ANALGESIC ACTIVITY IN A SERIES OF N-SUBSTITUTED ETHYL 4-PHENYLPIPERIDINE-4-CARBOXYLATES

BY

A.M.J.N. BLAIR, B.SC.

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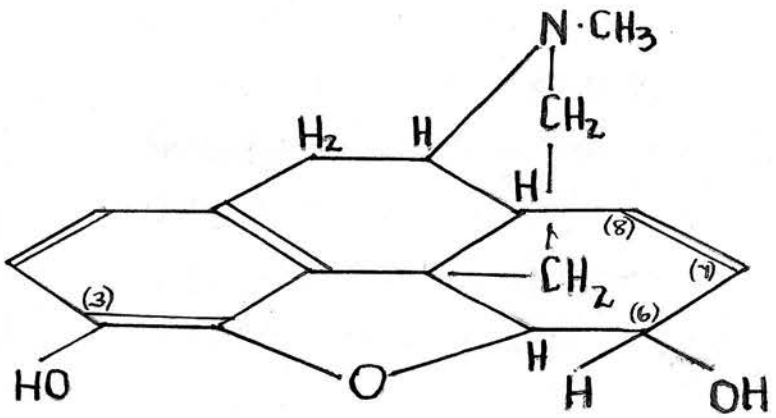
CONTENTS

The purpose of this work is to examine a series of pethidine derivatives synthesized by R. F. Anderson, P.M. Frearson and E.S. Stern in the Research Department of J.F. Macfarlan and Co., Ltd. The series consists of derivatives in which the N-methyl group has been substituted. This work is a continuation of that of Millar and Stephenson (1956).

Opium, which is still regarded by many as the best analgesic, has been used for over two thousand years. The first undisputed reference to opium is found in the writings of Theophrastus in the third century B.C. (Goodman and Gilman, 1955). Opium originates from Asia Minor where its use spread to Greece. Later, Arab traders introduced the drug to the Orient and China. By the middle of the sixteenth century, the uses of opium which are valid today were fairly well understood in Europe (Macht, 1915). Until well into the nineteenth century the opium preparations used medicinally were crude extracts. In 1806, Sertürner succeeded in isolating morphine from opium. Other alkaloids in opium were discovered later, for example, codeine by Rabiquet in 1832; papaverine by Merck in 1848. However, morphine is a dangerous drug being particularly liable to cause addiction. This property is shared by its close derivatives, such as "heroin", "dilaudid" and "eukodal" which

FIG 1

MORPHINE



also have been used as analgesics. Only comparatively recently have attempts been made to find equally active but less toxic synthetic compounds.

PAIN AS A PROTECTIVE REFLEX

Sir Charles Sherrington defined pain "as the pyschical adjunct to an imperative protective reflex". The pain reflex protects against damage of the tissues and the experience of pain warns against imminent dangers. Analgesics inhibit this protective function of pain by suppressing first the conscious experience of pain and in higher doses also the reflex response to painful stimuli.

There is a protective function in the regulation of respiration. It protects the body against acidosis. Analgesics block first the warning sensation of dyspnoea and finally in higher doses, the subconscious autonomic regulation of respiration as well. The effect on the gastro-intestinal tract is first to suppress the call to defaecate, then the defaecation reflex and finally, the entire propulsive motility. This latter aspect of the action of morphine has been reviewed by Vaughn-Williams (1954). Analgesics also block in high doses the regulation of both circulation and temperature. These functions are particularly sensitive to analgesics and have been collectively termed the

"protective system" by Schaumann (1956). He states that an important part of the relief given by analgesics rests on the ability to suppress the protective system with its dysphoric sensations and anxiety.

The term "analgesic" should refer to any drug which lessens the pain sensation but is used here to refer to the powerful "morphine-like" compounds. Drugs such as aspirin, phenacetin etc. also act by selectively depressing the central nervous system (Wikler, 1950). However, they differ from morphine in other respects being only useful in pains of low intensity and in not being drugs of addiction. They have therefore, been called "antalgics" by Fourneau (1938). All the morphine-like analgesics share certain effects and these are termed "specific effects". With the synthetic analgesics it is possible to separate specific from the non-specific effects. An effect is specific if it is common to all analgesics whatever their chemical structure, and if the potency in producing the effect runs parallel to their analgesic potency (Schaumann, 1956).

Schaumann has emmerated the specific effects of morphine-like analgesics, viz.

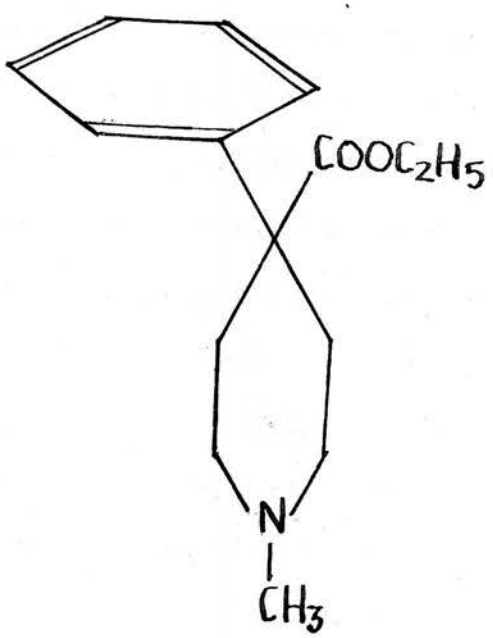
(1) analgesia
(2) the depression of respiration
(3) the effect on intestinal motility
(4) central excitation in cats and mice
(5) the hyperglycaemic effect
(6) the effect on body temperature and
(7) the liability to produce addiction.

Attempts to separate analgesic action from depression of respiration have repeatedly failed. Basil et al (1950) found that the analgesic activity of phenadoxone and allied substances was related to depression of respiration as did Green (1953) in a series of 3:3 dithienylalkenylamines. Eddy et al (1956) state that the analgesic activity of alphaprodine and betaprodine is increased relative to pethidine without any significant change in "addiction sustaining dose". Schaumann (1956) considers that it is not correct to regard depression of respiration, constipating effect and liability to addiction as side effects. He believes that they are inseparable from the analgesic action. It is hoped that at least a quantitative separation of analgesic activity from depression of respiration and liability to addiction may be obtained. Recent work supports the view that the constipating effect is associated with analgesic potency (Schaumann et al, 1952).

Trendelenburg (1917) showed that morphine has a paralysing action on the peristaltic reflex in isolated guinea-pig ileum. Recent work indicates that all strong analgesics have this property and their activity on this preparation runs parallel to their analgesic power (O. Schaumann et al, 1952;

FIG 2

PETHIDINE



W. Schaumann, 1954). It is also suggested that the analgesics exert their specific paralysing action by a method analogous to that which is responsible for their analgesic effects. If this is correct, the guinea-pig ileum is a suitable preparation on which to study the analgesic activity of pethidine and its derivatives.

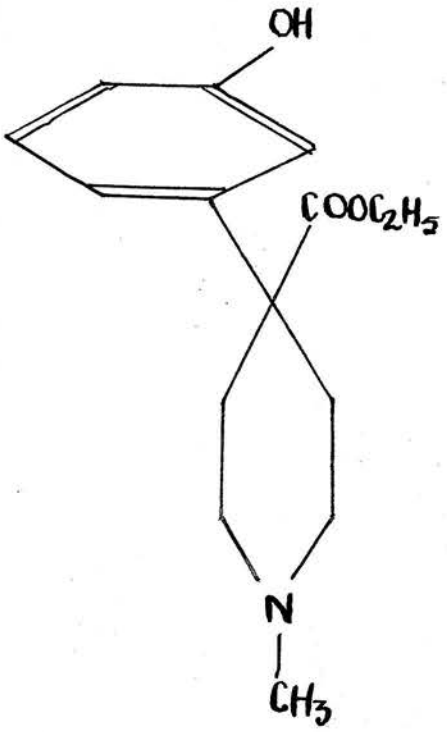
CHEMICAL CONSTITUTION AND ANALGESIC ACTION.

The earlier attempts to synthesize potent analgesic compounds without addictive properties and other undesirable effects, consisted of the screening of large numbers of compounds possessing one or more of the structural characteristics of the morphine molecule. Increased analgesic potency in the close derivatives is accompanied by increased toxicity, examples are 6 acetylmorphine, diacetylmorphine and dihydromorphinone (Beckett, 1952).

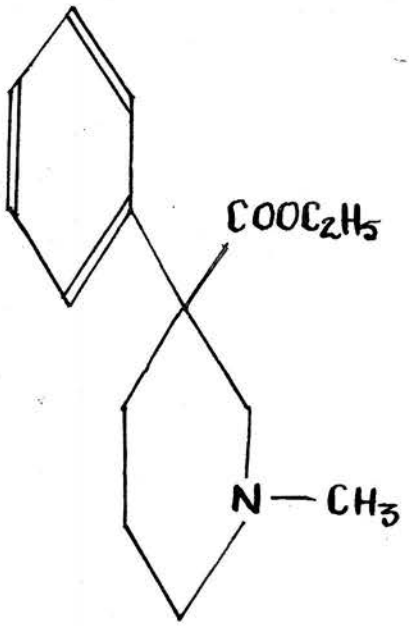
Progress was first made with the drug pethidine (Eisleb and Schaumann, 1939). This was originally introduced as a spasmolytic and its morphine-like analgesic properties were discovered accidentally. Morphine has a peculiar effect on mice making them hold their tails erect. Pethidine caused this effect, which is known as Straub's reaction and consequently it was tested for analgesic

FIG 3A

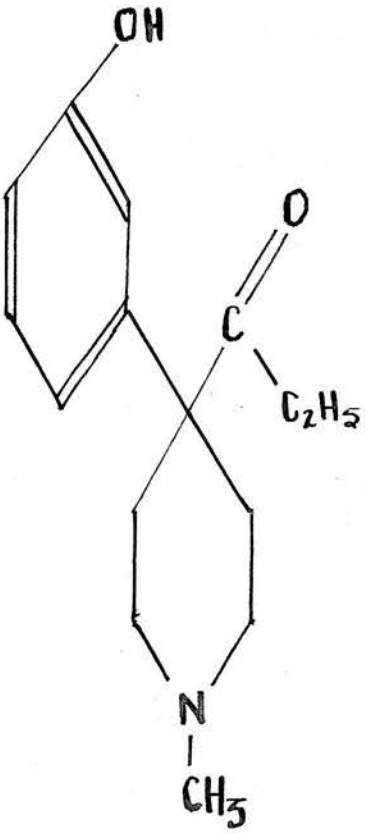
PETHIDINE LIKE COMPOUNDS



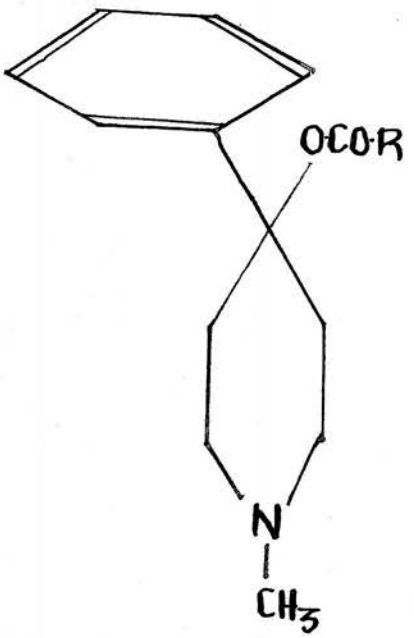
HYDROXYPETHIDINE



β- PETHIDINE



KETOBEMIDONE



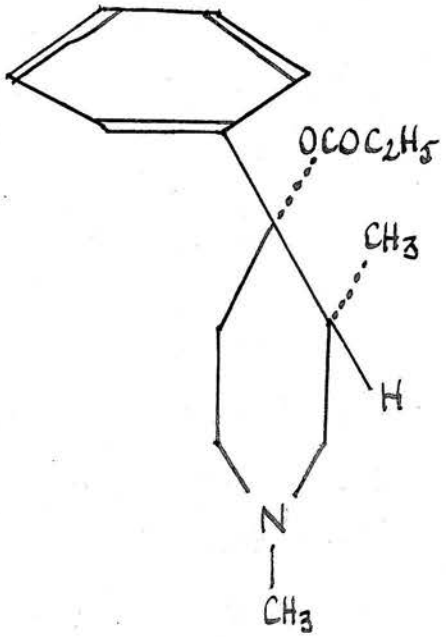
REVERSED ESTERS OF PETHIDINE

activity. Although pethidine is not so powerful an analgesic as morphine (about 1:5) it rarely depresses the respiratory centre and has far less addictive properties than morphine. Contrary to earlier reports, pethidine is a drug of addiction (Yonkman, 1948). Since the discovery of pethidine, much work has been done in preparing modifications of the molecule in attempts to increase the activity and lower the incidence of side effects. These modifications include moving, removing and substituting the phenyl group; substituting and breaking open the piperidine ring; replacing the N-methyl group by other groups, and replacing the ethyl ester by other ester groups, hydrogen, ketone and reversed ester groupings. This topic has been reviewed by Foster and Carman (1947).

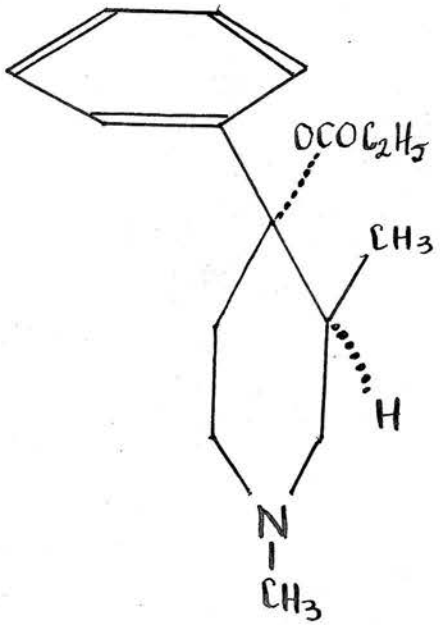
Macdonald, Woolfe, Bergel, Morrison and Rinderknecht (1946) prepared derivatives of pethidine with variations in the phenyl ring. They found that a m-phenolic group as in the compound hydroxypethine (Bemidone) did not alter activity. All deviations from the ethyl ester group which they tried resulted in considerable loss of analgesic activity. 3-phenyl-N-methylpiperidine-3-carboxylates were less active, although it was still the ethyl ester, β pethidine which was the most potent.

FIG 3B

PETHIDINE LIKE COMPOUNDS



ALPHAPRODINE



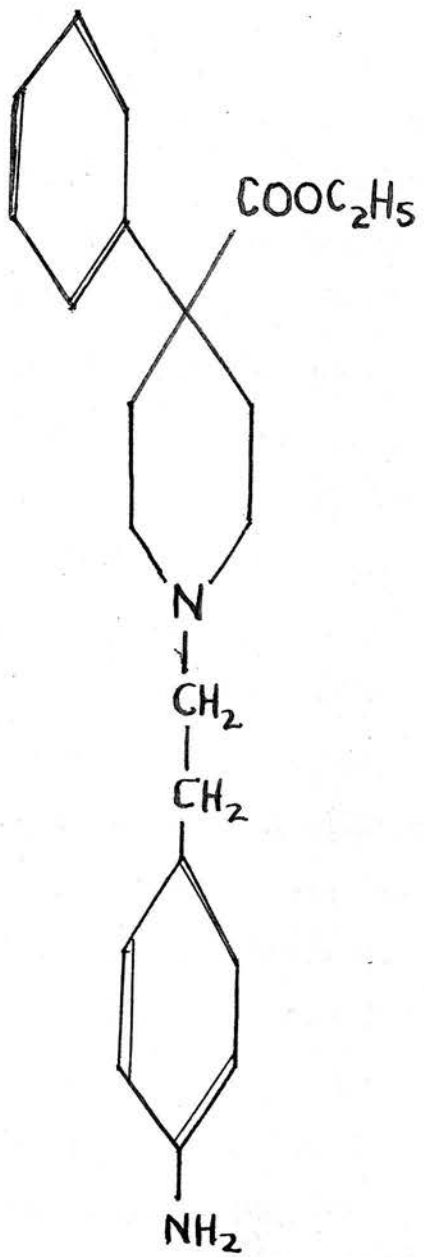
BETAPRODINE

β pethidine is less potent and less toxic than pethidine. 2-phenyl-N-methylpiperidine-2-carboxylates and lactones derived from 4-O hydroxyphenyl-N-alkylpiperidine-4-carboxylic acids were inactive. It was found that some ketones were more active than the esters. The ethyl compound corresponding to pethidine is about as active but the m-hydroxyphenyl compound, ketobemidone, corresponding to hydroxypethidine is about 2 to 3 times as active as morphine. Ketobemidone also has powerful addicting properties.

Compounds in which the ester groups of pethidine have been "reversed" were found to be analgesics. "Reversed" esters are esters of 4-phenylpiperidols. In 1943, Jensen et al reported that these compounds were more active than pethidine especially the propionoxy derivative ($R = C_2H_5$) which was stated to have 5 times the activity of pethidine. Independently, Ziering and Lee (1947) prepared similar compounds obtaining alphaprodine (cis-form racemate) and betaprodine (trans-form racemate). Randall and Lehmann (1948) found the ratio of potencies of morphine, alphaprodine and betaprodine to be 100:97:550. In man, the difference in action between the cis and trans racemates is not so pronounced (Gross et al, 1948). These

FIG 4

ANILERIDINE



drugs are said to be relatively less liable to addiction than pethidine (Eddy et al, 1956). In this type of compound, the best group on the nitrogen atom was found to be methyl.

It has been generally accepted that for analgesic activity a methyl group is the optimal radical on the nitrogen atom present in all potent analgesics (Braenden, Eddy and Halbach, 1955). Recent studies however, have demonstrated an increase in activity when the methyl group is substituted (Perrine and Eddy, 1956; Millar and Stephenson, 1956; Orahovats, Lehman and Chapin, 1957).

Perrine and Eddy (1956) substituted a phenethyl group for the methyl radical: the resultant compound is about three times as active as pethidine. Orahovats et al (1957) replaced the N-methyl group by N-(p-aminophenethyl). The resultant compound, anileridine, is about as active as morphine. Millar and Stephenson (1956) tested compounds where the N-methyl group was replaced by a tertiary aminoalkyl group. The most active of these morpholinoethyl-norpethidine is about as active as morphine. They found that the presence of an oxygen atom in the heterocyclic ring was very important since compounds without it were inactive.

To summarize, there is no clear understanding of the chemical constitution necessary for analgesic

action. Many of the workers engaged in the search for synthetic analgesics have tried to explain any analgesic activity in their compounds in terms of a relationship with the morphine molecule, for example, Macdonald et al, 1946. However, sometimes it is difficult to see any direct relationship, for example, analgesically active diethienylbutylamines are not structurally similar to morphine (Adamson and Green, 1951).

