Immunosympathectomy and avoidance behaviour in mice

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IMMUNOSYMPATHECTOMY AND AVOIDANCE BEHAVIOUR IN MICE.

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ABSTRACT.

W. B. Cannon and his co-workers during the 1930's had purported to have demonstrated the non-essential character of the sympathetic nervous system's role in mediating emotion, but later workers using behavioural studies have tended to implicate the system in emotional behaviour.

In this thesis an immunological technique was used to induce hypotrophic growth in the sympathetic nervous system of neonatal rodents. The injection technique used a split-litter design with the control neonates being injected with normal horse serum(NHS).

Following failure to obtain behavioural separation between the immunosympathectomized (IS) and the control (NHS) littermates using standard active and passive avoidance tasks, it was argued that the time course of these procedures allowed ample opportunity for internal compensatory mechanisms to mask any essential differences. Accordingly an avoidance task (startle response) with a very short time course was used to test the animals. This task also failed to show unequivocal behavioural separation. Measuring the catecholamine metabolism of the adrenals, some evidence was obtained which showed that IS mice metabolized larger amounts of the sympathetic nervous

system's transmitter substance, noradrenaline, when subjected to a novel or an avoidance task. Evidence was examined on the essential interaction between the sympatho-adrenal medulla and the pituitary-adrenal cortex axis. It was concluded that certain controls are essential if an unambiguous demonstration is to be made of these two important central nervous system's efferent outflows in emotional states.

IS mice were placed on free operant avoidance schedules in specially constructed apparatus. The final experiment reported concerns an attempt to further reduce the range of IS mice adaptive responses by the use of an inclined alleyway avoidance task interpolated between two equal periods of free operant avoidance responding. It was finally concluded that unequivocal behavioural separation, using avoidance tasks, has not been shown between IS and control littermate mice either in this thesis or other published work. Hypotheses are discussed as to why this should be so.

CONTENTS.

CHAPTER I.

INTRODUCTION.

Throughout the 1930's the monolithic theories of learning experienced considerable difficulties in explaining the acquisition and maintenance of avoidance behaviour. An animal escaping from a punishing situation could be explained by recourse to drive reduction following removal of the aversive stimulus; however, the paradox of the avoidance situation was that a successful avoiding response could not logically be reinforced in the absence of such aversive stimulation. In his now historic paper, Mowrer, 1947, finally crystallized a solution by his two factor theory. The two factor theory explained the acquisition and maintenance of avoiding behaviours by linking together two very highly improbably bedfellows, Freud and Pavlov. Mowrer having a psychoanalytic background, saw the connection between Freud's, 1926, revised theory, which stressed the warning and subjective function, of anxiety and the Pavlovian experimental paradigm. Pavlov, 1927, who worked within an evolutionary theoretical framework, had emphasized the signalling function of the conditioned stimulus which by its linking to the unconditioned response allowed the formation of adaptive behaviour. But Pavlov

made no use of motivational concepts, and it was this aspect that Mowrer supplied from Freud's theory.

Mowrer argued that in escape training, using farradic stimulation, the shock gave rise to pain which by its nature has a large associated anxiety component mediated by the autonomic nervous system. The pain was specific to the electric shock, but the anxiety could become conditioned or attached to a neutral stimulus presented concurrently with the shock. From a series of neutral stimulus and shock pairings the neutral stimulus came to invoke anxiety i.e., incipient autonomic respondents. Escape behaviour made at this point would prevent the onset of the shock, autonomic nervous system respondents, and this prevention constituted the rewarding factor in the situation. Basically, Mowrer was saying that the autonomic nervous systems respondents and the ~~sensori-motor~~ somatic movements evoked in the first instance by the shock became elicited by the originally neutral stimuli. Miller, 1948, supporting Mowrer's theory showed that fear could be used as a drive to motivate the learning of a new response. Since 1947 the two factor theory of avoidance learning has remained substantially unaltered despite suggested modifications by Schoenfeld, 1964, and Dinsmoor, 1954 and 1955. Schoenfeld

pointed at the subjective and connotational problems that arose from the use of the hypothetical term anxiety. He felt that avoidance was acquired by the conditioned stimulus acquiring secondary reinforcing properties from the primary aversive reinforcement of the shock i.e., for Schoenfeld the hypothetical term anxiety was to be replaced by a proprioceptive secondary reinforcer. Dinsmoor pointed out that a punished response was not an isolated incident but a member of a chain of responses which were linked by discriminative and, thereby, secondary reinforcing stimuli. Stimuli coming immediately before the punished response, both internal and external, are paired with the punishment and as a result of this pairing gain aversive properties in their own right.

