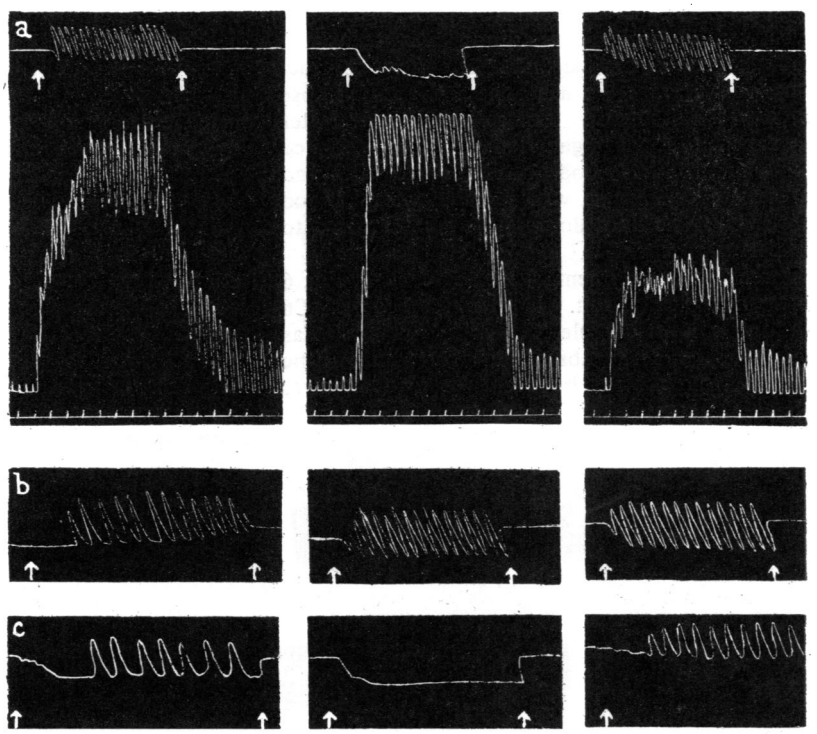

FIG. 4.—Trendelenburg preparations of rabbit ileum. Between the arrows, the intraluminal pressure was raised by 2.5 cm. of water. The effect of this on the circular muscle is shown on all records and the effect on the longitudinal muscle on the top set of records. (*a*) The first record shows a control contraction, the second the abolition of the reflex by hexamethonium bromide (2 mg./100 ml. organ-bath), and the third the restoration of the reflex after washing. (*b*) The ileal strip used in this experiment was taken from a C6-tolerant rabbit. The first record shows a control contraction. The second record shows a contraction after 5 mg. C6, and the third after 75 mg. C6 had been added to the bath. (*c*) Normal rabbit's ileum pre-treated *in vitro* for 3 hr. with 1 μg./ml. of C6 in Ringer-Tyrode. The first record shows a control contraction after washing, the second the effect of C6 (1 mg./100 ml.), and the third the effect obtained after the C6 had been washed out of the organ-bath.

rabbits were kept in Ringer-Tyrode solution containing C6 (1 mg./ml.) at room temperature for 2–4 hr. before being used for Trendelenburg preparations. After repeated washing of the strip, the dose of C6, or of another methonium compound, needed to abolish the peristaltic reflex was determined and compared with that required for untreated intestinal strips. A typical result is shown in Fig. 4c. Here a 1 mg. dose of C6 in the 100 ml. bath was sufficient to abolish the reflex contractions of a strip even after treatment *in vitro* for 3 hr. with C6-containing Ringer-Tyrode solution. Tolerance does not seem to have developed. Related compounds, tested on other preparations that had been treated *in vitro* with C6, gave similar results; the doses of these methonium compounds which abolished the circular muscle contractions of the treated strips were similar to the doses which abolished the circular muscle contractions of the untreated strips.

Tolerance to the Action of Methonium Compounds on the Superior Cervical Ganglion of the Cat

The development of tolerance after a single intravenous dose of C6 (5 mg./kg.) was studied in 6 cats. The drug was injected while the nictitating membrane was being kept contracted by stimulating the peripheral end of the pre-ganglionic cervical sympathetic nerve with spiked electrical shocks applied at the rate of 10/sec. When a second, equal dose of C6 was injected several hours later, it produced less blocking of the superior cervical ganglion (as judged from the contractions of the nictitating membrane) and a smaller fall of blood pressure. Such experiments indicate that in *intact* cats tolerance to the ganglion-blocking action of C6 develops even after a single intravenous dose. Tolerance could not be demonstrated within 2 hr. of an injection, but it was demonstrable within 3–4 hr.; after 24 hr. it was well marked (Fig. 5).