One of the major difficulties is that a number of chemically different compounds are analgesics suggesting that the mechanism of action is probably a physico-chemical one. Morphine, pethidine and amidone possess in common a tertiary nitrogen group, and a quaternary carbon atom separated by a CH_2-CH_2 linkage. Eddy (1950) has stated that a tertiary nitrogen seems to be essential for analgesic action and a CH_2-CH_2 link joining the tertiary N and the quaternary carbon seems to be desirable and perhaps optimal for analgesic action.

Beckett (1952) has summarized the evidence emphasizing the importance of the stereochemical configuration. The clearest examples are provided by the analgesics containing one assymmetric carbon atom, where in all cases in which the optical enantiomorphs have been prepared one enantiomorph is always very much more active than the

other. For instance, in methadone the l-isomer is 30 times more potent than the d-isomer.

Macdonald et al (1946) considered that "the shape or fit of the molecule as a whole is more important in determining its analgesic value than any precise duplication of any one fraction of the morphine structure".

METHODS OF MEASURING ANALGESIC ACTIVITY.

The lack of any clearly defined knowledge of what chemical constitution is required for analgesia has hindered the search for better analgesics. Innumerable substances have been synthesized by varying the chemical structure of active drugs. The pharmacologist is thus faced with the task of looking for analgesic activity in hundreds of compounds and of comparing the potencies of the active derivatives. This requirement is met by about 50 different methods which have been elaborated within the last fifteen years. The purpose of these methods is to provide exact figures for the analgesia produced and to allow a statistical evaluation of the results. This subject has been reviewed by Hardy, Wolff and Goodell (1952) and Beecher (1957).

One of the reasons for the multitude of methods

is that pain sensation has no specific stimulus. Any stimulus will elicit pain if applied with sufficient intensity. In general, the stimuli employed are: mechanical (sharp point, broad pressure), thermal (heat by conduction or radiation and cold by conduction), chemical and electrical (faradic and galvanic, condenser discharge etc.). The receptive fields to which stimuli are delivered include: skin, cornea, tail, conjunctiva, ear, lips, birth canal, scrotum and tooth. Standard responses which are measured consist of: verbal reports, lid reflex, sharp cry, defensive movements, squeaking, flight, galvanic skin responses, limb withdrawal, muscle twitch, tail flick, tension of neck muscles, pupillary reflex and movements of the whole body (Goetzl, Burrill and Ivy (1944).

The response to a painful stimulus is complex. The pain experience may be regarded as composed of a pain sensation accompanied by a more or less predictable pattern of associated sensations (such as heat, cold, pressure etc.) and emotion (anger, fear etc.). The reactions to pain include physiological changes, such as those in the electrical resistance of the skin, which vary independently in an unreliable manner to the intensity of stimulus. The "overall" reaction to pain, such as limb withdrawal,

is a manifestation of the "imperative protective reflex", (Hardy et al, 1952).

The mechanism of morphine analgesia is poorly understood. The locus of action is unquestionably central, probably on the cortex (frontal lobes) and diencephalon. Masson (1956) has evidence that morphine also acts on the spinal cord. This topic has been reviewed by Wikler (1950).

Hardy et al (1952) state that analgesic drugs act mainly by raising the pain threshold, reducing the perception of pain, and by reducing the reaction to pain. The latter is believed to be the most important aspect of the therapeutic action. A pain stimulus is usually applied to an animal until it tries to "avoid" the stimulating agent. Thus in animals, the effect of analgesics on the "avoidance reaction threshold" is studied.

In consideration of the various methods principal attention will be given to the limitations of each method.

METHODS USING A THERMAL STIMULUS

These methods are popular. The success of Hardy, Wolff and Goodell (1952) created further interest in this type of test. This method has been applied to the study of analgesia in animals (e.g. D'Amour and Smith, 1941).

The heat which actually constitutes the stimulus is difficult to measure in exact terms. Furthermore, the temperature of the receptive field is determined not only by the heat delivered to it but also by the circulation of the area. An assumption of direct proportionality between the heat stimulus and the opposing homeostatic mechanisms of the receptive area is not justified (Goetzl, 1946). Analgesics have a species difference in their effect on the temperature, e.g. morphine reduces the body temperature of dogs and raises the temperature of rats. Use of high levels of heat may lead to a persistent erythema and only a few readings on any one area are advisable (Winter and Flataker, 1953). The usual animal response is a "tail flick" which involves the spinal cord whereas in man, pain sensation is mediated through the thalamus and cortex (Bianchi and Franceschini, 1954). However, Masson (1956) has presented evidence that morphine as well as its effect on the brain, also acts on the first synapse in the afferent pathway of the spinal cord. Macdonald et al (1946) studied the reactions of mice when placed on a hot plate. The apparatus described by D'Amour and Smith (1941) is complex and the results quoted by various authors are conflicting, indeed the estimates of activity obtained

from quantal or graded responses are not identical (Bonnycastle and Leonard, 1950).

It seems that thermal stimuli are not particularly suitable for analgesic studies on animals.

METHODS USING ELECTRICAL STIMULI

The method of stimulation of pain by a faradic current was first introduced in 1851 by von Helmhotz. The favourite site for stimulation is a tooth since the only sensation produced is pain. Goetzl, Burrill and Ivy (1943) stimulated the amalgam fillings of dogs teeth. Attempts to repeat this work have failed (Miller, 1948). Evidence has been presented by Reynolds and Hutchins (1948) that painful stimulation produces a hyper-irritable central state which persists from months to years. What influence this might have on repeated determinations of pain threshold by electrical stimulation of teeth is not known. However, prolonged stimulation at high intensities results in irreversible damage to the tooth (Hardy et al, 1952).

Beecher (1957) states that in view of the remarkable inconclusiveness of the method of electrical shocks to teeth, it is difficult to accept work that depends upon this technique. Goetzl and his colleagues (1943 and 1946) however, consider the tooth pulp method to be the most promising of all

methods producing experimental pain.

METHODS USING CHEMICAL STIMULI

Hardy et al (1952) have suggested that critical stimulus for pain is the rate of tissue damage. The damaged tissue would release a hypothetical chemical which would be the specific stimulus for pain. This view is not accepted by Beecher (1957). However, if this suggestion is correct then the production of experimental pain with such a chemical would have considerable appeal. Actually, little quantitative work has been done using injurious chemicals to cause pain. It is extremely difficult to give a graded stimulus using chemical reagents. This method is unsuitable for animals but Armstrong et al (1951) claim good results with their technique in man.

METHODS USING MECHANICAL STIMULI

These methods are popular in man and animals.

Mechanical stimuli have been used to cause pain in various ways. Von Frey (1897) used weighted horse hairs in his classical experiments. Bishop (1948) has studied this method and concluded that the bending or stretching caused deformation and this, not pressure, is the form of stress to which

pain endings react.

Hydrostatic pressure has been used to simulate visceral pain (Gaensler, 1951). Balloons have been inserted into the oesophagus (Chapman and Jones, 1944) and into the biliary tree (Layne and Bergh, 1940). These methods are of special interest since it is possible that visceral pain is a different sensation from "superficial pain", being evoked by its own special set of stimuli and transmitted to the central nervous system through special pathways. However, both morphine and pethidine increase the pain of biliary colic by producing spasm of the sphincter of Oddi and this complicates the results (Hewer and Keele, 1948).

Experiments have been based on the pain evoked by muscle ischaemia which causes the pain to be slowly produced and sustained. This is in contrast to other methods where the experimental pain produced is usually sharp and fleeting. This technique is still being developed (Green and Beecher, 1957).

Another method is simply to squeeze a mouse's tail and note the presence or absence of a "cry response". This method was described by Haffner in 1929 and was that used by Schaumann (1940) when he discovered the analgesic action of pethidine.

Recent support for this method is given by Bianchi and Franceschini (1954). Green and Young (1951) used a plunger head of a hypodermic syringe to squeeze a rat's tail. The syringe is connected to a mercury manometer which measures the stimulus intensity.

In conclusion, it seems that mechanical stimuli are particularly suitable for evoking pain. An important factor is that the deeper structures are stimulated and not only those in the skin. The results using the method of Green and Young are reproducible in different laboratories (Millar and Stephenson, 1956; Green and Ward, 1956) and bear close relationship to clinical experience.

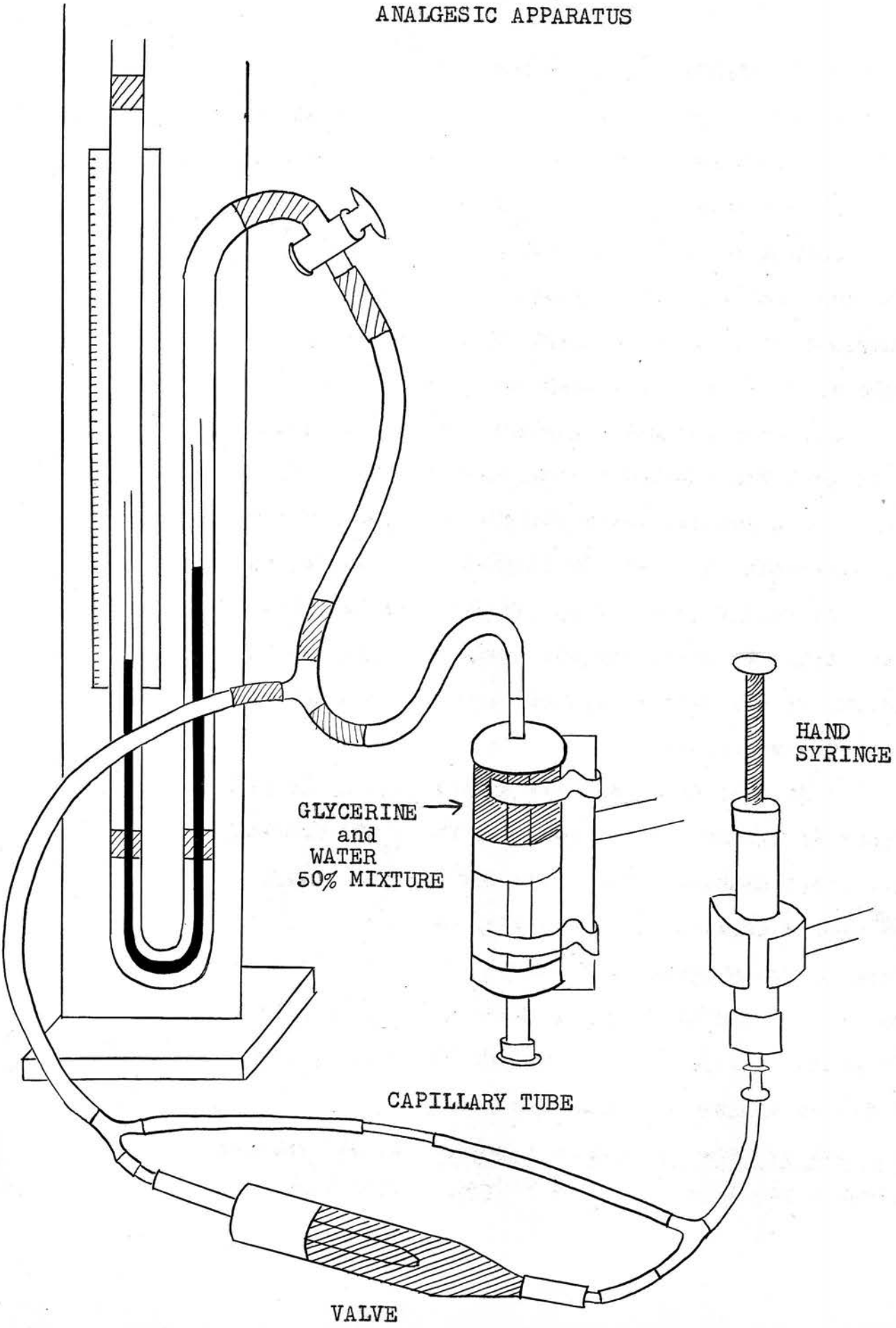
The method of Green and Young (1951) as modified by Millar and Stephenson (1956) was used to determine analgesic activity.

EXPERIMENTAL METHODS

MEASUREMENT OF ANALGESIA

The pressure was measured with a mercury manometer, the bore of which was 3 mm. It was transmitted to the tail by a strip of wood (12 x 4 x 5 mm.) which was cemented to the plunger head of a large vertical syringe and applied to the tail at right angles about 1 cm. from the tip. The small

FIGURE 5
ANALGESIC APPARATUS



horizontal syringe was operated by hand and was fitted with a spring under compression between the barrel and plunger head, to ensure a rapid release of pressure (see Figure 5). A narrow capillary tube with a valve in parallel was placed between the two syringes; this restricted the rate of increase of pressure without unduly affecting the rate of release. The syringes were of 20 ml. and of 2 ml. capacity and their cross sectional areas (and thus the applied forces) were in the ratio 6.5:1. A smaller ratio required more effort from the operator and a steady increase of pressure was more difficult to achieve. In practice, pressure was increased until the rat squeaked, when the height of the mercury column was noted and the pressure released.

This technique was used quantitatively in contrast to the method of Green and Young who favoured a quantal method. The difficulty is that, with effective doses of an analgesic, some rats fail to respond to any pressure, and an arbitrary value has to be allotted to obtain a mean value for a group. To avoid this, Millar and Stephenson took as an index of analgesia the ratio:

$$\frac{\text{Pressure required to induce a squeak before injection}}{\text{Pressure required to induce a squeak after injection.}}$$

A small index is an indication of a large effect, i.e., complete analgesia has the index 0.
If a rat did not squeak with a pressure three times the control figure the index was taken as 0, which, unlike infinity, can be included in a mean value for a group. Usually, in preliminary experiments, it was found that if a rat did not squeak at three times the control pressure it did not squeak at even six times that figure. The index was calculated from the pressure measured at ten minute intervals for an hour after injection of the test analgesic. The substances tested did not markedly differ in duration of action and I therefore calculated the mean of these six values to give a single index for the response of each rat. The inclusion of a control pressure might be expected to render the index independent of the dimensions of the apparatus and of the size of the rat's tail. Green and Young (1951) showed that the variation of the control pressure between rats of the same age was no greater than that between different trials on the same rat. On all occasions I have used, as numerator in each index, the mean control pressure of the rats used on that occasion.

Most of the substances tested were injected subcutaneously in 4 ml. saline/kg. body weight.

Substances which were prepared as bases were dissolved in the minimum amount of $\frac{N}{10}$ hydrochloric acid and the volume made up to 4 ml./kg. with saline. Other substances, which were insoluble in saline, were dissolved in the minimum amount of alcohol and the volume made up with saline. 50% by volume of absolute alcohol does not affect the activity of an analgesic to any significant extent or has any analgesic action by itself.

Newly weaned rats were used as test animals. Three week old rats are very sensitive to pressure stimulation, with thresholds of 2 - 4 cms. mercury. Green and Young (1951) have found that this sensitivity decreases rapidly with age but remains fairly constant from $3\frac{1}{2}$ to $8\frac{1}{2}$ weeks of age. Green and Young (1951) suggested that rats of about 4 to 5 weeks old show a minimum inter rat variance. Accordingly, rats about three weeks old were used for three weeks and then discarded. There is no significant difference between the sexes up to 8 weeks but here female rats have been used exclusively. Another advantage is that with young rats smaller amounts of test substances may be used.

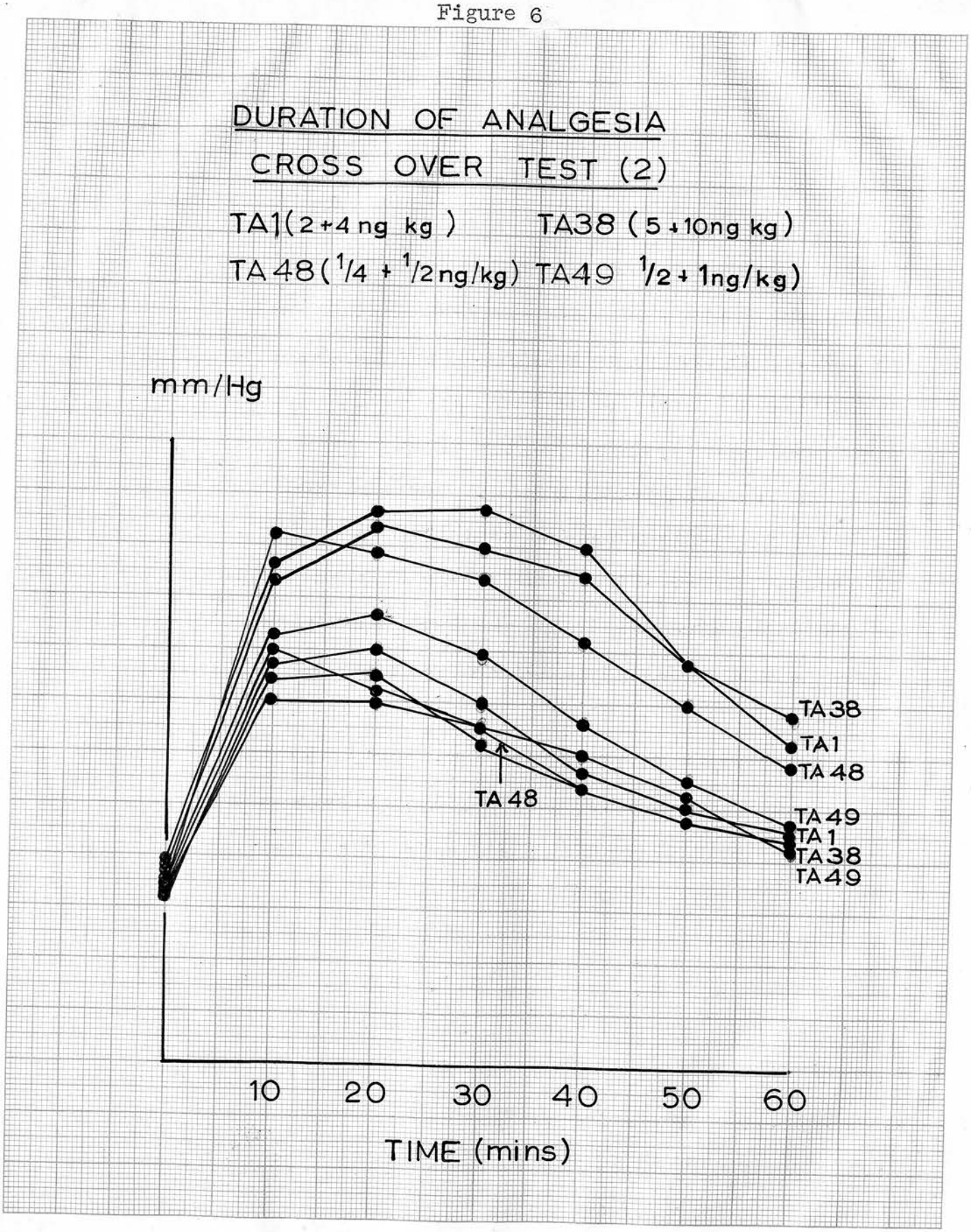
I am very grateful for a generous supply of rats from the Clinical Endocrinology Research Unit (M.R.C.) Edinburgh. These rats were only handled

by the technician who fed them and also helped me with my experiments, and myself. This avoided excitement due to handling by "strangers". These rats were sensitive and gave regular responses. All the animals used in the cross-over tests were from this source. The other rats used in routine tests were supplied by Tuck and Son, The Mousery, Rayleigh. Some of these rats were "insensitive" to pressure, that is, they struggled but did not squeak. These rats were discarded.