~~Thus~~, The largest single component of fear or anxiety was the efferent outflow of the autonomic nervous system and it was this outflow that mediated avoidance behaviour. Implicit in the two factor theory was the suggestion that the level of peripheral activation was positively correlated with the degree of fear or anxiety experienced by the animal. Mowrer and Keehn, 1958, in explaining how intertrial avoidance responses were reinforced, argued that when a conditioned trace was established a progressively increasing anxiety level occurred as the time period between trials elapsed. When this reached a critical level the animal made an avoidance response.

Learning theorists had firmly planted their theoretical foundations of affective states in the sympathetic nervous system. This was done despite Cannon and his colleagues (Cannon, Lewis and Britton, 1926; Cannon, Lewis and Britton, 1927; Cannon, Newton, Bright, Menkin and Moore, 1929) who, from a series of experiments using sympathectomized cats and dogs, purported to have demonstrated the non-essential character of the thoracolumbar autonomic outflow (SNS) in emotional rage and fear reactions. This was somewhat ironic, in that it was probably another of Cannon's concepts 'flight of fight' (1929) that was one of the most important factors in implicating the SNS in the mediation of emotion. Initially, physiological psychology experiments designed to test the mediational role of the sympathetic nervous system in emotion were carried out using either pharmacological agents to block the efferent thoracolumbar outflow, or removal of the chains of thoracolumbar ganglia by surgery. The latter technique usually involved several resection operations to complete the removal of all the SNS ganglia. In a series of experiments mainly using the surgical techniques, some use was made of pharmacological agents, Soloman and Wynne (Solomon and Wynne, 1953; Soloman, Kamin and Wynne, 1953; Solomon and Wynne, 1955; Wynne and Solomon, 1955) reported that sympathectomized dogs run in an active avoidance situation,

using a Mowrer-Miller avoidance box, tended to be slower to acquire and quicker to extinguish the avoidance response when compared with normal dogs. Their results have recently been held to at least partially confirm the two factor theory (Rescorla and Solomon, 1967). However, their findings were far from unequivocal. Solomon and Wynne admitted the problems of post surgical trauma but did not mention specifically the problem of neural sensitivity arising from the resection techniques (Cannon and Rosenblueth, 1949). Nor, apart from mentioning the source of their dogs, that their experimental animals were stray dogs obtained from the local dog compound, and if previously conditioned responses to aversive stimuli were an important variable, that it might be held that their dogs could have had considerable training for aversive discriminations. This factor may have been a confounding variable and to have resulted in the limited behavioural difference obtained by Solomon and Wynne between the normal and experimental animals.

Auld, 1951, used a drug, tetraethyl-ammonium (T.E.A.), as a pharmacological means of blocking the autonomic nervous system. His experiment was designed, "to secure evidence on the way a fear motivated habit is affected by a drug". Auld used rats in a Mowrer-Miller active avoidance box. The animals ran into a black compartment to avoid an electric shock which

was given in a white compartment. The drug treated rats had slower running times, both on the training and extinction days, than the non-treated animals. Auld concluded that the blocking of the efferent impulses through the autonomic ganglia resulted in his drug treated animals showing a reduction of fear. Brady, 1953, also using the drug T.E.A. to study its effects in rats, pointed out that Auld's study placed a premium on efficient sensori-motor behaviour. In his study using an operant technique, Brady showed that injections of the drug eliminated lever pressing of animals on a V.I. schedule of reinforcement using water. Brady felt that his results raised serious doubts about the fear reduction interpretation of the effects of T.E.A. Arbit, 1957, also showed that muscular asthenia was produced by injections of T.E.A. Injections of a similar drug, hexamethonium, ~~but~~ having less side effects revealed no significant differences in extinction rates between the hexamethonium treated and control animals. Davitz, 1953, using operant behaviour and a C.E.R. technique found that temporary blocking of the autonomic nervous system using T.E.A. inhibited rather than facilitated extinction of a C.E.R. Thus, the drug studies were no less unequivocal than the surgical studies. Interpretation of pharmacological studies are further confused by such complicating factors as: route of injection of the drug; amount of drug injected; drug vehicle; and last, but by no means least, the biological condition of the

animal. It is this type of non linear interaction that makes combined behavioural and pharmacological studies so difficult to interpret even if precise and exact details are given. Weiskrantz, 1964, has indicated some of the methodological problems in interpreting behaviour arising from drug action.

The finding by Solomon and Wynne, and to a lesser extent those of Auld, suggest that feedback from the peripheral autonomic nervous system may play a more important part in the elaboration of emotions than suggested by Cannon and his colleagues.