Vascularly Isolated Ganglion Preparations.—The first part of the next series of experiments was as above: after an initial control contraction had

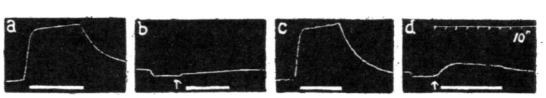

FIG. 5.—Cat. Pentobarbitone-urethane anaesthesia. Response of nictitating membrane to pre-ganglionic stimulation. The white line marks the period of stimulation. An arrow indicates the intravenous injection of C6 (5 mg./kg.). (*a*) Control contraction. (*b*) Block produced by first dose of C6. (*c*) Control contraction 24 hr. later. (*d*) Partial ganglionic blockade produced by second dose of C6. The smaller effect of C6 indicates the development of tolerance.

been recorded, an intravenous dose of C6 was given while another contraction was being recorded ; then, 24 hr. later, another control contraction and the effect of a second intravenous dose of C6 were recorded. Next, 3–4 hr. later, after the superior cervical ganglion had been isolated from the rest of the body but for its vascular and nervous connexions, it was perfused with a blood substitute. The third dose of C6 was injected into the perfusion cannula.

Three such experiments were performed ; Fig. 6 shows a typical result. The initial dose of C6 produced almost complete ganglionic blockade, as indicated by the near absence of contraction of the nictitating membrane ; but there was recovery after 24 hr. and the second dose did not produce such powerful blocking. The intra-arterial injection of C6 3–4 hr. later produced very little ganglionic blockade. Therefore, provided that the dose given intra-arterially (0.1–0.5 mg. of C6 according to the size of the animal) gives a concentration of C6 in the blood reaching the ganglion comparable to that obtained after the intravenous injections, it would seem that the tolerance to C6 which has developed in the intact animal remains, and can be exhibited in the isolated sympathetic ganglion preparation. Perfusion of the ganglion with a blood substitute—regarded as equivalent to washing the ganglion under pressure—did not remove the tolerance. In a further 2 experiments, the intra-arterial dose of C6 was increased to 1 mg. and was given immediately before the contraction of the nictitating membrane was elicited by stimulating the preganglionic nerve. The results of these 2 experiments were similar to those of the 3 preceding.

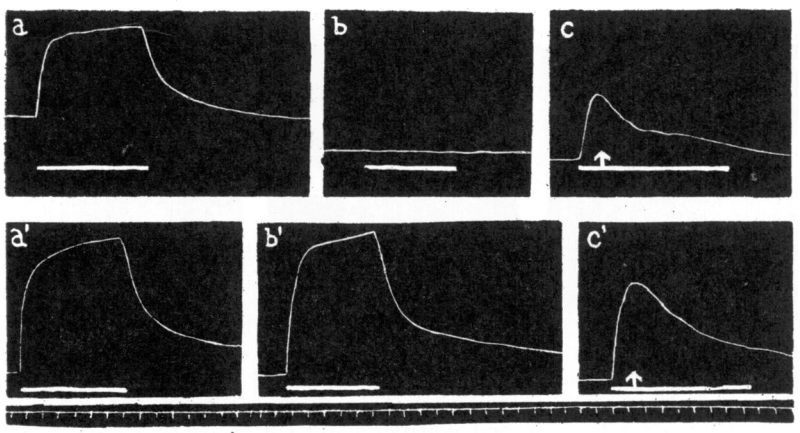

FIG. 6.—Cat. Pentobarbitone-urethane anaesthesia. The contractions of the right and left nictitating membranes (a,b,c and a',b',c' respectively) induced by stimulation of both pre-ganglionic cervical sympathetic nerves have been recorded simultaneously. The white lines mark the periods of stimulation, and the arrows the intravenous injection of C6 (5 mg./kg.). Time, 10 sec. (*a*, *a'*) Control contractions of nictitating membranes. (*b*) After 20 min. application of 1% C6 to the right superior cervical ganglion. (*b'*) After 20 min. application of isotonic saline to the left superior cervical ganglion. (*c*, *c'*) Records obtained simultaneously 24 hr. later. Meanwhile the animal had been continuously anaesthetized, the right ganglion treated with 1% C6 solution and the left ganglion with isotonic saline except during the last half-hour, during which period both ganglia were washed with normal saline.