It is important that any method used to label the rats should not damage the tail. Recognition by mutilation, cutting ears etc. is difficult with large numbers and unnecessary. A pen filled with a red dye and fitted with a felt pad nib was used. The markings last for a few days and cause no discomfort. The animals were labelled at the beginning of each experiment.

Green and Young (1951) performed statistical trials to determine the reproducibility of the pain threshold. The absence of systematic variation among repeated readings showed that with normal threshold pressures no damage was sustained by the tail. No obvious physical damage to the tail has been observed in rats subjected to 3 times the control pressure.

Figure 6


DURATION OF ANALGESIA
CROSS OVER TEST (2)
TA1 (2+4 ng kg)
TA38 (5+10ng kg)
TA48 (1/4 + 1/2 ng/kg)
TA49 1/2 + 1ng/kg)
mm/Hg
TA38
TA1
TA48
TA49
TA1
TA38
TA49
TA48
10
20
30
40
50
60
TIME (mins)

No difference was found in the duration of analgesia for doses of these derivatives of pethidine giving about the same mean analgesic effect (see Figure 6). The pressure required to elicit a squeak returned to the control level after an hour or before, in analgesic trials. The high dose of analgesics was selected so that very rarely was complete analgesia produced for the sixty minutes.

The test analgesics were all compared with TA1, the most effective substance described by Millar and Stephenson, because it seemed likely that the slope of the dose-effect curves of the test substances would be more parallel to TA1 rather than that of pethidine.

Three types of tests were performed. Preliminary tests were carried out to gain knowledge about the approximate shape of the log dose effect curve. In the routine tests two doses of the "unknown" substance were selected to give about the same mean analgesic effect as 2 mg./kg. TA1 (index 0.65) and 4 mg./kg. TA1 (index $\simeq$ 0.35). Two control readings of the pain threshold were observed for each rat. The four doses were then administered; four or five rats for each dose. At the same time, four or five rats were given 4 ml./kg. saline and these served as controls. The pain thresholds were measured at ten minute intervals for one hour. The mean analgesic

index for each dose was calculated. This test was repeated twice not necessarily on the same group of animals. Animals were allowed to "rest" at least two days. The log dose effect curves based on these results were drawn and the estimate of the relative potency was calculated from the horizontal distance between the two regression lines (see Figures 7, 8, 9, 10, and 11). The results of these tests are tabulated in Table I.

For more accurate analysis a cross-over technique was used. Cross-over tests for analgesics are not usual because of the possibility of tolerance being developed. Millar and Stephenson (1956) found no evidence of the development of tolerance. They used a (2 + 2) 4-way cross-over test in which twenty-four rats were used. Each rat received every dose in a different order so that all the 24 possible ways were used. They compared two drugs, TA1 and pethidine in this way.

It was decided, because of the large number of active drugs to be tested, to modify the cross-over technique so that four drugs could be compared. The test used was an eight (2+2+2+2) dose 4-way cross-over test in which twenty-four rats were used. There was a 2-day interval between each test. Each rat

received each of the four drugs and two high doses and two low doses, but not every dose. This design confounded the variance due to differences in slope and the variance due to differences between rats, because all the rats received different treatment.

The rats were divided into three cages of eight. Each cage represented a different environment and environment affects the response of each rat. To avoid errors due to the grouping of rats, each group was given the eight doses using a symmetrical latin square design. Thus each dose on any one day was given to three rats, one in each group.

A determination of potency should always include an estimate of its error computed as an integral part of the assay. Gaddum (1933) provided the basis for a biological assay which included an estimate of the precision of the result and showed that his formulae applied equally well to measured responses and to quantal data. Schild (1942) published an account of a null hypothesis assay conducted on statistically sound principles. A simplified version of this assay has been described by P. Holton (1948). Statistical methods in biological assay have been reviewed by Finney (1952) and Gaddum (1953a).

The mean response to each dose is plotted

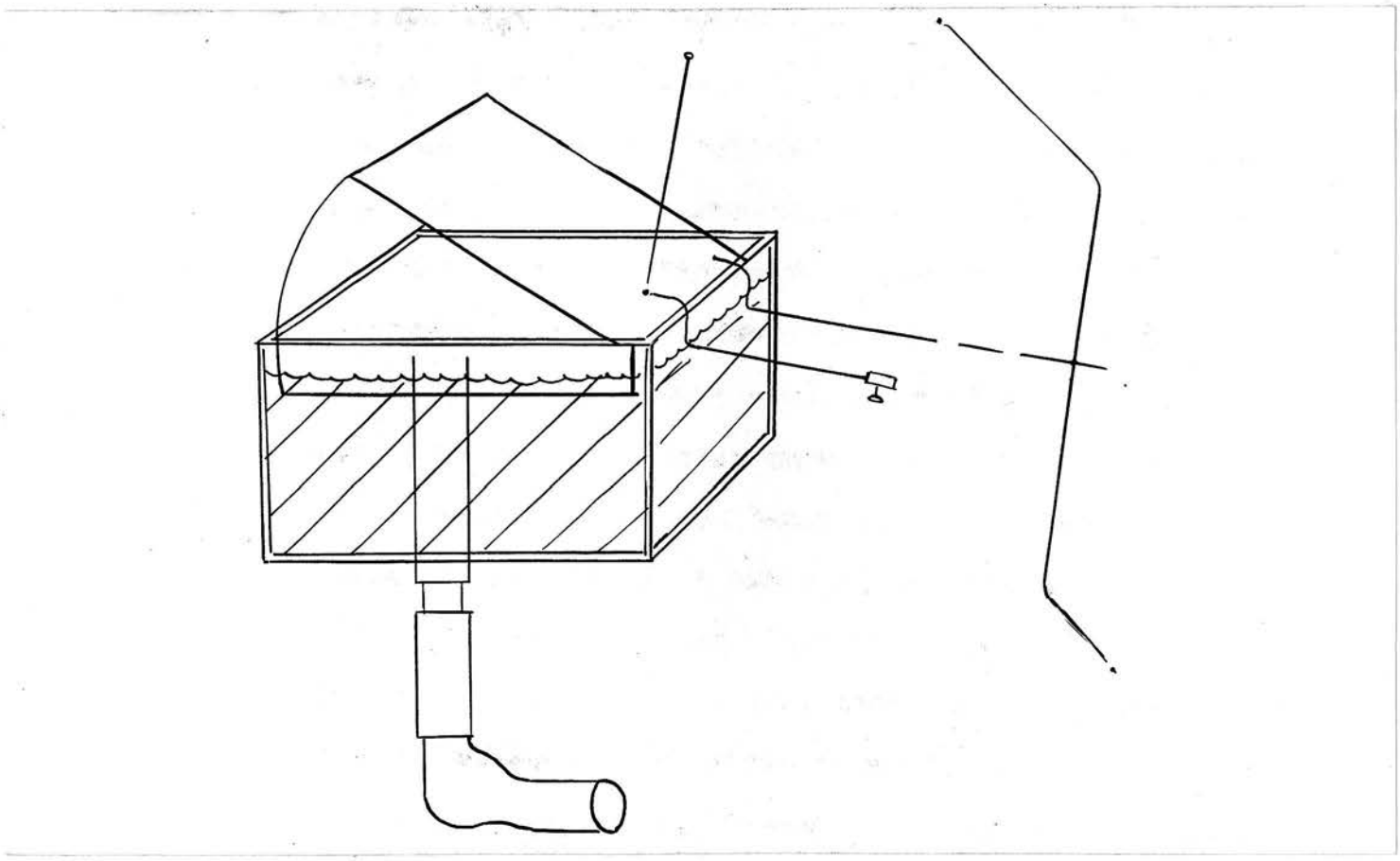
against the log dose. The null hypothesis states that there is no difference between the standard and the unknown drug, and so the standard log doses are used in plotting the points for the unknown. Since the unknown and standard solutions did not contain the same active principle the regression lines are unlikely to be exactly parallel (see Figures 7, 8, 9, 10 and 11). Therefore, in order to obtain a reliable estimate of the potency and an estimate of its error it is necessary to calculate the estimate rather than determine it graphically.

The methods of calculation and analysis of variance are those of Schild (1942) but differ in some ways from those described. An example of a typical assay is given in the section on results.

METHOD OF RECORDING ACTION OF TEST ANALGESICS ON RESPIRATION AND BLOOD PRESSURE

Male rats (weight 250 -300 g.) were anaesthetised with urethane 0.6 ml./100 g. of a 25% solution, injected subcutaneously and "reinforced" with ether in the initial stages, if necessary. The usual dose of urethane, 0.7 ml./100 g. 25% solution produced too deep a level of anaesthesia. I tried several combinations of anaesthetics including pentobarbitone and urethane (Amin, 1953). It seemed

Figure 12

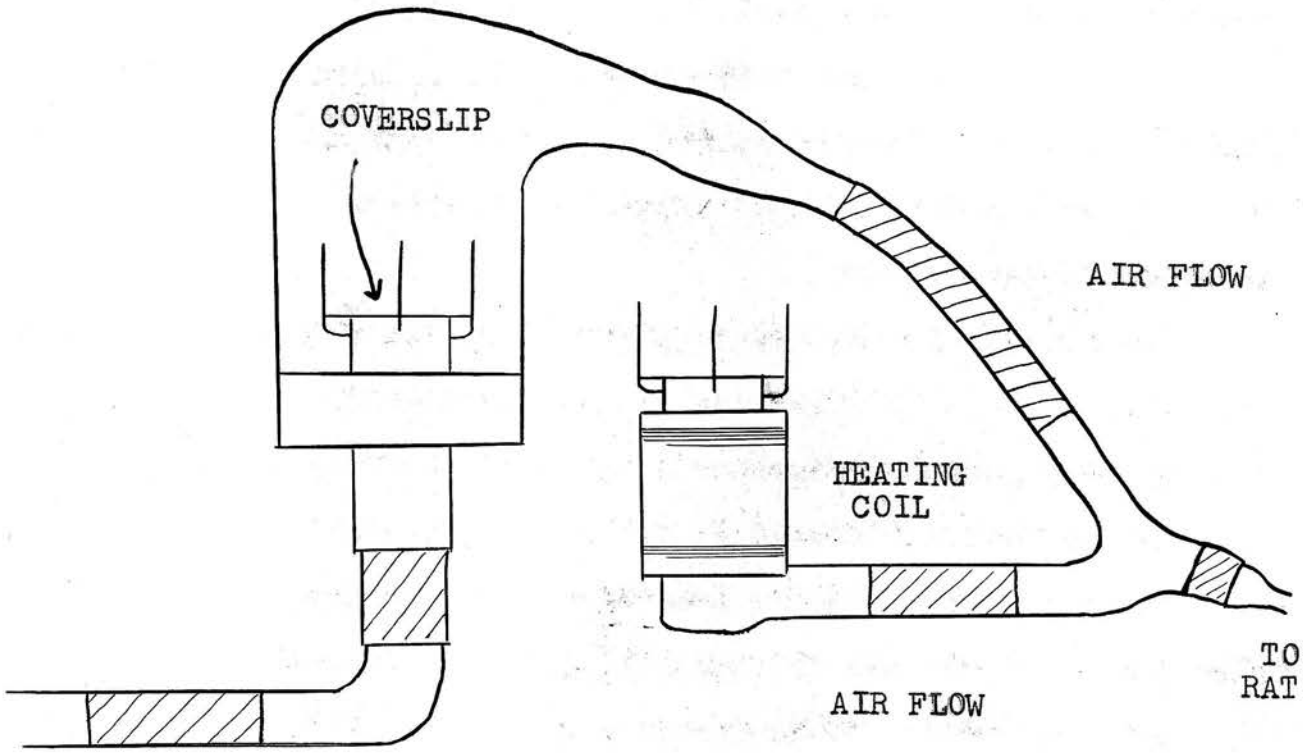


that ether was necessary to produce complete surgical anaesthesia and then the urethane was sufficient to maintain that level. If the level of anaesthesia was too deep the animal usually died after a few doses of the test analgesics.

The femoral vein was cannulated. This cannula was fitted with a piece of rubber tubing for injection and connected to a burette filled with saline. A solution of 100 u. of heparin per 100 g. was injected into the vein: this prevented clotting for the duration of the experiment. A blood pressure cannula was introduced into a carotid artery and connected to a narrow bore mercury manometer. The blood pressure was recorded on a smoked drum.

The drugs were injected into the venous cannula through the rubber tubing. The dose washed in from the cannula by a small volume of saline run in from the burette. The respiration was recorded using a modified version of Gaddum's method (1941). A rubber tube was attached to the tracheal cannula of the rat, which was thus made to breathe in through one valve and out through another. Air was passed to the apparatus through a small hole which acted as a resistance. The negative pressure inside the hole was recorded with a "pressure recorder" described by Paton (1949) (Figure 12). The recorder

FIGURE 13



had a frontal writing lever which wrote on a smoked drum and the height of the record above the base line was directly proportional to the minute respiratory volume. In addition, the recorder did not need to be calibrated after each experiment; and offered little resistance to respiration. It was possible to alter the sensitivity of this recorder.

The valves were coverslips which fell on brass hollow tubes thus forming an efficient seal (Figure 13). A heating coil around the expiratory valve kept the cover slip dry.

The valves were tested to ensure that they were leak free. I found rubber valves unsatisfactory because they quickly became moist and inefficient.

It was found difficult to use the rat to test the effect of the pethidine derivatives on the respiration. Nearly all the rats of suitable size and age have some degree of lung damage. The rats did not seem to be robust enough to withstand the depressant effect of the anaesthetic and the test analgesics. Some experiments were performed recording the respiration with a rubber tambour. These experiments were not any more successful.

METHOD OF RECORDING ACTION OF TEST ANALGESICS ON THE PERISTALTIC REFLEX

Guinea-pigs were killed by a blow on the neck. The lower ileum was used discarding the 10 cm. next to the caecum. Part of this strip of ileum was stored in Tyrode's solution at 5.5°C. for 24-hours. A suitable portion of the remainder was suspended in a magnesium free Tyrode's solution. The bath was aerated with 95% O_2 and 5% CO_2 and the temperature kept at 36 to 37°C. This method was described by Trendelenburg (1917). Changes in volume and isotonic contractions of the longitudinal muscle were recorded on a smoked drum. Peristaltic waves were elicited by a rise of intraluminal pressure of Tyrode's solution. For distension of the lumen the pressure was raised in steps of 1 cm., 2 cm., 3 cm. or 4 cm. to determine the minimum stimulus necessary to produce the maximum effect. In fresh, and in cooled preparations, the rise in intraluminal pressure was maintained for 40 sec. and followed by a rest period of two minutes. The drugs were added one minute before a rise in intraluminal pressure. All doses mentioned in the text refer to µg of the substances added to the 40 ml. bath.

The peristaltic reflex was easily obtained in the guinea-pig intestine on distension of the lumen. It consisted of two phases termed by Trendelenburg preparatory and emptying. The preparatory phase consisted of a contraction of the longitudinal muscle, the emptying phase, which followed, was a wave of contraction of the circular muscle spread aborally over the whole preparation. According to Feldberg and Lin (1949) different mechanisms were responsible for the two phases. Pethidine and its derivatives blocked both these phases. W. Schaumann (1955) suggested that morphine may prevent the action of released acetylcholine or act on some point in the reflex involved in the preparatory phase without the mediation of acetylcholine. This method of testing analgesics has been developed by Schaumann and his colleagues (1952).

Both phases were seen when studying the fresh preparation but only the preparatory phase could be elicited from the stored preparation. It was difficult to obtain consistent and "graded" results to different doses when studying the depression of the emptying phase. It was decided to study the effect of the analgesic compounds on the preparatory phase both before and after being kept in the refrigerator.

Only a few of the substances were tested on this preparation.

TOXICITY TESTS

ACUTE Drugs were injected intravenously into the tails of mice. All the drugs were given in 4 ml. saline/kg. Each dose was injected into five mice which were not used again. If possible, the LD50 for each drug was determined. Some of these compounds are relatively insoluble in saline and for the purpose of the analgesic tests were dissolved in alcohol. Alcohol was itself very toxic to mice and could not be used as a solvent. If the drugs were soluble in warm saline an attempt was made to determine the LD50. Since these drugs tend to come out of saline solution on cooling the figures given may be inaccurate. Some other drugs were quite insoluble in saline and could not be tested in this way.

SUBACUTE Two groups of ten rats were weighed and given subcutaneous injections daily over 12 days. The "test" group received five times the larger dose used in the analgesic tests in 4 ml. saline/kg. The control group received 4 ml. saline/kg each day. On the thirteenth day suitable doses were given to equal numbers of control and test animals and an analgesic test carried out. It was hoped that this would give an indication about the development of

tolerance. The animals were then killed and a blood smear taken. The main organs were dissected and prepared for examination. These included the liver, the kidney, the spleen, the heart, the lungs, the brain and the bone marrow. Unfortunately, some rats died during these tests and were eaten by the survivors.

RESULTS

Millar and Stephenson (1956) examined a series of pethidine derivatives in which the N-methyl group of pethidine had been replaced by a tertiary amino alkyl group. The most active analgesic was morpholinoethylmorpethidine (TA1). The log dose effect curves of TA1 and pethidine were not parallel and so they could not give an absolute value for the potency ratio. One cross-over test gave a potency ratio of 6.5 fiducial limits 4.51 and 9.47, P=0.95. The only substances showing appreciable activity contained the morpholino ring and the oxygen atom appeared essential since substances without it were inactive. The analogous compound to TA1 but containing sulphur in place of oxygen was quite active but less so than TA1 (TA1: TA18 1:0.33).