In a recent review article "Developmental Psychopharmacology" Young, 1967, has pointed out the potential value of using immunological techniques in psychology. Such techniques allow selected tissues to be destroyed without surgical trauma or the side effects of drugs; moreover, the destruction can take place before functional use of the system has developed. Mihailović and Janković, 1961, have reported attempts to prepare an antiserum against specific neurones within the central nervous system. Gluecksohn-Waelsch, 1957, demonstrated that emulsified brain extracts injected into pregnant mice resulted in anatomical brain abnormalities in a proportion of the offspring. Zamenhof, Mosley and Schuller, 1966, have demonstrated the proliferation of cortical neurones following prenatal treatment with growth hormone. Coggeshall and Maclean, 1958, have shown that the chemical compound

3-acetylpyridine when injected into rats and mice resulted in hippocampal lesions restricted to discrete areas. At present specific immunological or chemical lesioning within the central nervous system is no more than an interesting possibility. But the early work indicates that with increasing knowledge and technical sophistication, a valuable technique will be added to the armamentarium available for studying the development of behaviour.

In 1960 Professor Rita Levi-Montalcini began publishing a series of papers (Levi-Montalcini and Booker, 1960; Levi-Montalcini, 1964; Levi-Montalcini, Schenkein, Bueker, Crain, Benitez and Watter, 1964; Levi-Montalcini and Angeletti, 1966) describing a nerve growth factor (NGF) which was found to produce specific hypertrophic growth in the thoracolumbar ganglia. The NGF when isolated and purified was found to be a protein. Levi-Montalcini and Booker, 1960, have described the production of an antiserum from NGF. Injection of the antiserum into neonatal mammals produced hypotrophic growth specific to the sympathetic nervous system of treated animals. Thus, NGF and its related antiserum meant that a technique was available to study the value of peripheral feedback in fear and anxiety states in animals that had experienced no extensive conditioning of the afferent autonomic outflow of the sympathetic nervous system before the hyper

or hypotrophic changes were induced.

Zaimis, 1964, reviewing the technique of immunosympathectomy wrote, "animals experimentally deprived of their peripheral sympathetic system provide most valuable material on which to test the validity of current theories concerning the role of the sympathetic nervous system and the way in which structures innervated by it are affected". In her review, Zaimis was concerned with the pharmacology of the sympathetic nervous system; but her passage would have been equally relevant had she been discussing the psychological aspects of the sympathetic system.

Largely as the result of Cannon's work during the 1930's the sympathetic nervous system and the adrenal medullae (embryologically the adrenal medullae are part of the sympathetic nervous system) were recognized as important mediators of the mammalian adaptation to stress, and important concomitants of emotional response in animals. Selye's (1950) work on the stress syndrome has extended our knowledge of stress mediation within the organism by implicating and demonstrating the responsiveness of the pituitary-adrenal cortex axis to physiological stress.

Evidence has been produced to show that both of the systems are responsive to psychological stimulation and clearly the two systems interact to a large degree. Behavioural studies implicating the sympathetico-adrenal system have been quoted earlier

in this introduction. But other workers have specifically examined the pituitary-adrenal system. In particular, Mason and his colleagues have published a series of papers (Mason, Brady and Sidman, 1956; Mason, Brady and Sidman, 1957; Mason, Maugan, Brady, Conrad and McRioch, 1961) examining the concurrent levels of plasma 17-hydroxycorticosteroid and conditioned emotional behaviour in monkeys. Levine and Soliday, 1960; Levine and Jones, 1965, and Appley, 1965, have shown that the integrity of the pituitary-adrenal cortex axis was necessary for the acquisition of active avoidance behaviour in rats.

Published evidence appears to indicate that the hormonal pituitary-adrenal cortex axis is more sensitive to insult than the more direct and neural route of the sympatheticoadrenal route. This, despite the slower action of the former. Fortier, de Groot and Hartfield, 1959, quote a period of 2 minutes before plasma corticosteroids rose detectably above the baseline level following electric shock given to male mice. Clearly, in any adaptive response to aversive stimulation a biological system would appear to require an immediate adaptive response followed by a slower system that maintains flat gradients of generalization for the aversive stimulation. The obvious systems would appear to be the neural sympathetico-adrenal route mediating the

first stage while the slower hormonal pituitary-adrenal system mediates the second and maintaining stage. As mentioned earlier, if previous conditioned responses were a confounding variable, the use by earlier workers of adult animals might have resulted in the absence of clear-cut behavioural separations when sympathetico-adrenal blocking was used as the independent variable.

The use of NGF and its antiserum would allow for hyper or hypotrophic changes in the SNS to be induced before the neonate had any extensive conditioning to the neural outflow of the sympathetic nervous system. Prior to beginning the experiments reported in this thesis, only one behavioural study was found that had used NGF or its related antiserum. Francois and Sines, 1961, reported a study using immunosympathectomized (IS) rats which they subjected to immobilization stress for 12 hours. The rats were then killed and the stomachs dissected and examined for the presence of lesions. The authors reported that they found a significantly greater incidence of lesions in the stomachs of their IS rats. They also found a significant rank order correlation between the percentage reduction of the number of cells found in the SNS cervical ganglion and stomach lesion severity in the experimental animals. No reliable differences were

obtained between the experimental and control animals in any of the open field measures taken before the immobilization stress. However, the study pointed hopefully towards the possibility of obtaining behavioural differences between rats treated with NGF or its antiserum and control animals.