Local Application of C6.—Fig. 6 illustrates the result of an experiment in which both ganglia were exposed and isolated but for their nervous and vascular connexions, after which one ganglion was treated for 24 hr. with C6 and the other with saline. The initial control contractions were unequal, although the conditions on both sides seemed to be identical as regards both the balancing and magnification of the levers and the state of the membranes and ganglia. In 4 other experiments performed under the same conditions unequal contractions of the two nictitating membranes were also obtained. No explanation for the inequality could be found. After an intravenous injection of C6, the percentage reduction in the size of the contractions was the same for the treated and untreated sides. Thus, there seemed to be no development of tolerance on the treated side although the ganglion had been surrounded by pledgets of cotton-wool soaked in 1% C6 solution for 24 hr. This suggests that the local application of C6 to ganglia is not sufficient to give rise to tolerance. It is possible, however, that these results might be explained by the unphysiological conditions of the experiment. It was therefore decided to perfuse the ganglion with the blood substitute previously used, since it was thought that perfusion under pressure might bring the C6 into more intimate contact with the ganglion.

The first set of experiments, performed on 3 cats, differed from the preceding set in that the ganglion on one side was treated with C6 by perfusing it for 1 hr. with blood substitute containing 5 μg./ml. of C6 and then for another 30–60 min. with the simple blood substitute. A subsequent test dose of C6 produced much the same response as the initial test dose, indicating that tolerance had not occurred. The same result was obtained in the following 6 experiments: 3 differed from the last

only in that heparinized blood from untreated patients was used in place of the blood substitute; in 1 heparinized blood from an untreated cat was used in place of the blood substitute; and in 2 the C6 was given by injecting 5 mg. of the bromide dissolved in 5 ml. of 0.9% NaCl slowly and continuously into the perfusion cannula over a period of 15 min. by means of an electrically driven syringe, the blood substitute being perfused at the same time. It was always found that while the C6 solution was being perfused the contractions of the nictitating membrane in response to preganglionic stimulation were completely abolished; but subsequent perfusion with the blood substitute alone or with heparinized blood that did not contain C6 was followed by complete recovery of the ganglion sensitivity to C6 and to other methonium compounds.

Attempted Reversal of Tolerance by Local Washing.—The general plan of the next series of experiments was (i) to make the whole animal tolerant to C6, and then (ii) to wash one superior cervical ganglion repeatedly to see whether this procedure could restore the sensitivity to C6 of the washed ganglion, even though the rest of the animal remained tolerant as shown by the reaction of the other superior cervical ganglion and nictitating membrane.

In 3 experiments, cats were made tolerant to C6 by giving two 5 mg./kg. doses, the first 24 hr., and the second 20 hr., before the washing. Next, the right superior cervical ganglion was dissected and isolated from the rest of the body except for its nervous and vascular connexions. Contractions of the nictitating membranes were recorded at this stage, as before and after the test doses of C6. Finally, the right ganglion was washed externally with saline for 30–60 min., a third test dose of C6 (5 mg./kg.) was given intravenously, and contractions of both nictitating membranes were recorded. With each cat it was found that both ganglia exhibited approximately equal tolerance to the test doses of C6; the sensitivity of the washed ganglion was reduced to about the same extent as that of the control. In 2 of these experiments the post-ganglionic nerve was stimulated after a further dose of C6. This produced as big a contraction as the control stimulation, indicating that the block had occurred at the ganglion.

As it seemed possible that washing the ganglion from the outside with saline might not be adequate to remove a tolerance-producing substance, a further 3 experiments were performed in which the right common carotid artery was cannulated and the corresponding ganglion was perfused at a pressure of 140–160 mm. Hg for 1 hr. with the plain blood substitute, after control measurements had been made as before. Here, too, tolerance was unaffected by the washing, for the injection into the perfusion cannula of a 1 mg. dose of C6—calculated to be equivalent to a body dose of at least 5 mg./kg.—produced no greater degree of ganglionic blockade than was observed with the control ganglion after an intravenous dose of 5 mg./kg. of C6.

Effects of Blood or Plasma from C6-tolerant Animals or Patients.—The evidence already given suggested that, although tolerance to C6 develops at autonomic ganglia and cannot be removed by washing, it cannot be produced by applying C6 directly to an isolated ganglion. Presumably the ganglion is affected by a process that occurs elsewhere in the body. It therefore appeared of interest to see if an isolated ganglion could be made tolerant to C6 by treating it with blood obtained from C6-tolerant animals, for tolerance could be explained by the elaboration in one part of the body of a substance which in one way or another neutralized the effect of C6.