If the chain of carbon atoms linking the

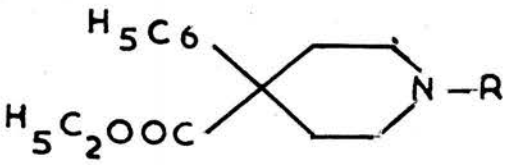
nitrogen atoms of the two rings in TA1 was lengthened or shortened, there was considerable reduction in activity. Millar and Stephenson found that branching also reduced activity though not sharply. A substance comprised essentially of two molecules of pethidine joined together had little or no analgesic activity. They found that simple derivatives of morpholine without the pethidine nucleus were not effective.

A surprising discovery was that the presence of oxygen or sulphur in the heterocyclic ring should be so important for analgesic action. Compounds without the oxygen (or sulphur) atom, but otherwise closely similar, were practically inactive despite the fact that nearly all the pethidine structure remained intact.

Analgesics (TA) numbers 20 to 63 were tested: the numbers indicate the chronological order of synthesis.

TABLE I

Analgesic activities of ethyl-4-phenyl piperidine-4-carboxylates relative to TA1.



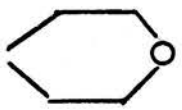
TA No.	R	Chemical nature and solvent	Relative analgesic activity	Intravenous LD50 mg/kg
Pethidine	-CH_3	HCl salt soluble in saline	15-30	60
1	-$CH_2.CH_2.N$ 	HCl salt soluble in saline	100	20
24	-$CH_2CH_2-O\,CH_2\,CH_3$	base soluble in $\frac{N}{10}$ HCl	125-103-85	20
25	-$(CH_2)_3-O\,CH_2\,CH_3$	Hbr salt soluble in alcohol	46-38-31[2]	15[1]
26	-H NORPETHIDINE	Hbr salt soluble in saline	4	60

TABLE I Contd:

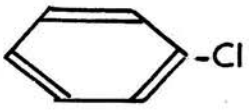
TA No.	R	Chemical nature and solvent	Relative analgesic activity	Intravenous LD50 mg/kg.
27	$-CH_2.CH_2.OPh$	Hbr salt soluble in saline	169-140-115	15
28	$-CH_2.CH_2.OCH_2Ph$	Hbr salt soluble in saline	177-145-117	$7\frac{1}{2}$
29	$-CH_2.CH_2.OMe$	base soluble in $\frac{N}{10}$ HCl	43-35-28[2]	20
30	$-CH_2.CH_2.O$ 	Hbr soluble in saline	15	20
31	$-CH_2.Ph$	HCl salt soluble in saline	5	25
32	$-(CH_2)_5-OC_2H_5$	base soluble in $\frac{N}{10}$ HCl	94-73-56[2]	$17\frac{1}{2}$
33	$-(CH_2)_4-OC_2H_5$	base soluble in $\frac{N}{10}$ HCl	368-265-191[2]	15

TABLE I Contd:

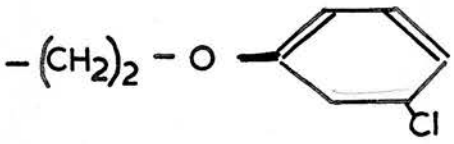
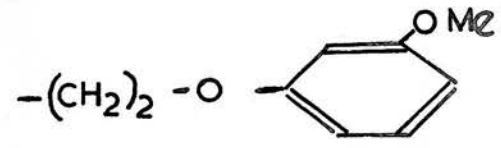
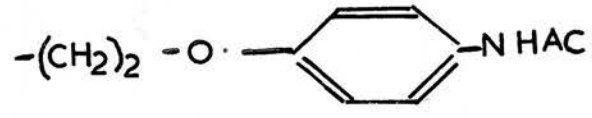
TA No.	R	Chemical nature and solvent	Relative analgesic activity	Intravenous LD50 mg/kg
34	$-(CH_2)_2-OPr^n$	base soluble in $\frac{N}{10}$ HCl	54- 44- 36[2]	22½
35	$-(CH_2)_2-OPr^i$	base soluble in $\frac{N}{10}$ HCl	50	12½
36	$-(CH_2)_2-OBu^n$	base soluble in $\frac{N}{10}$ HCl	50- 42- 35[2]	15
37		Hbr salt soluble in saline	40	30
38	$-(CH_2)_2-O-(CH_2)_2OEt$	base soluble in $\frac{N}{10}$ HCl	50- 39- 30	20
39		Hbr salt soluble in saline	40	15
40		Hbr salt soluble in saline	30	15

TABLE 1 Contd:

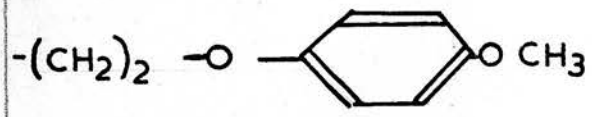
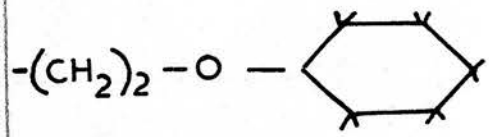
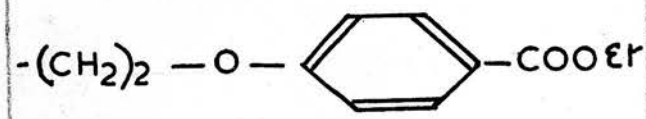
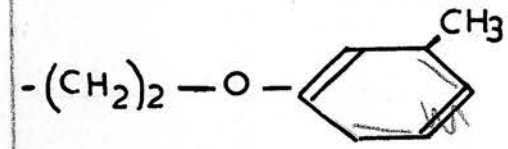
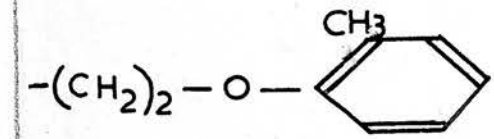
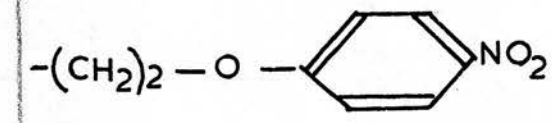
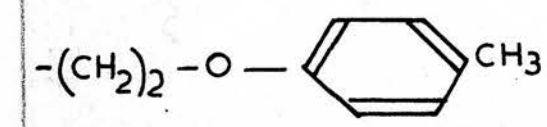
TA No.	R	Chemical nature and solvent	Relative analgesic activity	Intravenous LD50 mg/kg
41		Hbr salt soluble in saline	20	25
42		Hbr salt soluble in saline	25	12½
43		Hbr salt soluble in saline	5	40
44		Hbr salt soluble in alcohol	47-31-21	12½[1]
45		Hbr salt soluble in alcohol	17-12-9	20[1]
46		Hbr salt soluble in alcohol	10	25[1]
47		Hbr salt soluble in alcohol	20-19-18	15[1]

TABLE I Contd:

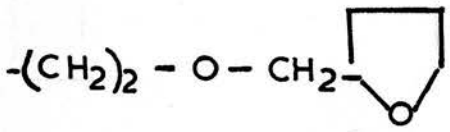
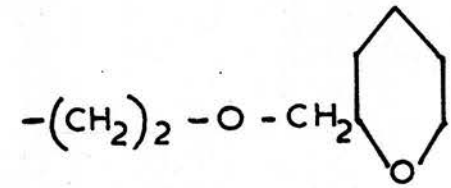
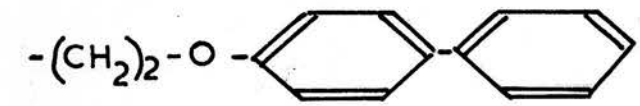
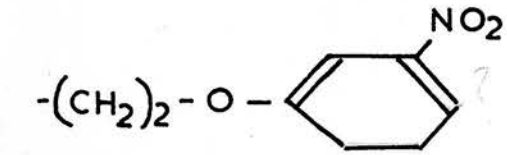
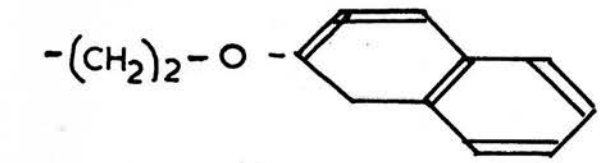
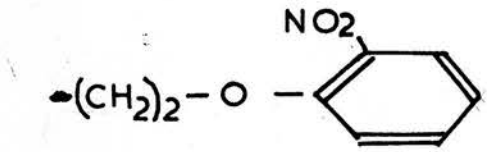
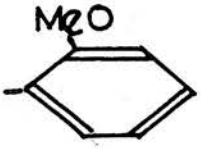
TA No.	R	Chemical nature and solvent	Relative analgesic activity	Intra-venous LD50 mg/kg
48		base soluble in N/10 HCl	960-729-564	25
49		base soluble in N/10 HCl	420-265-167	10
51		Hbr salt soluble in saline	15	40
53		Hbr salt soluble in saline	7	50
54		Hbr salt soluble in alcohol	5	-
55		Hbr salt soluble in alcohol	5	-
56	$-(CH_2)_2 . S\,Ph$	Hbr salt soluble in alcohol	10	-

TABLE 1 Contd:

TA No.	R	Chemical nature and solvent	Relative analgesic activity	Intravenous LD50 mg/kg
57	$-CH_2-$ 	base soluble in $\frac{N}{10}$ HCl	15	45
58	$-(CH_2)_2.SEt$ $-(CH_2)_2SET$	base soluble in $\frac{N}{10}$ HCl	5	50
59	$-(CH_2)_2.SMe$ $-(CH_2)_2SMe$	base soluble in $\frac{N}{10}$ HCl	15	40
60	$-(CH_2)_2-O-$ 	base soluble in $\frac{N}{10}$ HCl	10	30
61	$-(CH_2)_2-O-(CH_2)_2OPh$	Hbr salt soluble in saline	30	20
62	$-(CH_2)_4OPh$	Hbr salt soluble in saline	40	$17\frac{1}{2}$
63	$-(CH_2)_4-O-CH_2$ 	base soluble in $\frac{N}{10}$ HCl	125	$7\frac{1}{2}$

[1] In order to determine the intravenous toxicity of these hydrobromides they were dissolved in warm saline. These salts, however, come out of solution on cooling and the values given here may be inaccurate.

[2] These values for analgesic activity are relative to TA24 which is regarded as 100.

PROTOCOL

The results of the cross-over tests are listed in TABLE I and the analysis of variance for each cross-over test is also given. An example of the method of calculation is given below.

The results are listed in two separate tables A. and B. to enable different totals to be obtained more easily. The figures are the total analgesic indices for each rat multiplied by a hundred. As mentioned before, the pain threshold is observed at ten minute intervals for an hour. The analgesic index for each reading is calculated and the six indices added. In Table A. the results are listed so that the total response of any one cage of rats on any one day may be obtained and also the mean response for each dose. Table B. gives the total response of all the rats on any one day and the total response of each rat over the test.

Since the solutions compared do not contain the same active principle the estimate of the potency of the unknown solution has to be calculated. An estimate of the potency of the unknown solution can be made by using the two regression lines. The horizontal distance (M) between the lines is the difference between log doses producing the same effect and is therefore the log of the ratio of un-

known to standard. By elementary geometry

$$M = \frac{\bar{Y}u - \bar{Y}s}{b}$$

where $\bar{Y}u$ and $\bar{Y}s$ are the mean responses to test analgesics and standard respectively.

"b", the regression coefficient, is the slope of each line.

CALCULATION OF RESULT

(1) $$M = \frac{\bar{Y}u - \bar{Y}s}{b}$$

$$= \frac{\Sigma[(U_H + U_L) - (S_L + S_H)] \times d}{\Sigma[(U_H + S_H) - (U_L + S_L)]}$$

where U_H = response to the higher dose of unknown etc. and
d = log high dose - log low dose.

$$M_{3s} = \frac{[(2183+4645)-(4472+2201)] \times 0.301}{[(2183+2201)-(4645+4472)]}$$

$$= 0.0099 \text{ or } \bar{1}.9901$$

R antilog M = 0.98

$M_{4s} = \bar{1}.9596$ $\quad R_{4s} = 0.91$

$M_{4g} = \bar{1}.8213$ $\quad R_{4g} = 0.66$

(2) CALCULATION OF ERROR

The regression coefficient "b" is the slope of each line and is therefore equal to

$$b = \frac{\text{sum of responses to high doses.} - \text{sum of responses to low doses.}}{\frac{1}{2}n \times (\text{log high dose} - \text{log low dose})}$$

(If the deviation from parallelism is significant the regression coefficients of each comparison are calculated separately. Otherwise the responses

are "pooled" to give a composite estimate of "b").

In this case:

$$b_{38} = \frac{(2183+2201) - (4645+4472)}{48 \times 0.301} = \underline{327}$$

$b_{48} = \underline{300}$

$b_{49} = \underline{207}$

In order to make a statement about the reliability of the estimate R, the standard error (Sm) of M must be calculated.

The error of the assay can be obtained through an analysis of variance.

Sum of squares of deviations attributable to the variation.

1) between days

$= \frac{1}{4}$ (FIRST DAY TOTAL^2 + 2nd DAY TOTAL^2 etc.) - $\frac{\text{Sum of all day totals}^2}{\text{Total no. of observations}}$

$= \underline{47354}$

2) between high doses and low doses (regression)

$$= \frac{(\Sigma \text{ high doses} - \Sigma \text{low doses})^2}{n}$$

$= \underline{591419}$

3) between drugs

$$= \Sigma \frac{(\text{high+low doses of each drug})^2}{24} - \text{mt} \quad \left(\text{where mt} = \frac{29211^2}{96}\right)$$

$= \underline{81231}$

The quantity

$$\frac{\Sigma \text{dose total}^2}{12} - mt = 749995$$

is equal to the variance due to ~~days~~ always, regression and deviation from parallelism. The variance due to deviation from parallelism is obtained by subtraction.

Variance due to deviation from parallelism = 77345

Variation due to rats

is equal to the sum of the squares of the deviation of the responses of each rat, from the total of the mean responses to the same four doses, divided by 4

= 277992

4) The quantity

$$\frac{\Sigma \text{cage totals on each day}^2}{8} - mt = 193087$$

is the sum of the variance due to differences between cages and days and the interaction of days and cages. The interaction between days and cages is given by subtraction.

Variance due to differences between cages

$$= \frac{\Sigma \text{cage totals for four day}^2}{32} - mt = 91958$$

Variance due to interaction days and cages

= 193087 - 91958 - 47354

= 53775

5) The quantity

$$\frac{\Sigma(\text{total responses for each dose on each day}^2)}{3} - mt$$

consists of the variance due to differences between doses and days and the interaction between days and doses.

Interaction days and doses = 102185

The sum of squares of deviations of all the observations from the common mean = total sums of squares

= (Σ(each observation^2) - mt = 1552768

When all the sums of squares due to known causes have been subtracted from the total the remainder is the residual variance of the assay.

If certain known sources of variation are not significantly different from the residual variance they are added to that sum to give the error of the assay.

Variance $S^2Y = \frac{663461}{83} = 7994$

When "g", the index of significance of b, is less than 0.1 it can be neglected and the standard error of M can be calculated from Schild's formula.

$$g = \frac{\text{variance of b } t^2 \text{ (students t)}}{b^2}$$

$$\text{Variance of b} = \frac{S^2Y}{nd^2}$$

$G_{38} = 0.03$: $G_{48} = 0.04$: $G_{49} = 0.08$

Schild's formula

$$S^2M = \frac{4S^2Y}{nb^2}\left(\frac{M^2}{d^2} + 1\right)$$

for TA38

$$S^2M = \frac{4 \times 7994}{96 \times 327^2}\left(\frac{0.01^2}{0.09} + 1\right)$$

$$S^2M = 0.003117$$

$$Sm = 0.0558$$

Limits of M = $M \pm sm \times t$

t for d^oF 83 and P = 0.05 is 2

$\therefore$ Sm x t = 0.1116

Limits of M_{88} = 0.1017 and $\bar{1}$.8785

Limits of R_{88} = 1.26 - 0.98 - 0.76

Compared with TA1 (adjusted for differences in dosage)	50.4	- 39.1	- 30.4%
TA48	960	- 729	- 564%
TA49	420	- 265	- 167%

TABLE A

PROTOCOL OF CROSS-OVER TEST

Drug	Dose mg /kg.	First Day			Second Day			Third Day			Fourth Day			Sum	Mean total for each dose	Mean analgesic index for each dose
		Cage 1	2	3	Cage 1	2	3	Cage 1	2	3	Cage 1	2	3			
TA1	2	371	331	510	277	483	298	293	366	365	381	401	396	4472	373	0.62
	4	189	285	97	91	278	230	0	276	155	192	179	229	2201	183	0.30
TA38	5	308	315	426	370	383	436	313	244	581	465	392	412	4645	387	0.64
	10	72	37	323	136	00	404	266	0	430	185	136	194	2183	182	0.30
TA48	¼	243	344	277	456	422	563	243	352	506	402	426	431	4665	389	0.65
	½	164	325	123	307	159	242	0	92	324	231	237	387	2591	216	0.36
TA49	½	272	358	369	537	372	292	379	437	404	414	330	427	4591	383	0.64
	1	209	320	288	348	308	338	310	435	276	346	320	365	3863	322	0.54
		1828	2315	2413	2522	2405	2803	1804	2202	3041	2616	2421	2841	29211		