CHAPTER II.

PREPARATION OF NGF AND ITS ANTISERUM. BREEDING AND WEANING. INJECTION TECHNIQUE. INITIAL BEHAVIOURAL STUDIES USING IMMUNOSYMPATHECTOMIZED MICE.

This chapter deals with the early work which falls under two main headings: (a) the preparation of the Levi-Montalcini nerve growth factor (NGF) and its related antiserum, (b) the initial behavioural studies which were designed to reduce possible confounding variables that might have arisen from the litter handling procedures that were required in order to inject the active biological material.

Preparation and Injection of Nerve Growth Factor.

At the outset of this work it was hoped to be able to produce sufficient quantities of purified NGF to allow induction of hypertrophy of the sympathetic nervous system of newborn rats. Initial extractions and purification followed the technique published by Cohen, 1959 and 1960; however, the technique was found to give very poor yields of the biologically active protein and it was not of a sufficient purity to eliminate side-effects. A description of the cortisone-like side-effects has been reported by

Bueker and Schenkein, 1964, as part of a symposium on NGF (Levi-Montalcini et al., 1964). Consultations with Dr. D. C. Edwards, Wellcome Research Laboratories, indicated that his team had also obtained poor yields from the technique published by Cohen. In 1966 the method used by Edwards and Fenton (personal communication) was used with considerably more success. This method of Edwards and Fenton is reported in the biological appendix.

The new technique together with a gift of NGF protein from the Wellcome Research Laboratories enabled the author to collect sufficient active material to inject several litters of rats. Eighteen rats were injected daily with 100, 250 or 500 units/gm. body weight of the protein for nine consecutive days following birth. Matched litter-mates injected with saline served as controls. All the animals were tested at forty days in an active avoidance task using an automated shuttle box based on the design reported by King, Achenbach, and Levine, 1961. The rats were given ten trials per day with an intertrial interval of 2 minutes. No significant differences were recorded between the NGF injected animals and their litter mates, either for running speeds or number of trials required to reach the avoidance and extinction criteria. Dissections and weighings of thoracic and cervical ganglia revealed that there was no significant difference in size

between the ganglia from the NGF injected and their control litter mates. Maintenance of the initially induced hypertrophy was obviously dependent upon a constant injection regimen. This lack of maintenance of the initial hypertrophy had also been noted by Dr. D. C. Edwards (personal communication). The twin problem of obtaining the large quantities of the NGF protein required for daily injections over 30 - 40 days (considerable side-effects could be expected from the high dose levels required) and the problems that would arise from handling the animals every day in order to inject the active material precluded the possibilities of continuing this line of research. The rats injected with NGF, apart from a small initial body weight drop during the injection programme, did not show any gross physiological changes at any time during the study.

Nerve Growth Factor Antiserum.

At this point the author decided to concentrate upon the production of NGF antiserum. Earlier reports (Levi-Montalcini and Booker, 1960) had indicated that the hypotrophic response induced by the antiserum was permanent. The NGF was extracted and purified and an antiserum with a titre of approximately 3,000 anti-units/ml was produced in a rabbit (see appendix for full

details). The antiserum produced reductions in the sympathetic ganglia of rats of about 15 - 20%. This was not as high as those reported in the earlier literature (Levi-Montalcini and Booker, 1960; Cohen, 1960; Francois and Sines, 1961).

During 1967 the Wellcome Research team at Beckenham, Kent, succeeded in producing a high titer NGF antiserum (9600 anti units/ml) in a horse. After testing, some of this freeze dried antiserum was made available to the author. As the Wellcome NGF antiserum produced a relatively greater degree of hypotrophy in mice than rats the following experiments reported in this thesis concerned experiments made with a genetically heterogeneous strain of mice maintained in the Department of Psychology at Durham University.

The first complete experiment using immunosympathectomized (IS) mice and matched litter mates injected with normal horse serum (NHS) is reported in Chapter III. The remainder of this chapter is used to describe the injection technique and method of breeding and rearing. Certain experiments designed to examine the possibility of selective maternal behaviour towards the IS mice and the effects of handling or "gentling" are also mentioned.