Single vascularly isolated ganglion preparations were used for the remaining 11 experiments. They were perfused initially with the blood substitute. During this part of the experiment the effects of various test doses of C6 injected at the height of contraction of the nictitating membrane were recorded. At least 15 min. rest period was allowed between two successive recordings and the volume of all injections into the perfusion cannula was kept constant. Next, the perfusion medium was changed for 15–60 min. to the heparinized whole blood of cats treated previously with C6 (5 experiments) or to blood substitute containing plasma obtained from hypertensive patients under treatment with C6 (6 experiments). Lastly, the simple blood substitute was perfused again while C6 was injected at intervals in the test doses used previously.

The result of a typical experiment is shown in Fig. 7. During the first period of perfusion with the blood substitute no tolerance developed at the ganglion. After perfusion of the ganglion with the blood or plasma obtained from the C6-tolerant cats or patients, tolerance now developed at the ganglion. The second series of test doses of C6 produced much smaller effects than the control injections, even no effect on occasion. In addition, cross-tolerance to other methonium compounds developed. Evidently the blood of C6-tolerant patients or animals can confer on autonomic ganglia the property of tolerance to C6 and related

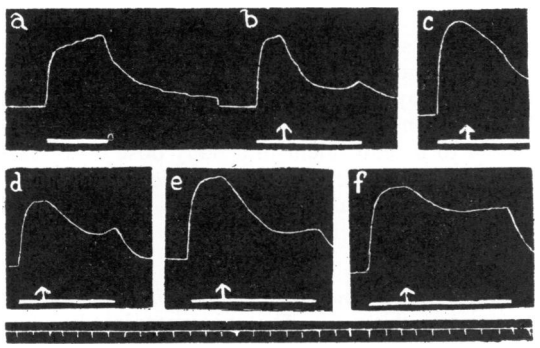

FIG. 7.—Cat. Pentobarbitone-urethane anaesthesia. Perfused superior cervical ganglion-nictitating membrane preparation. Contractions of nictitating membrane obtained by pre-ganglionic stimulation. The white lines show periods of stimulation, the arrows the injection of various C6 solutions into the perfusion cannula. Time, 10 sec. (*a*) Control contraction. (*b*) Effect of C6 (30 μg.) in 2 ml. of saline. (*c*) C6 (20 μg.) in 2 ml. of saline. (*d*) C6 (30 μg.) in 2 ml. of blood plasma of a C6-tolerant patient. (*e*) C6 (100 μg.) in 2 ml. of blood plasma of a C6-tolerant patient. (*f*) After 15 min. perfusion of the ganglion with blood substitute containing 1 part in 4 of plasma from a C6-tolerant patient. C6 (200 μg.) in 2 ml. of plasma injected at the arrow.

drugs. When such tolerance was produced, it it could not be removed by local washing or by perfusion under pressure for 1–2 hr.

DISCUSSION

The experiments on mice show that when hexamethonium (C6) is given repeatedly, considerable tolerance to its mydriatic action develops. However, all the actions of C6 are not affected to the same extent, for it has been found that the LD50 was not raised to a significant extent by the prior administration of substantial doses of C6. The signs shown by the dying animals suggest that C6 killed mainly—if not entirely—by a curariform action. Whatever the action of C6 that causes death in mice, little or no tolerance develops to it.

Tolerance has been demonstrated for four actions of C6, all of which depend on its ability to block autonomic ganglia—mydriasis, impairment of the peristaltic reflex, lowering of blood pressure, and reduction of the response of the nictitating membrane to stimulation of the cervical sympathetic chain. There seems little doubt when all four actions are considered that tolerance to them represents a change in the sensitivity of the ganglion rather than, say, a compensatory cardiovascular mechanism of the type suggested by Paton and Zaimis (1952) and Morrison (1953).

The rate of development of tolerance and cross-tolerance in the intact animal seems to be similar to that observed clinically (Smirk and Alstad, 1951 ; Smirk, 1952, 1953). Tolerance seems to develop slowly. It reaches a maximum in weeks rather than days and, when this point has been reached, the degree of tolerance remains more or less constant provided that the administration of the drug is continued. The development of tolerance is not only slow and progressive but appears to be preceded by a lag period. In the cat, tolerance could not be demonstrated until at least 3 hr. had elapsed after an intravenous dose of C6. Similarly, although tolerance could be demonstrated in mice after 24 hr., it was much more noticeable at the end of a week.