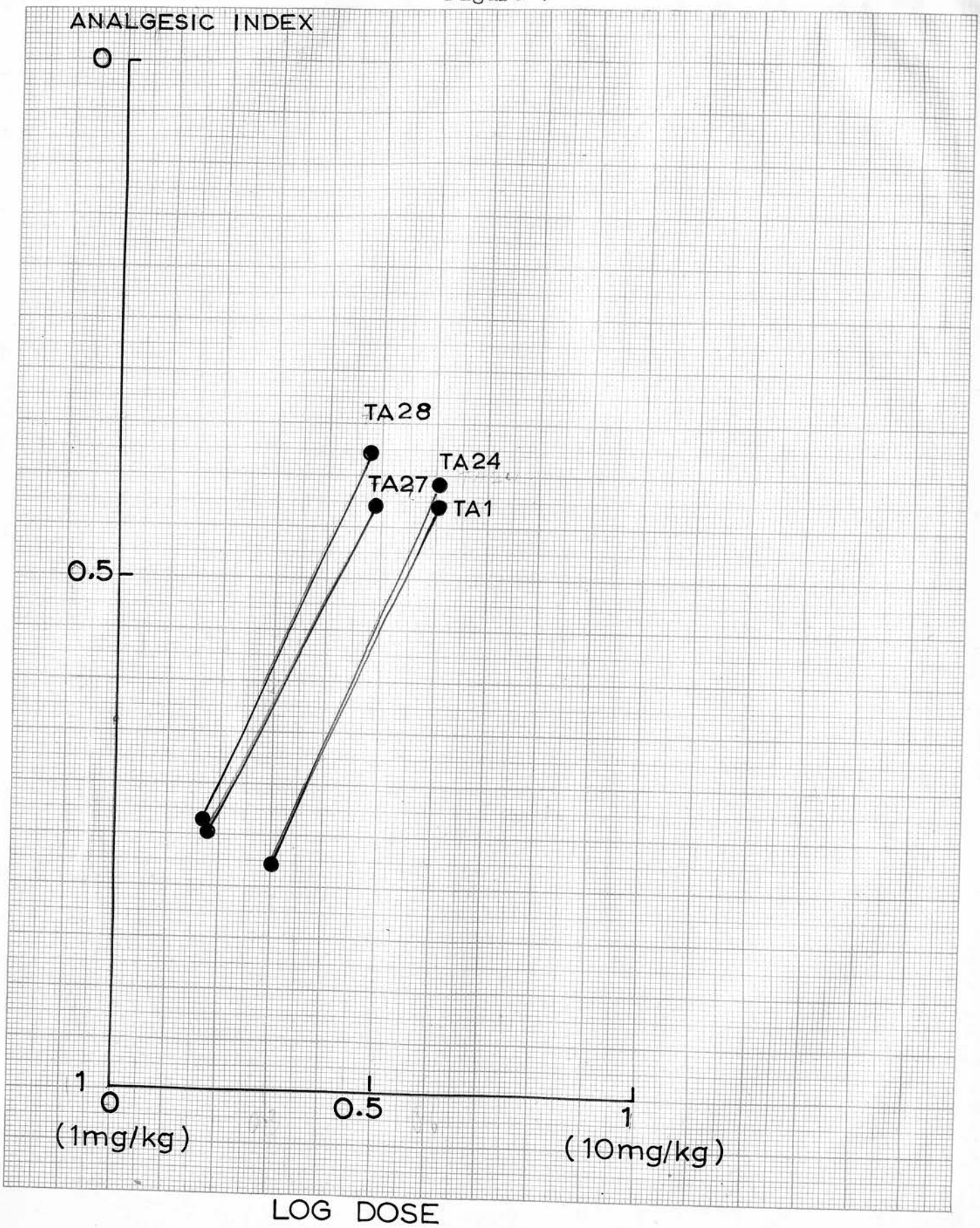
TABLE B

(see text - p.34)

Cage	Rat No.	1st DAY		2nd DAY		3rd DAY		4th DAY		SUM
	1	1:4	189	38:5	370	48:¼	243	49:1	346	1148
	2	48:¼	243	38:10	136	49:½	379	1:2	381	1139
	3	48:½	164	49:1	348	38:5	313	1:4	192	1017
1	4	38:5	308	1:4	91	49:1	310	48:¼	402	1111
	5	1:2	371	49:½	537	38:10	266	48:½	231	1405
	6	49:½	272	1:2	277	48:½	0	38:10	185	734
	7	38:10	72	48:½	307	1:2	293	49:½	414	1086
	8	49:1	209	48:¼	456	1:4	0	38:5	465	1130
	9	1:4	285	49:½	372	48:½	92	38:5	392	1141
	10	48:¼	344	1:2	483	49:1	435	38:10	136	1398
	11	38:10	37	48:¼	422	1:2	366	49:1	320	1145
2	12	38:5	315	48:½	159	49:½	437	1:4	179	1090
	13	49:1	320	38:10	0	48:¼	352	1:2	401	1073
	14	1:2	331	49:1	308	38:10	0	48:¼	426	1065
	15	48:½	325	1:4	278	38:5	244	49:½	330	1177
	16	49:½	358	38:5	383	1:4	276	48:½	237	1254
	17	38:5	426	48:½	242	49:1	276	1:2	396	1340
	18	1:2	510	38:5	436	48:½	324	49:1	365	1635
	19	49:½	369	1:4	230	38:10	430	48:¼	431	1460
3	20	49:1	288	48:¼	563	38:5	581	1:4	387	1819
	21	38:10	323	1:2	298	49:½	404	48:½	229	1254
	22	48:¼	277	49:½	292	1:4	155	38:10	194	918
	23	48:½	123	49:1	338	1:2	365	38:5	412	1238
	24	1:4	97	38:10	404	48:¼	506	49:½	427	1434
			6556		7730		7047		7878	29211

The figures in blue refer to drug number and the dose in mg./kg. (for example: 48:¼ means TA48, ¼ mg./kg.)

Figure 7



LOG DOSE RESPONSE LINES OF TA1, TA24, TA27 and TA28
CROSS-OVER TEST NO.1

COMPARISON OF TAI, 24, 27 and 28: ANALYSES OF VARIANCE

CROSS-OVER TEST NO.1

Source of variation	Sum of Squares	d.f.	Variance	F	P
Drugs	9020	3	3007	4.7	$> 5\%$
Regression	1056301	1	1056301	74.8	$< 0.1\%$
[1] Deviation from parallelism	3384	3	1128	11.0	$< 0.1\%$
Doses	1068705	7			
Days	26567	3	8856	1.59	$> 20\%$
Cages (rat inter cage variation)	57152	2	28576	2.02	$> 10\%$
Cages (rat intra cage variation)	200780	21	9561	1.48	$> 10\%$
Rats	257932	23	11214	1.26	$> 20\%$
Interaction days and cages	193724	6	32287	2.29	$< 5\%$
Interaction days and doses	76968	21	3665	3.85	$< 1\%$
Residual variance	494137	35	14118		
Total sums of squares	2118033	95			

[1] For calculation of the variance ratio for deviation from parallelism the sum of squares due to the variation between rats in a cage was added to the residual variance.

Residual variance plus intra rat cage variance	694917	56	12409		

RESULT OF TEST (compared with TA1)

TA24 125 - 103 - 84.6% (L = 5.9)

TA27 169 - 140 - 115%

TA28 177 - 145 - 117%

Figure 8

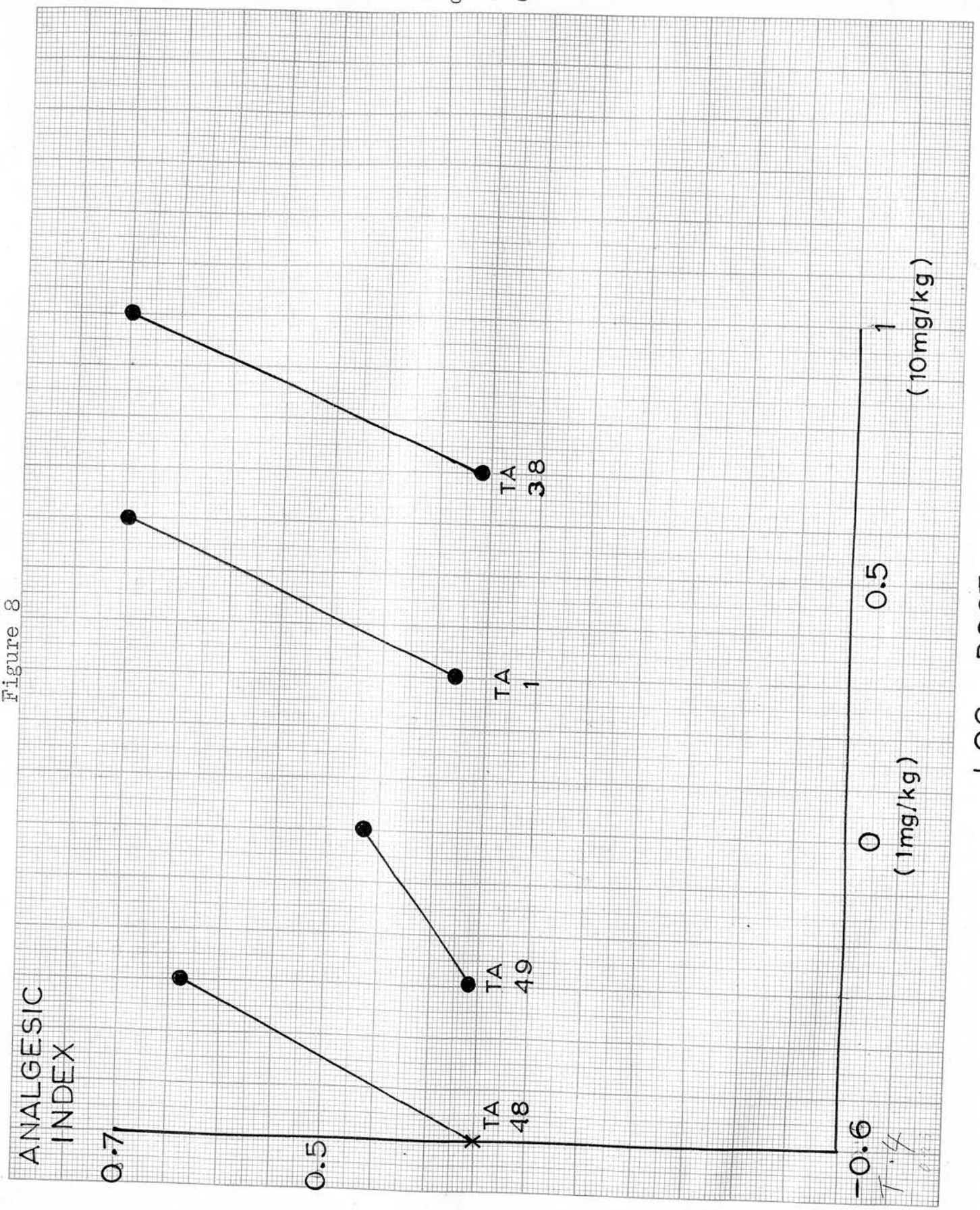


Figure 8

LOG DOSE RESPONSE LINES OF TA1, TA38, TA48 and TA49
CROSS-OVER TEST NO.2

COMPARISON OF TA1, 38, 48 and 49: ANALYSES OF VARIANCE

CROSS-OVER TEST No.2

Source of variation	Sum of Squares	d.f.	Variance	F	P
Drugs	81231	3	27077	2.95	<5%
Regression	591419	1	591419	64.4	<1%
[1] Deviation from parallelism	77345	3	25782	2.85	5%
Doses	749995	7			
Days	47354	3	15784	1.7	<20%
Cages (rat inter cage variation)	91958	2	45979	5.0	>1%
[2] cages (rat intra cage variation)	186034	21	8859	1.04	>20%
Rats	277992	23	12087	1.32	>20%
[2] Interaction days and cages	53775	6	8962	1.02	>20%
[2] Interaction days and doses	102185	21	4866	1.89	5%
Residual variance	321467	35	9185		
Total sums of squares due to error	1552768	95			

[1] For calculation of the variance ratio for deviation from parallelism the sum of squares due to the variation between rats in a cage is added to the residual variance.

Residual variance plus intra rat cage variance	507501	56	9063		

[2] The sums of squares of error due to differences between rats in a cage, interaction days and cages and interaction days and doses are not significant and are therefore added to the residual variance.

Error variance	663461	83	7994		

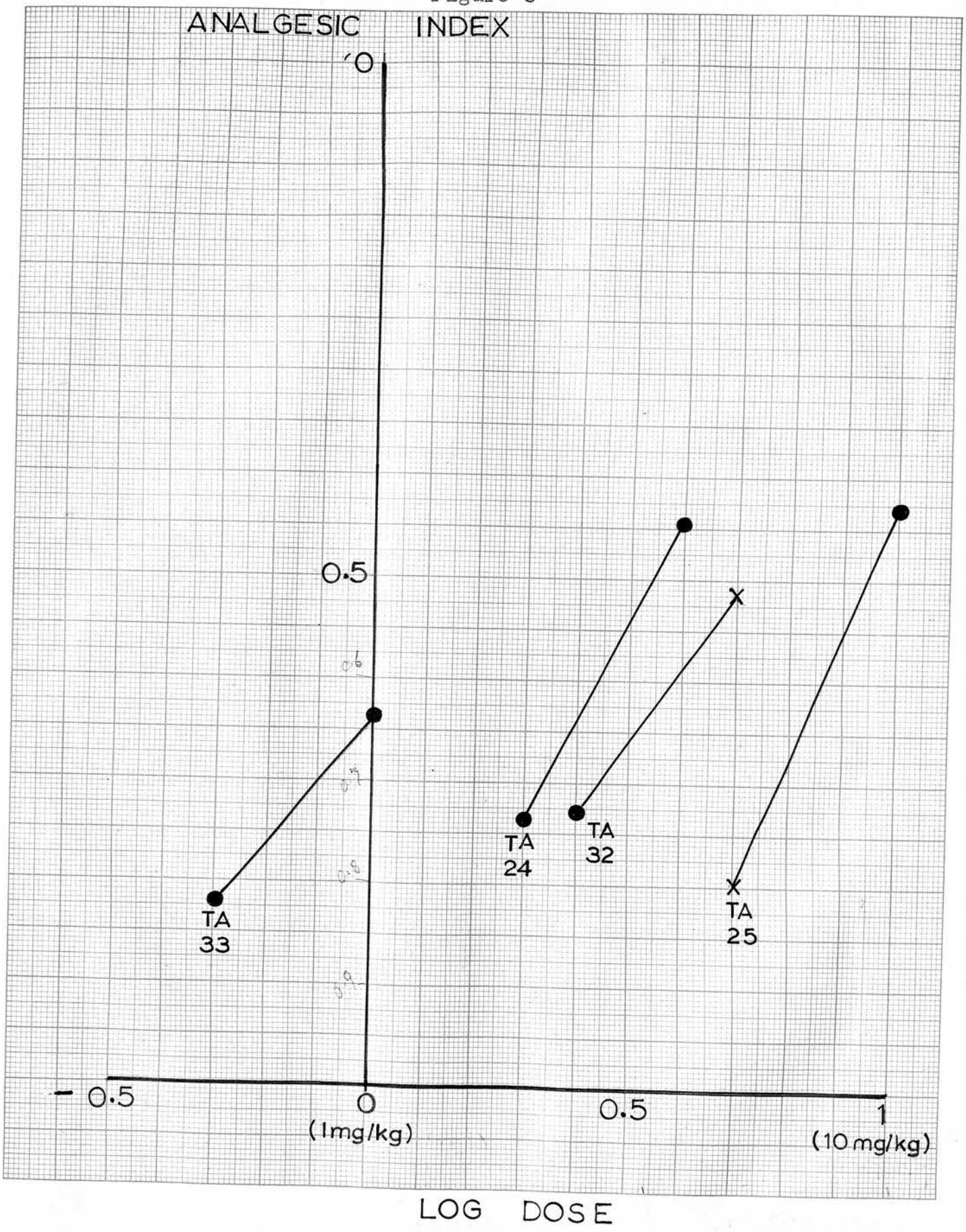
RESULT OF TEST (compared with TA1)

TA38 50.4 - 39.1 - 30.4% (L=3.5)

TA48 960 - 729 - 564% (L=3.2)

TA49 420 - 265 - 167% (L=2.1)

Figure 9



LOG DOSE RESPONSE LINES OF TA24, TA25, TA32 and TA33
CROSS-OVER TEST NO.3

COMPARISON OF TA24, 25, 32 and 33: ANALYSES OF VARIANCE

CROSS-OVER TEST NO.3

Source of variation	Sum of Squares	d.f.	Variance	F	P
Drugs	99052	3	33017	10.7	<1%
Regression	593304	1	593304	192	<1%
[1] Deviation from parallelism	44283	3	14751	1.60	<20%
Doses	736609	7			
Days	140596	3	46853	15.1	<1%
Cages (rat inter cage variance	48601	2	24301	7.84	<1%
Cages (intra rat cage variance)	408975	21	19475	6.29	<1%
Rats	457576	23	19895	6.42	<1%
Interaction days and cages	125071	6	20845	6.7	<1%
Interaction days and doses	397816	21	18944	6.1	<1%
Residual variance	108422	35	3098		
Sum of squares due to error	1966090	95			

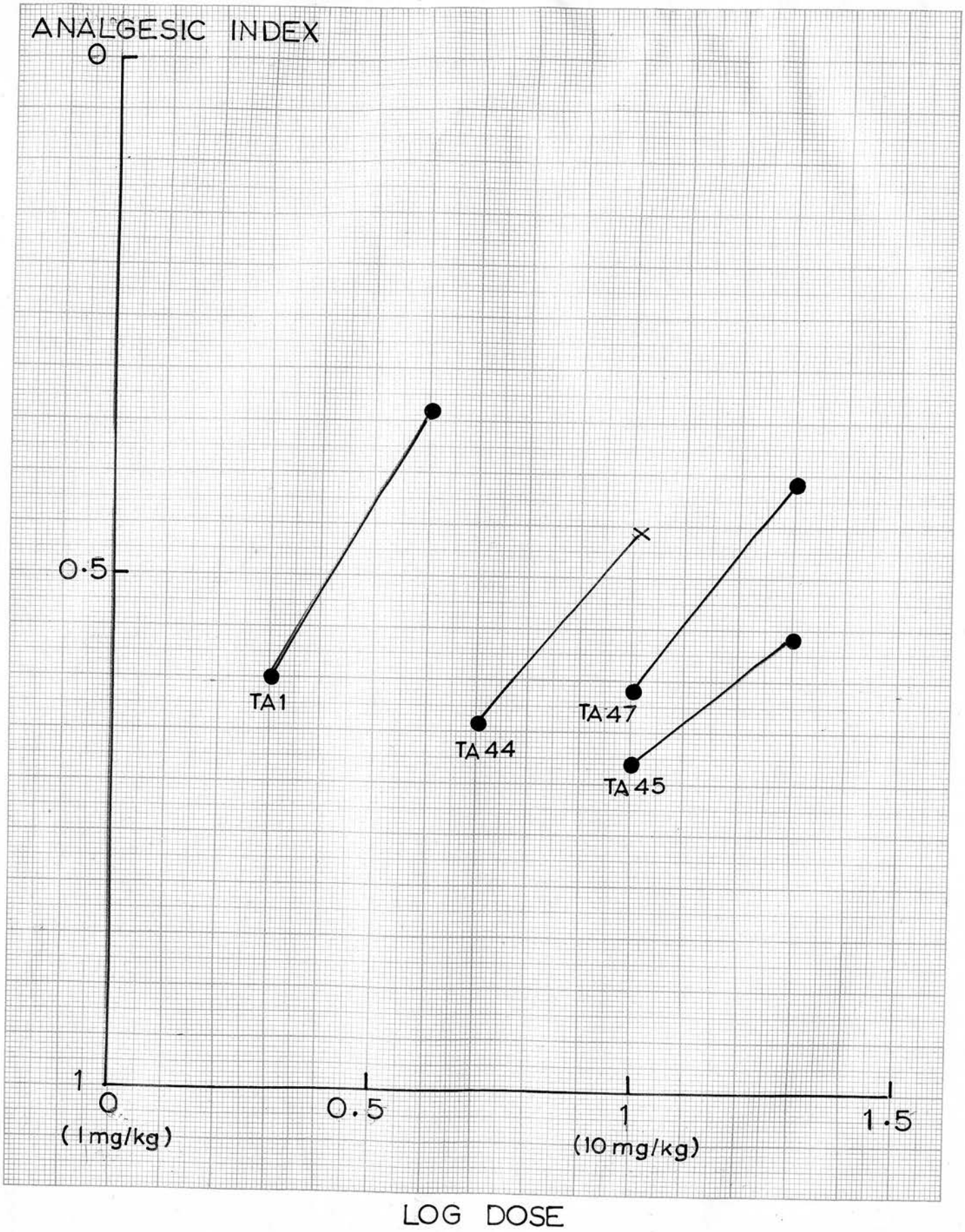
[1] For calculation of the variance ratio for deviation from parallelism the sum of squares due to the variation between rats in a cage is added to the residual variance.