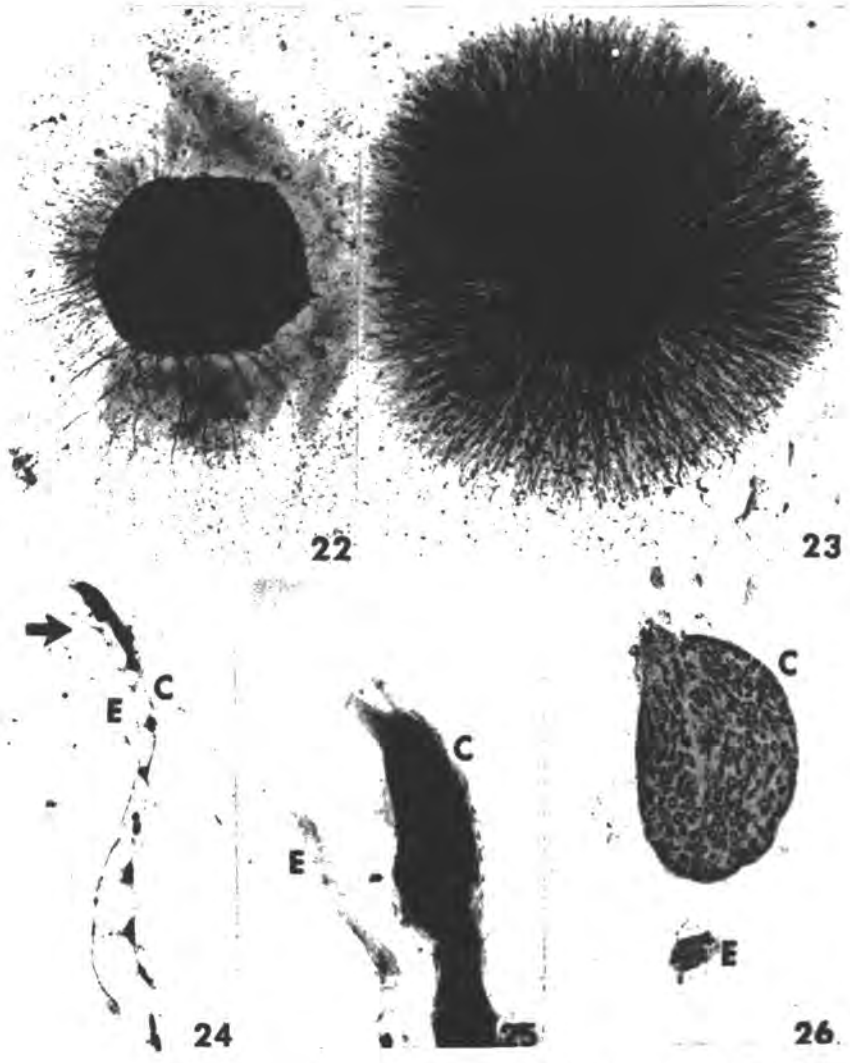
Figures 1 and 2 are included to show a comparison between the hypotrophy reported by other workers (Levi-Montalcini, 1962)

and those obtained during the present study using the immunosympathectomy technique. For a detailed discussion of the effects of NGF antiserum, both in vitro and in vivo, see Sabatini, Pellegrino, Iraldi and De Robertis, 1965.

Breeding and Rearing.

The mice were bred and reared in an isolated shed away from the main animal rooms and attempts were made to reduce disturbances in the shed to a minimum. Litters used for the injection of the NGF antiserum were from multiparous females. The procedure followed was to allow a batch of young, normal female mice to have their first litters and to select only those females who showed the necessary maternal behaviour patterns required to successfully rear a litter. Primiparous females who did not successfully rear their first litters were not used in any subsequent breeding programme. The use of multiparous females reduced the initial high mortality rate of neonates.

At the start of a breeding programme a single buck was placed in a mouse stock box, 50 cm x 35 cm x 12.5 cm, containing from nine to sixteen females. After one week the buck was removed and replaced by another male mouse. The second male was removed at the end of the second week. Towards the end of the third week



Figs. 22, 23. Microphotographs of 7-day sensory ganglia of chick embryo after 24 h *in vitro*. Figure 22, ganglion in a control medium. Figure 23, ganglion in a medium containing the salivary NGF. Silver impregnation.

Fig. 24. Thoracic sympathetic chain ganglia of a control (C) and of an experimental (E) 1-month old mice. Experimental mouse injected for 5 days after birth with the antiserum to the salivary NGF. Arrow points to stellate ganglion.

Fig. 25. Stellate ganglion of Fig. 24 at higher magnification to show the near total atrophy of experimental (E) ganglion. C, control.

Fig. 26. Transverse section through the superior cervical ganglia of control (C) and experimental (E) 8-month old mice. Experimental mouse injected for 3 consecutive days after birth with the antiserum to the purified salivary NGF factor.

(Figure 23 from W. D. McElroy and B. Glass (editors), *A Symposium on The Chemical Basis of Development*, Johns Hopkins, Baltimore, 1958, p. 657).

Sci. Repts. Ist. Super. Sanità, 2 (1962) 345-368

FIG. 1

Figs. 22-26, reproduced from an Elsevier Pub. Co. reprint, "Analysis of a specific nerve growth factor and of its antiserum" by R. Levi Montalcini. Sci. Repts. Ist. Super. Sanita, 2, (1962), 345-368.