The impression was gained from animal experiments that the degree of development of tolerance is related to the size and frequency of the dose. So long as the dose is not sufficiently large to injure the animal, the development of tolerance could be hastened by increasing the size and frequency of the dose. This observation is consistent with the clinical observations of Freis (1951), who thought that the development of tolerance to C6 was delayed by giving injections at intervals of 12 hr. instead of more frequently. Similar phenomena have been noted by Smirk (personal communication). In animals as in man, tolerance and cross-tolerance to methonium compounds wear off after the treatment has been stopped for about two weeks.

The results of experiments performed with isolated ganglion preparations taken from tolerant animals indicate that tolerance and cross-tolerance can be demonstrated in preparations containing either parasympathetic ganglia (intestinal strips) or sympathetic ganglia (nictitating membrane preparation). The tolerance and cross-tolerance could not be reversed by washing the isolated preparation, even by washing under pressure as in the perfusion of an isolated superior cervical ganglion for periods of up to 2 hr.

By contrast, the local action of C6 applied to or perfused through isolated ganglion preparations did not give rise to any tolerance or cross-tolerance. This happened also when the superior cervical ganglion was perfused with homologous animal or human blood to which C6 was added after bleeding, indicating that the presence of normal blood does not play a part in the development of tolerance at the isolated ganglion. When, however, the ganglion was perfused with the blood of animals or patients previously treated with C6, tolerance rapidly developed and, as with the isolated ganglion preparations obtained from tolerant animals, it could not be removed by washing or by perfusion for periods of up to 2 hr.

It seems reasonable to conclude that in the intact animal, or in man, the development of tolerance and cross-tolerance is not a local reaction of autonomic ganglia to the presence of C6, but that

the administration of C6 to the whole animal is followed after an interval of a few hours by the appearance in the blood of a substance which reduces ganglionic sensitivity to C6 and related compounds. No similar substances seems to be present in the blood of untreated patients or animals. Evidence as to the site of formation of this substance will be presented in a later paper.

Summary

1. Tolerance to the ganglion-blocking action of hexamethonium and related substances follows their administration to intact laboratory animals. However, the LD50 of hexamethonium for mice was not raised significantly by the prior repeated administration of substantial doses of the drug. Death in mice from hexamethonium is attributed mainly to a curariform as distinct from a ganglion-blocking action.

2. Tolerance can be demonstrated in isolated ganglion preparations obtained from animals previously treated with hexamethonium.

3. The local application of hexamethonium to isolated ganglion preparations from untreated animals does not give rise to tolerance.

4. The application of blood from tolerant patients or animals, to isolated ganglion preparations from untreated animals, is followed by the development of tolerance at the ganglia.

References

Arnold, P., and Rosenheim, M. L. (1949). *Lancet*, **2**, 321.

Burn, J. H. (1952). *Practical Pharmacology*, 1st ed., p. 57. Oxford: Blackwell.

Campbell, A., and Robertson, E. (1950). *Brit. med. J.*, **2**, 804.

Freis, E. D. (1951). *Lancet*, **1**, 909.

Gallagher, D. J. A. (1954). *Brit. J. Pharmacol.*, **9**, 129.

Ing, H. R., Dawes, G. S., and Wajda, I. (1945). *J. Pharmacol.*, **85**, 85.

Litchfield, J. T., and Fertig, J. W. (1941). *Bull. Johns Hopk. Hosp.*, **69**, 276.

McMichael, J. (1952). *Brit. med. J.*, **1**, 933.

Mohanty, J. K. (1954). *Nature, Lond.*, **174**, 184.

Morrison, B. (1953). *Brit. med. J.*, **1**, 1291.

Paton, W. D. M., and Zaimis, E. J. (1949). *Brit. J. Pharmacol.*, **4**, 381.

—— —— (1952). *Pharmacol. Rev.*, **4**, 219.

Restall, P. A., and Smirk, F. H. (1950). *N.Z. med. J.*, **49**, 206.

Saville, S. (1950). *Lancet*, **2**, 358.

Smirk, F. H. (1950). *N.Z. med. J.*, **49**, 637.

—— (1952). *Lancet*, **2**, 1002.

—— (1953). Ibid., **1**, 457.

—— and Alstad, K. S. (1951). *Brit. med. J.*, **1**, 1217.

Trendelenburg, P. (1917). *Arch. exp. Path. Pharmak.*, **81**, 55.

Turner, R. (1950). *Lancet*, **2**, 353.