Residual variance plus intra rat cage variance	517397	56	9239	

RESULT OF TEST (potency in terms of TA24)

TA25 46.5 - 38.3 - 31.5% (L=5.9)
TA32 94.4 - 73.2 - 56.4% (L=4.5)
TA33 368 - 265 - 191% (L=4.2)

Figure 10



LOG DOSE RESPONSE LINES OF TA1, TA44, TA45 and TA47
CROSS-OVER TEST NO.4

COMPARISON OF TA1, 44, 45 and 47: ANALYSES OF VARIANCE

CROSS-OVER TEST No.4

Source of variation	Sum of Squares	d.f.	Variance	F	P
Drugs	105910	3	35303	6.8	<1%
Regression	300832	1	300832	58.4	<1%
[1] Deviation from parallelism	20429	3	6809	1.2	>20%
Doses	427171	7			
Days	158341	3	52780	10.25	<1%
Cages (rat inter cage variation)	66416	2	33208	6.45	<1%
Cages (intra rat cage variation)	277116	21	13196	2.56	<1%
Rats	343532	23	14936	2.9	<1%
Interaction days and cages	187808	6	31301	6.1	<1%
Interaction days and doses	239960	21	11427	2.2	<5%
Residual variance	180220	35	5149		
Sum of squares due to error	1537032	95			

[1] For calculation of the variance ratio for deviation from parallelism the sum of squares due to the variation between rats in a cage was added to the residual variance.

Residual variance plus intra rat cage variance	457336	56	8167		

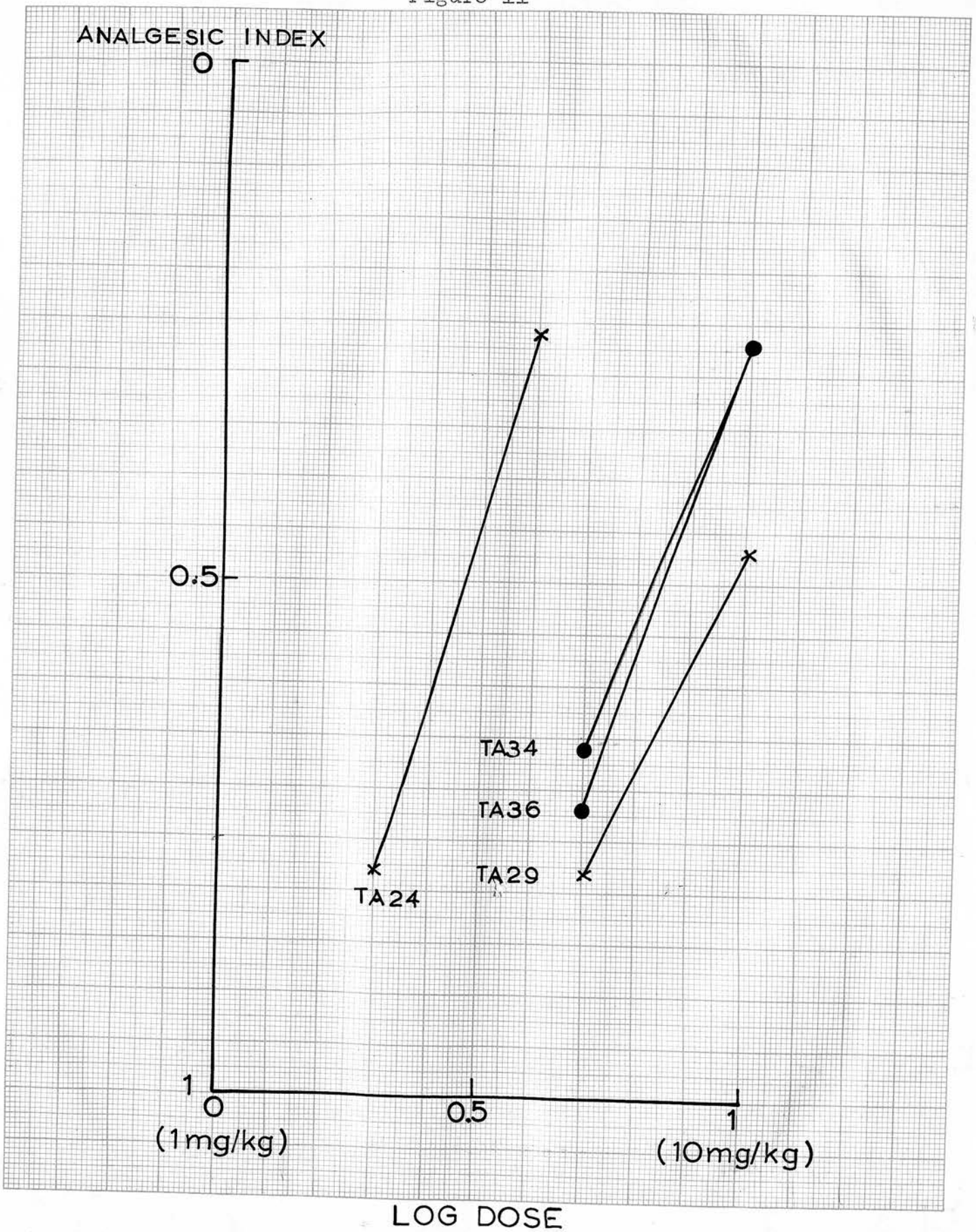
RESULT OF TEST (compared with TA1)

TA44 46.6 - 31.0 - 20.7% (L=3.0)

TA45 17.0 - 12.4 - 9.2% (L=2.6 "g" is significant)

TA47 20.4 - 19.5 - 17.8% (L=3.1)

Figure 11



LOG DOSE RESPONSE LINES OF TA24, TA29, TA34 and TA36
CROSS-OVER TEST NO.5

COMPARISON OF TA24, 29, 34 and 36: ANALYSES OF VARIANCE

CROSS-OVER TEST No.5

Source of variation	Sum of Squares	d.f.	Variance	F	P
Drugs	101802	3	33934	5.1	$< 1\%$
Regression	1549146	1	1549146	233	$< 1\%$
[1]Deviation from parallelism	44415	3	14805	1.90	$> 10\%$
Doses	1695363	7			
Days	50434	3	16811	2.53	$< 10\%$
Cages (rat inter cage variation)	31672	2	15836	2.38	$> 10\%$
[2]Cages (cage intra rat variation)	204840	21	9754	1.47	$< 20\%$
Rats	236512	23	10283	1.55	20%
Interaction days and cages	122922	6	20487	3.08	$> 1\%$
[2]Interaction days and doses	237217	21	11296	1.7	$> 5\%$
Residual variance	232491	35	6642		
Sum of squares ~~due to error~~	2574939	95			

[1] For calculation of the variance ratio for deviation from parallelism the sum of squares due to the variation between rats in a cage is added to the residual variance.

Residual variance plus intra rat cage variance	437331	56	7809		

[2] The sums of squares of error due to differences between rats in a cage and interaction between days and doses are not significantly different from the residual variance. They are added to the residual variance to give the error variance for the assay.

Error variance	674548	77	8760		

RESULT OF TEST (compared with TA24)

TA29 43.2 - 34.8 - 27.6% (L=4.6)
TA34 53.6 - 43.6 - 35.6% (L=4.9)
TA36 49.7 - 41.6 - 34.8% (L=5.2)

For convenience the series is divided into groups.

Group I - compounds with aliphatic side chains.

Subgroup A consisting of:

$\diagdown$N-$(CH)_2$OR where

R =	Me	Et	Pr^n	Pr^i	Bu^r	Cyclohexyl ring
Drug number =	TA29	TA24	TA34	TA35	TA36	TA42
Relative activity =	35	100	44	50	42	25

The optimum alkyl group seems to be ethyl.

Subgroup B consisting of:

$\diagdown$N-$(CH_2)_n$OEt. where

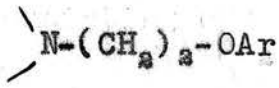
n =	2	3	4	5
Drug number =	TA24	TA25	TA33	TA32
Activity =	100	38	265	73

The two carbon unit seems to have some special significance since substances with n equal to odd numbers are less active than even numbers. The optimum distance of the oxygen atom from the nitrogen may be more than four carbon atoms.

TA26 is norpethidine the starting substance for the synthesis of these compounds. TA38 has a "repeating" ether oxygen group and is quite active.

Group II - compounds with aromatic side chains.

Subgroup A consisting of:



Ar =	Ph	CH_2Ph	Ph-Ph
Drug number =	TA27	TA28	TA51
Activity =	140	145	15

TA31 has a short chain (one methylene group) and an unsubstituted benzene ring. The ether oxygen atom is not present. This substance is inactive.

Subgroup B compounds where the benzene ring is substituted.

In cases of disubstitution on the benzene ring the primary substituted group R_1, in this case oxygen, not only determines the effectiveness of the second substitution but also the rate. Since oxygen has a "partial negative charge" there is a tendency for R_2 to be directed into the ortho and para positions. In other words, oxygen "repels" electrons into the benzene ring.

Hey (1952) found that the polarity of the "ether" oxygen atom of choline derivatives is an important factor in determining the nicotine-like stimulant actions of these compounds, and that

reduction of the electron density is associated with increased activity. It was hoped that the activity of TA27 could be increased by alteration of the electron density by introduction of a substituent on the benzene ring. A large number of compounds of this type were prepared and they were all less active than TA1. Electron repelling groups were better as second substituents. Substitution in the meta position gave the best analgesics of the substituted benzene derivatives.

Substitution of the benzene ring is deleterious to activity.

Group III - compounds where an oxygen atom has been replaced by a sulphur atom.

Sulphur compounds	analogous and oxygen compounds	Relative activities
TA18	TA1 (Millar and Stephenson, 1956)	33:100
TA58	TA24	5:103
TA59	TA29	15:35
TA56	TA27	10:140

Substitution of sulphur for oxygen leads to a marked reduction in activity.

Group IV - Cyclic ethers and compounds with more than one ether group.

a) substances with one oxygen atom in an ether unit:

TA24 $\diagdown$N-$(CH_2)_2$OEt Activity 103

TA62 $\diagdown$N-$(CH_2)_4$OPh Activity 40

b) substance with one oxygen atom in a cyclic ether group:

TA57 Activity 15

c) substances with 2 oxygen atoms both contained in ether oxygen units:

TA38 $\diagdown$N-$(CH_2)_2$-O-$(CH_2)_2$OEt Activity 40

TA61 $\diagdown$N-$(CH_2)_2$-O-$(CH_2)_2$OPh Activity 30

d) substances with 2 oxygen atoms, one contained in an ether unit and the terminal unit being a cyclic ether function.

TA48 Activity 730

TA49 Activity 265

TA63 Activity 125

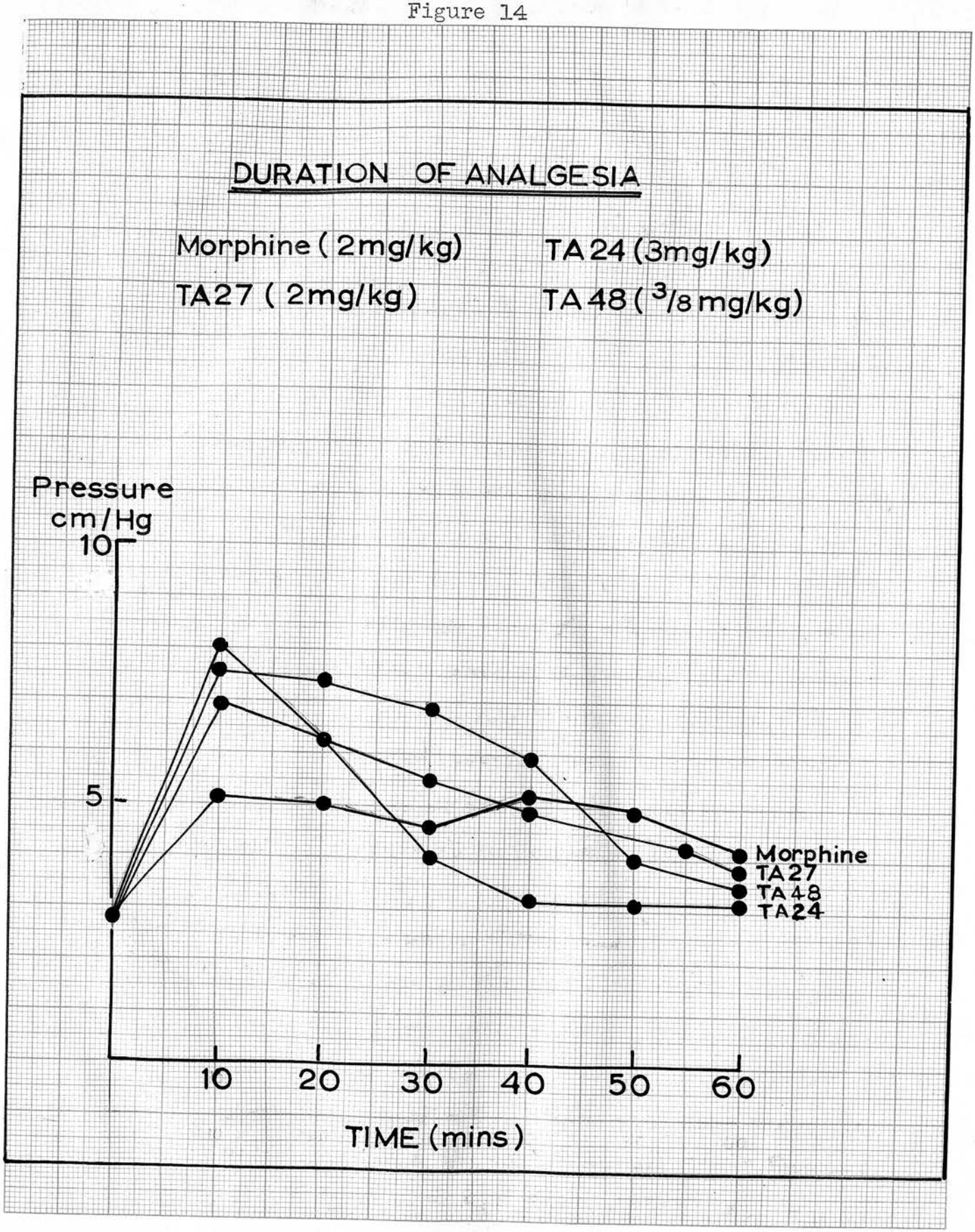
It seems that there are two important positions for the oxygen atom: 1) when it is itself the third

atom or 2) about the fifth atom away from the nitrogen (compare TA24 with TA33 relative activities 2:5). Incorporation of the second oxygen atom as a cyclic ether function leads to a marked increase in potency (compare TA24 with TA48 and TA49 ratio activities 1:7:2½). However, with the first oxygen atom contained in a cyclic ether function there is a reduction in activity (compare TA57 to T24, ratio of activities 1:6). This indicates a difference in the role of the two oxygen atoms.

On the basis of these results three further compounds were prepared by Dr. Stern and his colleagues. TA61, analogous to TA38, has a repeating ether oxygen unit with a terminal phenyl group similar to TA27.

TA62 has four methylene units analogous to TA33 but with a phenyl group instead of the ethyl. TA63 has four methylene units plus a cyclic ether unit analogous to TA48. Replacement of the alkyl group leads to a reduction in activity (compare TA33, TA62 ratio activities 5:1, and TA38 with 61 ratio activities 4:3) in contrast to earlier findings (compare TA24, TA27 103:140). It is interesting that TA63 with a terminal cyclic ether unit, is three times as active as TA62. This confirms that introduction of a second oxygen as a cyclic ether

Figure 14


DURATION OF ANALGESIA
Morphine (2mg/kg)
TA24 (3mg/kg)
TA27 (2mg/kg)
TA48 (3/8 mg/kg)
Pressure
cm/Hg
10
5
Morphine
TA27
TA48
TA24
10
20
30
40
50
60
TIME (mins)

function increases activity.

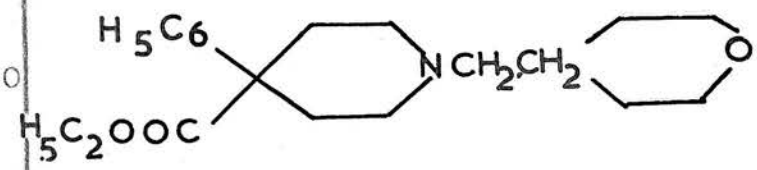
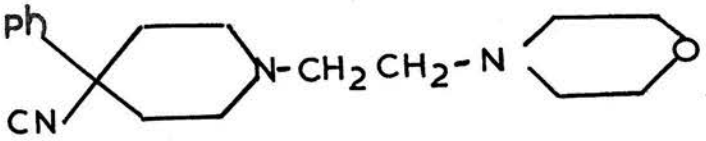
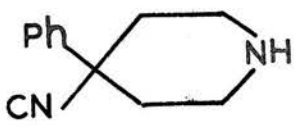
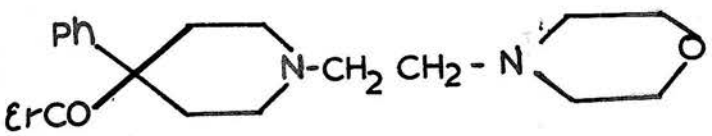
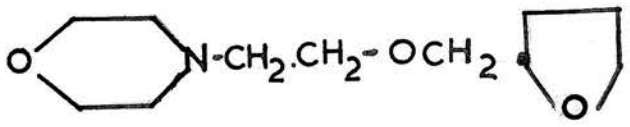
Some other drugs were tested (Table II).

TA20 is analogous to TA1 but the "morpholino" ring lacks the nitrogen atom (ratio activities TA20: TA1, 9:10). TA21 and TA23 are analogous to TA1 but the 4-acyloxy group is substituted. Both these compounds are inactive. TA22 has a substituted 4-acyloxy group and is inactive. TA50 has three oxygen atoms, one in a morpholino ring, another in an ether oxygen unit and the last in a cyclic ether function. It is inactive. TA22 and TA50 lack the pethidine nucleus and might be expected to be inactive. Many workers have shown that substitution on the 4-acyloxy group of pethidine is deleterious to activity (Goodman and Gilman, 1955).