FIG. 2

Showing differential size of sympathetic nervous system (SNS) ganglia of immunosympathectomized (IS) and normal horse serum (NHS) injected littermate control mice.
Top left: cervical and thoracic ganglia dissected from 20 day old mice; Left, ganglia from IS mouse; right, ganglia from NHS mouse. Magnification approximately x5.
Top right: pairs of cervical and throacic ganglia (outer pairs, NHS mouse; inner pairs, IS mouse) dissected from littermates selected at random from animals used in adrenal catecholamine assays reported in chapter VI. Magnification approximately x25.
Below: serial histological sections (top, NHS; below, IS) taken from mid-point of thoracic ganglia, from two littermates selected at random from the mice used in the Startle Response Experiment reported in Chapter V. Magnification approximately x165.

the females were checked daily and any female showing signs of pregnancy was placed in an individual breeding box. The breeding boxes were 45 cm x 22.5 cm x 15 cm with a totally enclosed nesting compartment at one end containing sufficient woodwool or paper to allow the female to make a nest. After being placed in a breeding box the females were checked at 12 hourly intervals, or less, for the presence of young.

Following the birth of a litter, the female was gently ushered out of the nest box which was then sealed off using a metal plate to prevent the female from returning during the initial sorting or injection of her litter. Litters were sorted into males and females, each sex being placed into a separate paper-lined (10 cm x 10 cm x 6 cm) metal enclosure. To reduce any detrimental effects caused by body temperature loss whilst handling the neonates (Hutchings, 1963; Schaefer, 1963) the metal enclosures were insulated with paper hand towels and they were placed on a warming table designed to maintain the body temperature of a rat under surgery. The neonates were then weighed and sorted into two further subgroups consisting of mice of approximately equal body weight. Two of the four subgroups, one male and one female, were then tail-clipped for identification purposes. Clipped tails in any litter might indicate either immunosympathectomized (IS) or control normal horse serum (NHS) neonates;

this was decided before the injection programme was started by random allocation. Litters consisting of more than eight young were culled to eight by removing the neonates with the lowest body weights. Following the initial sorting and marking procedures on day one, the neonates were placed back into the nesting box and the female allowed to return to her litter until all the litters born in the previous 12 hour period had been sorted and marked.

All the information concerning any female and her litter was entered onto a litter record sheet. Litters were numbered in order of birth rank, 1, 2, 3.....etc. prefixed by the month during which the injection programme was started.

Injection technique.

At the start of any injection programme a 10 ml phial containing freeze dried NGF antiserum was dissolved in 10 mls of sterile distilled water (experiments reported from Chapter VI onwards consist of mice who were injected with X2 concentrated antiserum which was prepared by dissolving 10 mls of freeze dried NGF antiserum in 5 mls of sterile distilled water). During an injection programme the NGF antiserum and the normal horse serum were stored at 3 - 4°C. Normal horse serum was used as a control material to eliminate for effects due solely to gamma globulin.

Before injection the sera was allowed to warm up to room temperature. The actual volume of sera required on any particular day was drawn, under sterile conditions, into sterilized 1 ml TB syringes mounted on No. 1 Record hypodermic needles.

After again confining the female to the outer part of the breeding box the neonates were sorted into tail-clipped and unclipped groups by placing them into the insulated metal enclosures. Injections were made subcutaneously into the nape of the neck and along the midline of the back. It was found that if the tip of the needle was gently directed away from the midline of the back into the fold of skin in front of the hind limb, ~~that~~ this prevented leakage of the serum after the needle had been withdrawn. Injections were given at 24 hourly periods for three consecutive days post partum and were of the following volumes: day one and day two, 0.05 ml/animal; day three, 0.1 ml/animal. After being injected, the neonate was replaced in the nest and not disturbed by the experimenter until the next injection period 24 hours later.

Total Body Weights.

Total body weights were recorded on day ~~one~~ 1, 10, 21 and following the behavioural test. No significant differences were

found between the body weights of IS, NHS, or minimally handled (MH) mice from the early injection programmes. When the X2 concentrated NGF antiserum was used, the IS mice were consistently lighter than the NHS mice.

Ear Punching.

At ten days the distinguishing mark made by clipping the tail was replaced by an ear punching identification mark. IS mice were distinguished by a hole placed in their left ear pinna and NHS mice by a hole placed in their right ear pinna. This identification marking was standard throughout the experiments reported and allowed for rapid identification of the treatment received by any animal.

~~Weaning and Breeding.~~ Box Cleaning.

Litters wereweaned at 21 days when the maternal female was removed to a female mouse stock box. The litters were then left undisturbed until the behavioural test. Breeding boxes were cleaned by replacing the old sawdust with new sawdust on days 10 and 21. Care was taken to minimize the disturbance of the litters when cleaning the boxes.

Timing of Behaviour Tests.