Morphine has been compared to TA1. The log dose effect curves appeared to be parallel. The potency ratio of morphine to TA1 was 1.2. However, this figure may be misleading because if the mean analgesic index was calculated beyond an hour morphine would appear more potent. Morphine took rather longer to reach its peak effect, 20 to 30 min. but maintained this level. The test analgesics, including TA1, reached their peak effect in about 10 min. This was not maintained and the effect diminished relatively rapidly (see Figure 14). The

TABLE II

Relative analgesic activities of other substances tested

Name of drug	FORMULA	Chemical nature and solvent	Relative analgesic activity to TA1 (100)	Intra-venous LD50 mg/kg
MOR-PHINE	See FIGURE 1	HCl salt soluble in saline	120	90
TA20		base soluble in N/10 HCl	90	30-40
TA21		HCl salt soluble in saline	2	70
TA22		HCl salt soluble in saline	2	20
TA23		HCl salt soluble in saline	NIL	60
TA50		base soluble in N/10 HCl	NIL	> 80

relative potency of morphine to other drugs was as follows: Morphine 100%; TA24 80%; TA48 700%; TA27 100% and TA1 100%.

TA48 and TA1 were given by mouth and tested for analgesic activity. Three separate tests were carried out on 24 rats. A high and a low dose of each drug was given subcutaneously and orally so that for each route a particular dose was given to 3 rats.

TA48 was found to be almost as active by mouth as by the subcutaneous route. The analgesic indices were, for the subcutaneous route:

TA48 $\frac{1}{2}$ mg./kg. 0.39; TA48 $\frac{1}{4}$ mg./kg. 0.65

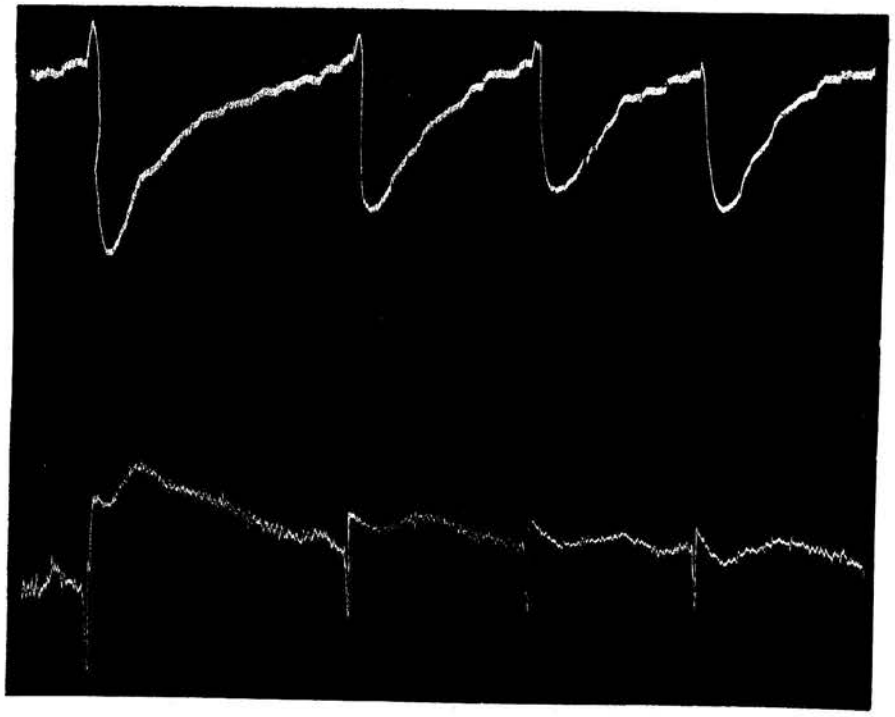
for the oral route:

$\frac{1}{2}$ mg./kg. 0.41; $\frac{1}{4}$ mg./kg. 0.68

The peak analgesic effect was found to occur 10 minutes after the subcutaneous injections and 20 minutes after oral administration.

TA1 is about a fifth as active by mouth in comparison to subcutaneous administration. Tests were carried out giving saline by mouth and by injection. Both procedures only cause temporary and minor discomfiture and the pain thresholds did not alter.

Figure 15



Action of Pethidine derivatives on the respiration and blood pressure of the rat. Upper record is the minute respiratory volume and lower record is the blood pressure.

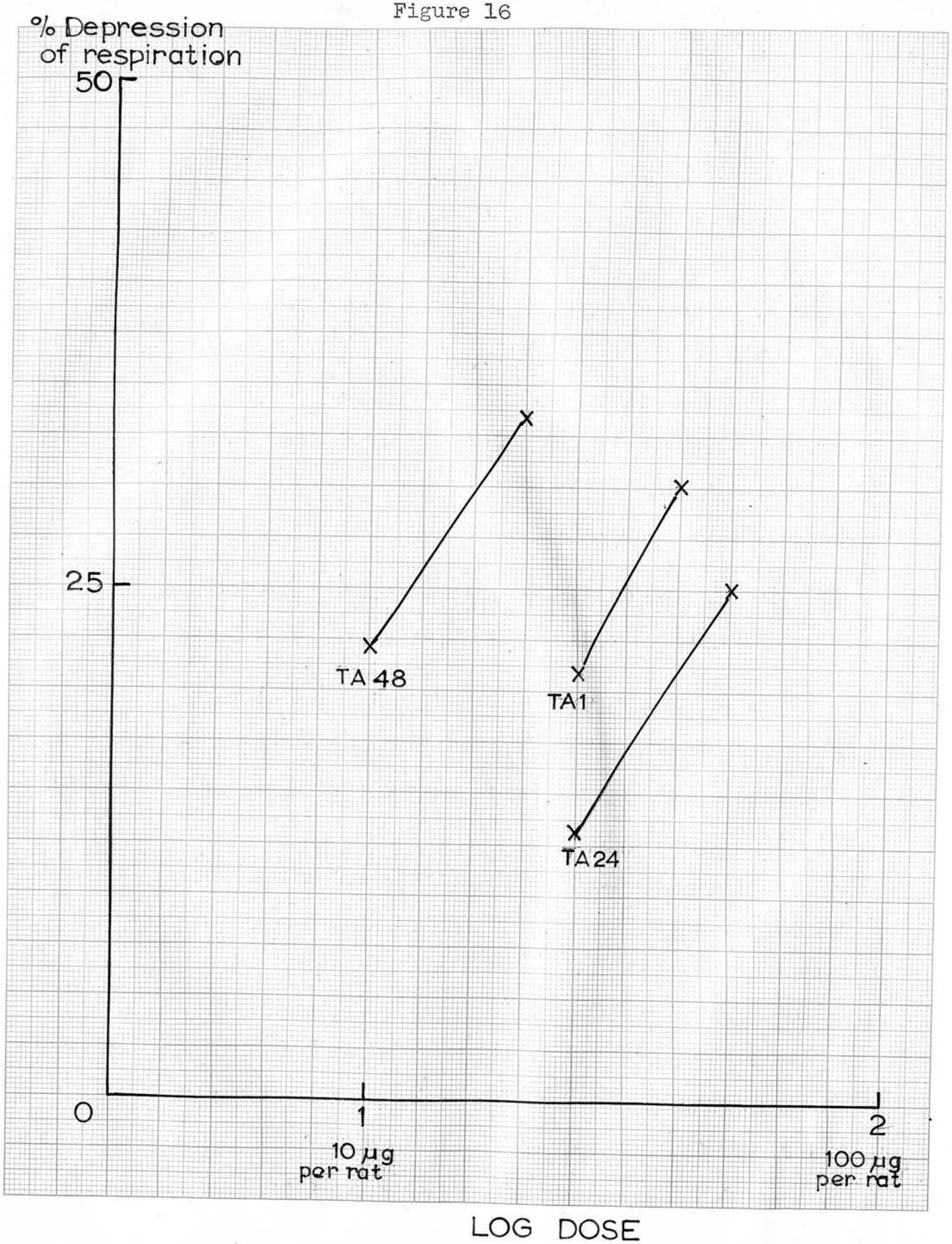
RESULTS OF RESPIRATION EXPERIMENTS

It is not usual to use the rat for the study of respiratory depression. The cat is usually the animal chosen. However, cats are expensive and difficult to obtain.

It was found difficult to use the rat for this purpose. Rats were susceptible to even slight ineffectiveness of the valves. The rubber valves became moist and inefficient and were discarded. Cover slip valves were used. The apparatus used to record the respiration seemed to impose a load on the rat since its respiratory rate and minute volume increased. This happened despite the fact that only a very small effort was required to lift the cover slip valves. These valves were very efficient and formed a perfect seal when necessary. The respiratory minute volume usually increased continuously until the respiration suddenly stopped. Perhaps this rise was due to an increase in aveolar CO_2 until the respiratory centre could respond no longer and failed. The animals that died in this way showed gross oedema of the lungs on post mortem examination.

Only occasionally was a preparation suitable for this work. Pethidine and the test analgesics have a depressant action on the respiration. Initially, the minute respiratory volume was slightly

Figure 16



RELATIONSHIP BETWEEN LOG DOSE OF THE TEST ANALGESICS AND THE RESPIRAION OF A RAT.

increased but was quickly followed by a rapid fall with a quick recovery (Figure 15). The rate of breathing was also diminished.

All the drugs were compared to TA1. Doses were selected to give a depression of respiration of about 40 to 20%. 25 and 50 µg. of TA1 per rat were the usual doses administered. The depression of respiration was calculated directly from the record. The relative activities of the test analgesics on the respiration was as follow.

TA1:	Pethidine:	TA27:	TA28:	TA33:	TA38:	TA48:	TA49
1:	0.25 :	0.8:	1 :	1.5:	1.25:	2 :	1.25

The log dose effect curves of the various test analgesics were nearly parallel. Rats did not seem to vary a great deal in their response to these substances (Figure 16).

Morphine seemed to have a different action on the respiration. A dose of 500 µg. per rat was required to depress the respiration even slightly (about 10%). However, this depression persisted for at least 90 minutes. This is in contrast to the pethidine derivatives which gave a marked rapid and temporary depression.

The most active analgesics were generally the

most powerful respiratory depressants. The only exception in the drugs tested was TA27. However, there was a quantitative separation of analgesia and respiratory depression, e.g. TA48 is 7 times more active than TA1 as an analgesic but only as twice as active as a respiratory depressant. The difference in route of administration may account for at least some of this "separation".

The effect of the pethidine derivatives on the blood pressure was slight but varied a great deal. The usual response was a small initial rise followed by a very short lasting depression (Figure 15). Morphine caused the blood pressure to fall with a relatively slow recovery (about 5 minutes).

TABLE III

Figure 17

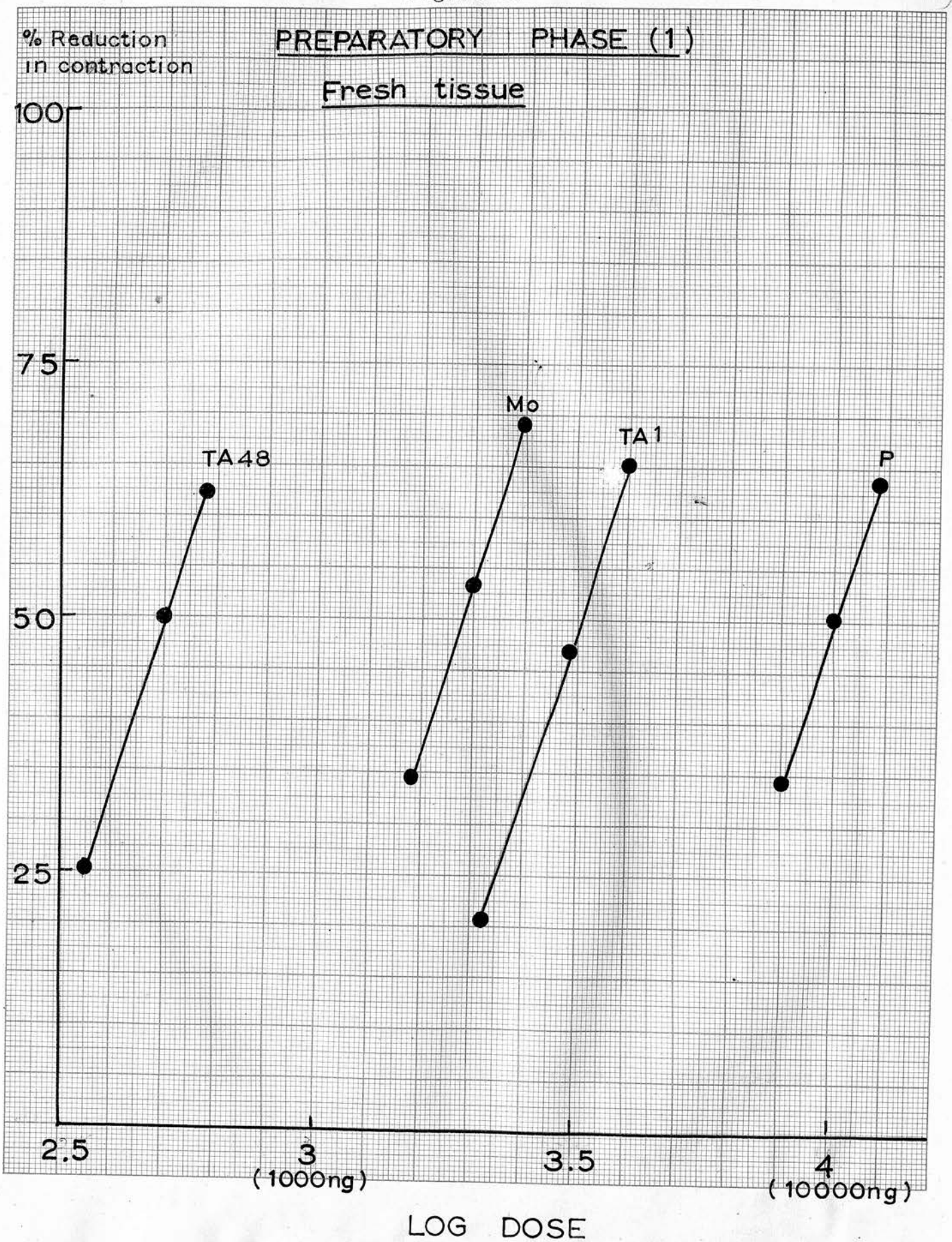

% Reduction in contraction
PREPARATORY PHASE (1)
Fresh tissue
100
75
50
25
TA48
Mo
TA1
P
2.5
3
(1000ng)
3.5
4
(10000ng)
LOG DOSE

TABLE III

RELATIVE ACTIVITIES OF ANALGESICS ON THE PERISTALTIC REFLEX

[1] PREPARATORY PHASE (I) fresh preparation

All drugs compared to TA1

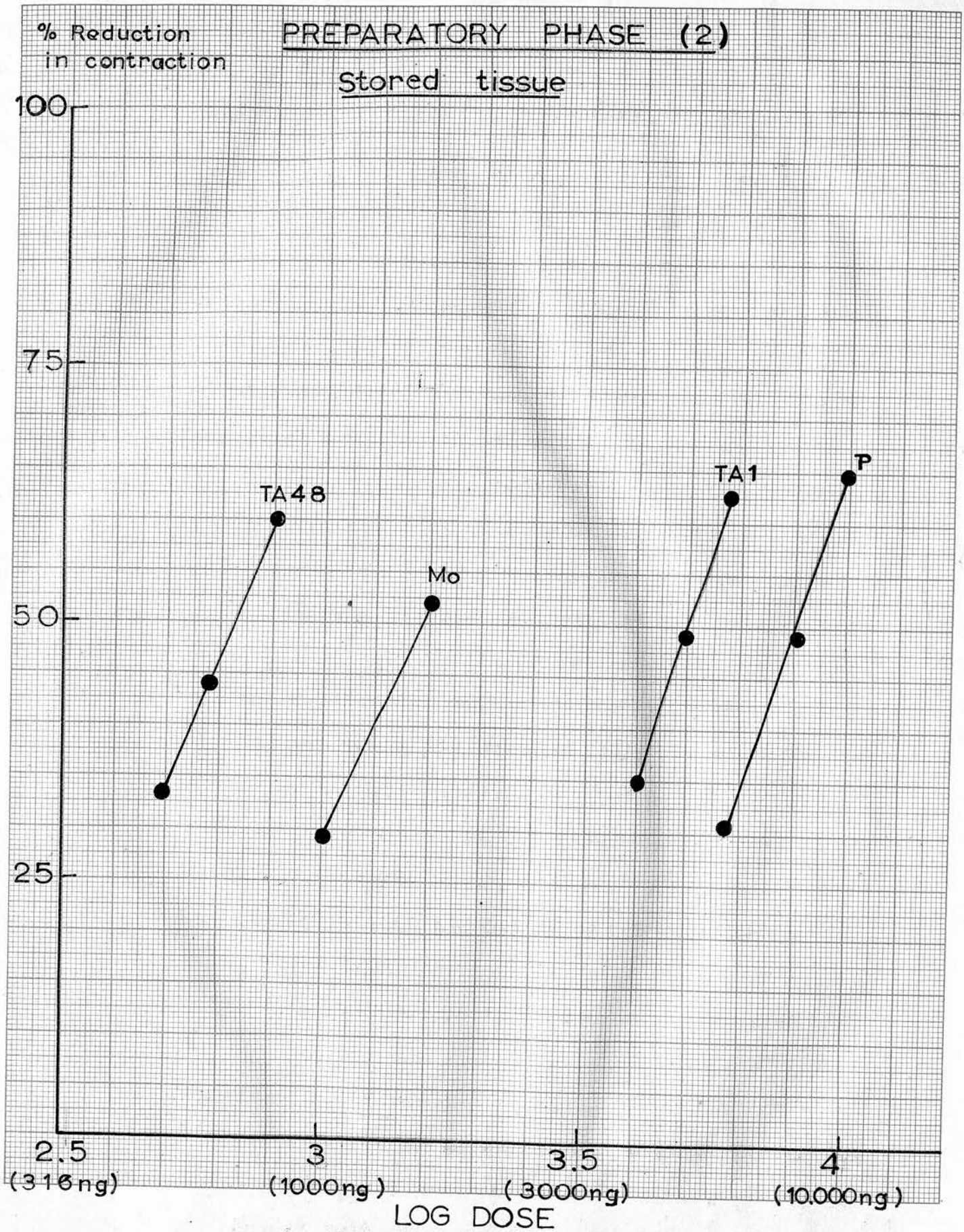
Pethidine	0.38 - 0.32 - 0.28
Morphine	1.92 - 1.65 - 1.28
TA25	0.52 - 0.41 - 0.36
TA27	1.24 - 1.07 - 0.86
TA28	1.27 - 1.09 - 0.88
TA33	3.0 - 2.6 - 2.1
TA48	7.8 - 6.5 - 5.3

[1] PREPARATORY PHASE (II) stored preparation

Pethidine	0.82 - 0.73 - 0.65
Morphine	6.1 - 5.0 - 4.0
TA25	0.67 - 0.58 - 0.46
TA27	1.6 - 1.3 - 1.0
TA28	1.7 - 1.4 - 1.1
TA33	3.5 - 3.1 - 2.6
TA48	9.0 - 7.8 - 6.7

[1] for explanation see text.