Except for the open field experiment reported in Chapter III, all behavioural tests were carried out at a mean age of 30 days. Scott, 1966, in a review of agonistic behaviour in rodents, has reported that in young mice there is a complete absence of playful fighting within the litters. Mice between the ages of 32 - 36 days would fight a strange mouse from another litter, but intra-litter fighting did not begin until a much later age. Testing at the mean age of thirty days would appear to have reduced any extensive conditioning of affective states occurring from intra-litter agression. In all experiments some minimally handled mice were tested and on no occasion, unless reported, was a significant difference found between the minimally handled and NHS mice. Mice remaining after an experimental design was completed were separated into the two sexes and kept. Similar behavioural tests to those carried out on their sibs were applied to these animals when they reached between 60 - 90 days. In no case did these tests on the older mice reveal profiles that were essentially different from those found in 30 day old animals.

Handling or "Gentling".

The effects of early stress on later behaviours have been the subject of a number of reviews (King, 1958; Ader, 1959; Levine, 1962; Denenberg, 1962). The main thesis of these reviews is that neonatal stress (this may range from mild handling to the use of electric shocks) results in these animals, when adult, showing different emotional behaviour from animals that were left undisturbed postnatally. It is proposed here to discuss this neonatal handling phenomenon only in so far as it might affect the behavioural experiments carried out during the studies reported in this thesis. For example; Levine, 1962, considered that the discrepancies reported in neonatal stressing experiments arose from neglect of the "de-emotionalizing effect of handling in infancy". Grey, Levine and Broadhurst, 1965, found that a single injection of an inert substance, arachis oil, markedly increased emotionality in rats as defined by the open field test and a technique used by Bindra and Spinner, 1958, called "time sampling".

Apart from the behavioural experiments reported above, Bovard, 1954, proposed a physiological theory to account for the effects of early experience on the viability of albino rats subjected to immobilization stress. Bovard suggested that handling caused a reduction in the sympathetic nervous system's

activity and a decrease in the effectiveness of the pituitary adrenal axis. This induced change resulted in permanent hypoactivity of the adrenal glands of gentled animals. However, Weininger, 1954, presented physiological findings which appear to contradict Bovard's theory. Weininger found increased metabolic rate and greater distension of cardiac blood vessels in handled animals. Jailer, 1950, using physiological measures, found that the maturation process of the pituitary adrenal axis took eight days postnatally. However, Rinfret and Hane, 1954 were not able to confirm Jailer's findings in a later study. Evidence from both behavioural and physiological studies, without being able to agree on precisely what is involved, indicates that caution and suitable controls are required in experiments that involve handling neonates. As indicated earlier in this chapter Hutchings, 1963, and Shaefer, 1963, reported that a critical, and often overlooked factor, in the handling phenomenon is a drop in body temperature. In all of the studies reported in this thesis attempts were made to minimize any hypothermia resulting from removal of a neonate from the nesting box.

Young, 1965, reported that a neglected and possibly an important confounding variable in neonatal treatment was the maternal behaviour. He suggests that the lactating female might

behave preferentially towards non-treated neonates, i.e, the female displayed differential behaviour which resulted in the treated animal receiving less attention than its undisturbed littermate. At the beginning of the studies reported in this thesis attempts were made to discover if the females behaved selectively towards her IS or NHS young. One particular problem was that if the IS neonate suffered from any form of muscular asthenia, would this result in it fairing less well than the NHS littermate in the competition for nourishment? As part of undergraduate theses Powell, 1966, and Bayfield, 1967, were unable to show any evidence of preferential selection by the maternal female between IS or NHS young; nor were they able to show preferential treatment ~~towards~~ between the sera injected and non-injected control animals. The technique used by the two undergraduates was to measure various aspects of maternal behaviour in a specially built retrieval arena. The apparatus used was very similar to the design reported by Young, 1965.

Immunosympathectomy and Muscular Asthenia.

From the outset of these studies it was realized that it must be clearly demonstrated that any inferiority shown by IS

animals, both during the acquisition and maintenance of avoidance learning, was not due to any muscular weakness arising from the hypotrophic sympathetic nervous system of IS animals. The studies of Auld, 1951, and Brady, 1953, quoted in the introductory chapter, clearly indicate the need for clarification on this point. Cannon (1929) and his associates, described some aspects of the physiology and behaviour of cats and dogs surviving complete sympathectomy. Their main conclusion was that the sympathetic nervous system in cats and dogs was not essential for life. Once the initial effects of the surgery was over, little or no sensori motor impairment was apparent. Brouha, Cannon, and Dill, 1936, as part of their analysis of the heart-rate of sympathectomized dogs during rest and exercise, concluded that the capacity for intense exercise was not diminished in their sympathectomized dogs. The dogs were tested in a treadmill using an endless belt arrangement.