Figure 18


% Reduction in contraction
PREPARATORY PHASE (2)
Stored tissue
100
75
50
25
TA48
Mo
TA1
P
2.5 (316ng)
3 (1000ng)
3.5 (3000ng)
4 (10.000ng)
LOG DOSE

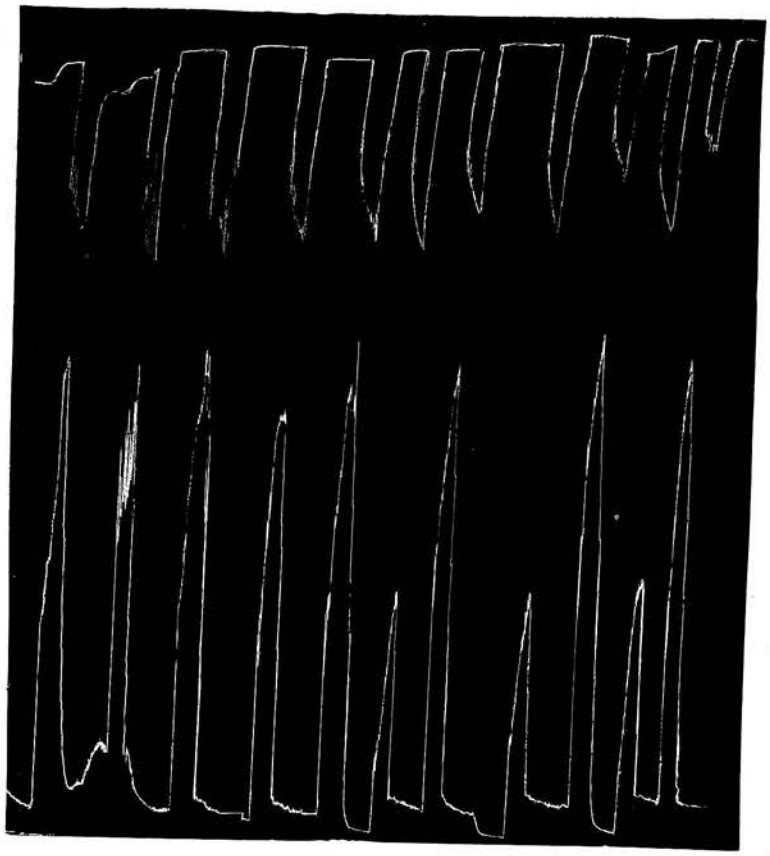
EFFECT OF TEST ANALGESICS ON THE PERISTALTIC REFLEX

The pethidine derivatives depressed both the emptying phase and the preparatory phase of the peristaltic reflex. It was decided to test the effect of the substances on the preparatory phase only. Depression of the preparatory phase would be expected to contribute towards the depression of the emptying phase. The emptying phase was particularly refractory to the depressant action of these drugs. It was difficult to depress the emptying phase without depressing the preparatory phase. It was not possible to obtain consistent graded depression of the emptying phase.

All the drugs were compared to TA1. Doses were selected to give a 20 and 50% reduction (Fig. 19 and 20). The log dose effect curves of all these substitutes were parallel in any one test (Fig. 17 and 18). However, each preparation varied in its sensitivity and in its log dose effect curve. The behaviour of any particular preparation probably depended on the amount of damage sustained during manipulation.

The results are listed in Table III. Morphine and pethidine were over twice as active relative to TA1 on the stored preparation than the fresh preparation. The results with the pethidine deriva-

Figure 19



Action of Pethidine derivatives on the preparatory phase of fresh tissue - 3IP refers to the stimulus 3 cm of intraluminal pressure. The drugs were allowed to act for a minute before the stimulus was applied.

tives were not dissimilar. The slopes of the log dose effect curves of pethidine and TA1 were not parallel in the analgesic tests of Millar and Stephenson (1956) and this difference in results may have been due to a similar cause. On the other hand, the action of pethidine and morphine on the emptying phase, which contributed to the results of tests on the fresh preparation, may not have run parallel to their effects on the preparatory phase.

The results with the N substituted pethidine derivatives run parallel to their relative analgesic potencies. This test was a satisfactory one for analgesic compounds and a useful supplement to analgesic tests proper.

TOXICITY TESTS

Acute - The acute intravenous LD50 of most of the pethidine derivatives is listed in Table I. Unlike pethidine they did not cause violent convulsions in mice. Respiratory depression seemed to be the main toxic action in this species. Other signs in mice included such morphine-like effects as tail erection, analgesia, mydriasis and bursts of hyperactivity.

Figure 20

Effect of analgesics on preparatory phase of stored guinea-pig's intestine. Drugs are given one minute before the stimulus, which was 1 cm rise in intraluminal pressure (see text).

Subacute - The results of these are listed in Table IV. None of the pethidine derivatives caused any retardation of growth.

Only one of the test rats died after the administration of 2½ mg./kg. TA48 subcutaneously for 12 days. No abnormal changes were found in the brain, liver, kidney, pancreas, spleen, myocardium, lymphoid tissue, blood or bone marrow. Terminal aveolar haemorrhages were found in one of the test animals and three of the controls.

20 mg./kg. of TA24 were injected subcutaneously into ten rats for 12 days and killed two rats on the first day and one rat on the third day. No abnormal histological features were found in the tissue of the survivors.

15 mg./kg. of TA27 killed one rat on the fifth day of treatment. The following features were found in the tissues of the control animals: minor foci of collapse of the lung and terminal aveolar haemorrhages (3 rats); a slight to moderate degree of periportal cellular infiltration of the liver (2 rats); a focus of round and polymorphononuclear cells in the brain (1 rat). In the test animals, the following histological features were found: terminal aveolar haemorrhages of the lungs (3 rats); numerous giant cells in the spleen (1 rat); bronchopneumonia (1 rat); numerous large reticulum cells

TABLE IV

TOXICITY TESTS

GROUP	DRUG	DOSE	INITIAL MEAN WEIGHT GRAMS	FINAL MEAN WEIGHT GRAMS	INCREASE
Control	Saline	4 ml./kg.	50	81.3	31.3
Test	TA27	15 mg./kg.	54	81.1	27.1
Deaths	Test group One on 5th day: Control group - none.				
Control	Saline	4 ml./kg.	46.1	76.4	30.3
Test	TA24	20 mg./kg.	50.4	85	34.6
Deaths	Test group Two on 1st day, One on 3rd day. Control group - none.				
Control	Saline	4 ml./kg.	36.6	71.3	34.7
Test	TA48	2½mg./kg.	38.5	71.0	32.5
Deaths	Test group One on 7th day: Control group - none.				
Control	Saline	4 ml./kg.	47.7	87.1	39.4
Test	TA20	20 mg./kg.	48.3	80.6	32.3
Deaths	Test group Two on 11th day: Control group - none.				
Control	Saline	4 ml./kg.	57.6	77.4	19.8
Test	TA28	15 mg./kg.	57.0	83	26.0
Deaths	Test group Five deaths - Three on 1st day, Two on 5th day. Control group - none				
Control	Saline	4 ml./kg.	50.4	69.2	18.8
Test	TA33	5 mg./kg.	51.4	86	30.6
Deaths	Test group One on 7th day, One on 8th day. Control group - none.				

in the spleen (1 rat), liver with an occasional focal granulomata (1 rat), an abscess in the brain (1 rat).

20 mg./kg. of TA20 killed two rats on the eleventh day of the test. In the brain of one control rat was found eosinophilic smudge* cells. The lungs of two other control rats were found to be damaged. Pathological findings in the treated animals included: terminal aveolar haemorrhages of the lungs (2 rats), early pneumonia (1 rat), a highly cellular nodule situated within a cerebral ventricle (1 rat) and eosinophilic "smudge" cells in the brain (1 rat)+.

5 mg./kg. TA33 per day killed one rat on the seventh day and one on the eighth day of the test. Terminal aveolar haemorrhages (2 rats) and early pneumonia (1 rat) were seen in the tissues of the control animals. In the test rats, the following features were observed: terminal aveolar haemorrhages (3 rats), early pneumonia (3 rats), a highly cellular nodule situated within the cerebral ventricle (1 rat) and eosinophilic "smudge" cells in the brain (1 rat).

15 mg./kg. TA28 per day killed 3 rats on the first day of the test and two on the fifth day of

+"Smudge" cells are the deep stellate cells of the molecular layer of the cerebellar cortex.

the test. Terminal haemorrhages of the lungs (2 rats) and two small cysts in the cortex of the kidney (1 rat) were found in the tissues of the control rats. In the test rats terminal haemorrhages of the lungs were also seen (2 rats) as well as eosinophilic "smudge" cells and indefinite signs of acute cell damage.

None of the control rats died during these tests.

Analgesics are only palliatives and there should be a very wide safety margin between the therapeutic dose and the toxic dose. Although the effects of drugs on man are not always the same as their effects on other animals, experiments on animals have proved a very useful guide to new human remedies and a very useful guard against new human poisons. It is unjustifiable to administer a new drug to man unless it is known not to have dangerous effects when administered to animals of several different species (Gaddum, 1953b).

A "therapeutic" dose of a mild drug may only give an abnormal reaction in one out of a hundred animals. In order to detect such a frequency with confidence nearly three hundred animals would have to be used (Barnes and Denz, 1953).

In this experiment the drug has been given re-

peatedly and the growth of weaning rats studied. This should detect any cumulative effects. A high dose was selected that would kill some of the animals but not all. The tissues of the survivors would be expected to show any pathological damage which might have been caused by the drug.

The main difficulty in toxicity tests is that of distinguishing naturally occurring disease and the pathological consequences of ageing from the effects due to the drug. The pathologist must be experienced to distinguish "normal" lesions from "abnormal" lesions. In this report, pathological findings have been mentioned which almost certainly have nothing to do with the drug administered.

Rats are very susceptible to lung infection and almost all the animals show some degree of lung damage. This finding was observed with equal frequency in both control and treated animals.

The histology of rat spleen is very variable. The presence of giant cells indicates a response to infection.

The liver responds to infection by a slight to moderate degree of periportal cell infiltration as well as an occasional focal granulomata. An abscess in the brain is a result of infection. The nature of the highly cellular nodule found within the

cerebral ventricle of one of the rats treated with TA20 is unknown.

The large eosinophilic "smudge" cells found in the brain are believed to be the first stage of the response to asphyxia. After the administration of all these drugs most of the animals were only semi-conscious. It is possible that this "response" is an acute one, i.e., takes place when the animal is being killed for examination. This change was found in one control animal and three treated animals and is of relatively little importance.

In conclusion, ninety rats were examined post mortem and 499 tissues prepared for microscopic examination. I am very grateful to Dr. A.E. Stuart, Department of Pathology, University of Edinburgh, who examined the sections. The pathological changes noted were diverse and included pneumonia, brain abscess, cystic kidneys, miliary granulomata of the liver and agonal pulmonary haemorrhage. The majority of tissues were healthy and the above changes were rarely encountered. The histological examination showed no signs of kidney, liver or bone marrow damage.

The drugs appear to be relatively harmless.

After each toxicity test, analgesic trials were performed, i.e., on the thirteenth day. The results

of these "tolerance" tests are listed in Table V, These indicate that tolerance to the analgesic effect was developed in rats. However, the tests were primarily performed as subacute toxicity tests and not to study the development of tolerance. These results suggest that if a series of larger doses were given clear-cut tolerance would develop.

TABLE V/

TABLE V

TESTS FOR DEVELOPMENT OF TOLERANCE TO ANALGESIC EFFECT

daily dose 1-2 days.

Drug	Dose	CONTROL ANIMALS	TEST ANIMALS
		Mean analgesic index	Mean analgesic index
TA27	1½ mg./kg.	0.72	0.86
TA27	3 mg./kg.	0.43	0.56
TA24	2 mg./kg.	0.75	0.81
TA24	4 mg./kg.	0.39	0.43
TA48	¼ mg./kg.	0.66	0.85
TA48	½ mg./kg.	0.34	0.59
TA20	2 mg./kg.	0.80	0.94
TA20	4 mg./kg.	0.44	0.79
TA28	1½ mg./kg.	0.77	0.98
TA28	3 mg./kg.	0.39	0.72
TA33	½ mg./kg.	0.83	0.96
TA33	1 mg./kg.	0.61	0.73

DISCUSSION

I have found the pressure method of Green and Young (1951) to be a useful technique for observing the effects of analgesic drugs. The "index of analgesia" described in the section on method is a convenient extension of the original procedure.

TA48 N-(tetrahydrofurfuryloxyethyl)4-phenyl-4-ethoxycarbonyl piperidine was superior to any of the compounds in the series. In addition TA27, TA28, TA63, TA33 and TA49 were more active than TA1, the most active substance described by Millar and Stephenson. TA24 was as active as TA1.

Millar and Stephenson emphasized the importance of the ether oxygen unit and this has been confirmed. It is interesting that compounds with an ether oxygen unit and a terminal cyclic ether function were very active. The role of these two oxygen atoms is unknown. Pfeiffer (1948) has suggested that the presence of an oxygen atom 7-9°A from the methyl on the nitrogen is important and that there should be 'one or more blocking moieties', the nature of which he leaves open. He considers the distance quoted to be independent of the 'general ring structure'. These ideas may be taken to imply electrostatic

forces between the oxygen atom and the biological structure concerned. It is possible that the three oxygen atoms, if correctly related to one another in space, are involved in the linkage of the drug to the receptor.

The cross-over technique was found to be a practical method for comparing analgesics. In the present tests, no evidence of tolerance was observed. In three of the tests the differences between cages were significant confirming that it was important that each cage of rats should receive the eight doses using a symmetrical latin square design. In two out of the five tests the variance due to differences between rats in a cage was significant. The doses were injected in an irregular order down the list of rats, this eliminated the possibility of systematic effects due to the order of injecting and testing, and also made the actual testing of the threshold more objective, since the observer did not remember at the time of testing which dose each rat received. This made it unnecessary to take special precautions to ensure that the observations were unbiased which otherwise would have been very desirable because of the occasional difficulty in deciding the precise end-point.

When different substances are compared in a biological test it frequently happens that the dose effect lines are not parallel. Millar and Stephenson (1956) found the difference between the slopes of TA1 and pethidine sufficiently large to make it reasonably certain that the difference was a true one. Thus it was not possible to say that TA1 was "R" times more potent than pethidine.

A difference between the dose effect lines may indicate a qualitative as well as a quantitative difference between two drugs. It is generally believed that some of the analgesics used clinically produce a maximal effect larger than that produced by others. It is possible that this difference is analogous to the difference of slope between TA1 and pethidine. There is thus some reason for supposing that the active test analgesics, e.g. TA48, may be more useful than pethidine in the treatment of severe pain. Moreover, TA48 was found to have a larger peak effect than morphine which, however, is not maintained. The treatment of severe pain with a combination of TA48 and morphine is worth investigation.

Millar and Stephenson found that TA1 20 mg./kg. killed only one rat in a subacute toxicity test conducted over eleven days. Pethidine 60 mg./kg.

killed six rats in a similar test. Neither drug had any effect on the growth of the rats. TA24, 27, 28, 33 and 48 did not kill as many rats as pethidine in recent tests. It is reasonable to suppose that the increase in potency of the active analgesics is not accompanied by as great an increase in toxicity. The absence of marked convulsions in mice after intravenous injection of the test analgesics makes close comparison with pethidine irrevelant. In rats death follows a period of depression with both pethidine and its derivatives and a comparison of toxicity has some value. Millar and Stephenson suggested that TA1 was less than three times as toxic as pethidine. TA48 is no more toxic than TA1 in tests on mice, whilst it is seven times more potent as an analgesic. Since TA1 is three to seven times more potent an analgesic than pethidine, it is possible to calculate that TA48 is about twenty to fifty times more powerful than pethidine but less than three times as toxic.

Schaumann (1956) believes that depression of respiration and the constipating effect of analgesics are inseparable from their analgesic action. I have found that the potency of the test substances in depressing the preparatory phase of the peristaltic reflex runs parallel to their analgesic

potency. However, a quantitative separation of analgesic activity from respiratory depression has been achieved. TA48 is only twice as active as TA1 on the respiratory centre but seven times more potent as an analgesic. It is interesting that another N- substituted pethidine derivative anileridine (Orahovats et al, 1957) is about five times more powerful than pethidine as an analgesic but only equally potent as a respiratory depressant. Anileridine, like TA48, is very effective orally, especially in higher doses. Another interesting action shared by morphine, anileridine and the more active pethidine derivatives is that they produce catalepsy in rats. Whether there is any relationship between catalepsy and analgesia is not known.

Substitution of the N methyl group of pethidine has produced some analgesics considerably more potent than pethidine and their toxicities are not correspondingly higher.

SUMMARY

1. A series of compounds in which the N methyl group of pethidine was replaced - by tertiary aminoalkyl and alkyloxyalkyl groups, both carrying a further ether oxygen function, alkyloxyalkyl and aryloxyalkyl groups - has been tested for analgesic activity in rats.

2. The method used for determining the analgesic effect was that of Green and Young (1951) as modified by Millar and Stephenson (1956).

3. A design for a cross-over test is described.

4. Acute and subacute toxicity tests have been performed.

5. The effects of the more active derivatives have been studied on the respiration of the rat. Whilst the order of potency was the same as found in the analgesic tests, the difference in potency relative to TA1 was not so marked. TA48 is seven times more powerful than TA1 as an analgesic but only twice as active as a respiratory depressant.

6. Structure action relationship is discussed.

7. The effect of the more active test analgesics have been studied on the peristaltic reflex of guinea-pig ileum. The results of the test analgesics on this reflex ran parallel to their relative analgesic potencies.

8. Many substances were found to be more active than pethidine. One of these, TA48, N-(tetrahydrofurfuryl oxyethyl) 4-phenyl-4-ethoxycarbonyl piperidine is twenty to fifty times more potent than pethidine and is less than three times as toxic.

9. Some of these derivatives, TA24, TA27, TA28, TA33 and TA48, have been selected as suitable for clinical trials.

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