Appley, 1964, in an address to the Canadian Psychological Association, entitled "Endocrine factors in Avoidance Learning" examined the role of the pituitary-adrenal axis in avoidance learning in rodents. He took account of what he called the "debilitation hypothesis" in his hypophysectomized rats and attempted to show that his experimental animals did not possess

a marked muscular disadvantage compared with the control animals. He rightly concluded that any debility effects would in varying degrees range over all aspects of performance, but only tested for debility in the avoidance situation that he used. For the purposes of the present studies it was decided to attempt to analyse the problem of muscular weakness over a wide range of conditions. To this end, after several aborted attempts, a collaboration with A. de Sa, Lecturer in Electronics, University of Newcastle upon Tyne, produced a continuously recording apparatus that would record the home cage activity of rodents over several consecutive days. The continuous recording activity cage that was developed during this collaborative study has been reported by Van-Toller, and de Sa, 1968. Activity tests were made on both rats and mice for periods of four or five consecutive days. Activity was recorded on an Elliott micro amp meter which uses a pen recording chart. Quantification of the data involved counting the pen excursion from the base line. The pen traces were added to one of the four categories of pen movement used and finally a histogram of activity for each 24 hour period was plotted. For further details see the paper reported above. Apart from a sex difference, no essential differences were observed in a study involving the following animals:

10 IS,10NHS, and 6 MH rats; 8 IS, 10 NHS, and 7 MH mice. However, it should be noted that IS animals always tended to show slightly more activity. No significant differences were observed between food and water consumption within the two species used. However, it was not possible to differentiate between gnawing and eating of food pellets.

Reproduction and Rearing by Immunosympathectomized Mice.

Cannon, Newton, Bright, Menkin, and Moore, 1929, reported that they found no adverse effects on the reproductive and rearing abilities of sympathectomized female cats. They also reported that they had no evidence concerning the reproductive abilities of male cats following sympathectomy. The author designed an experiment in which he examined the reproductive capacities of both male and female IS mice, and also the rearing ability of IS female mice. Mice of reproductive age, one male and one female, were placed into breeding boxes. The initial experiment consisted of the following design:

3 IS females	placed with 3 IS males.
3 IS females	placed with 3 normal males.
3 normal females	placed with 3 IS males.
3 normal females	placed with 3 normal males.

At the end of two weeks the male mice were removed from the

breeding boxes and the females observed daily until their litters were weaned on the twenty-first day, post partum. All the females gave birth to litters and there were no significant differences between the IS and normal females' litters, either in mean number or individual body weight of the neonates.

The experiment was then repeated, using a continuous breeding technique. During the 24 hours following parturition, female mice undergo a false estrous period during which reproduction usually takes place if the breeding buck is left in with the female. It was thought that this more demanding reproduction and rearing programme might reveal differences between the IS and normal females. The continuous breeding experiment used the same basic design but different animals. Each female was allowed to produce and rear three litters before the experiment was terminated. Litters were removed from the breeding boxes nineteen days following birth. In the first experiment two of the six IS females failed to produce a second litter from i.e., did not become fertilised during the false estrous period occuring immediately after parturition. A further eight IS females were placed in breeding boxes; four with IS males and four with normal males. These females all gave birth to three litters at 21 day intervals. It was concluded that in the strain of mice used

both male and females IS mice showed similar reproductive and breeding patterns to those found in the normal mouse of the strain.

CHAPTER III.

OPEN FIELD BEHAVIOUR IN IMMUNOSYMPATHECTOMIZED MICE.

This chapter was published in the journal "Physiology and Behaviour", 1968, 3, 1 - 4 and was the first complete study made. Supplies of the NGF antiserum had been obtained from the Wellcome Research Laboratories. This source of freeze dried antiserum, although still limited, meant that it was possible to concentrate on the behavioural aspects of immunosympathectomized rodents. One of the limitations of earlier studies stressing the role of the sympathetic nervous system in mediating behavioural responses to aversive situations, may have been the use of adult animals giving increased chances of vicarious functioning. The injection of the nerve-growth-factor antiserum to produce immunosympathectomized animals has the advantage that the hypotrophy of the sympathetic nervous system occurs before the animal has received any extensive conditioning to the widespread and diffuse effects of the efferent neural sympathetic outflow. This study reports an experiment in which immunosympathectomized and control litters of mice were subjected to minimal disturbances until 40 days when they were tested in an open field situation.

METHOD.

Subjects:

Twelve litters were obtained from multiparous females taken from a genetically heterogeneous population of mice maintained in the Department of Psychology, University of Durham. Each litter was culled shortly after birth to the six heaviest neonates and randomly allocated to one of three treatment conditions. This study is primarily concerned with the male animals; final number 38. A number of deaths, which did not reach significance, were recorded for each group. Litters were weaned at 21 days.

Weights:

Total body weights were recorded on days 1, 10, 21, and following the open-field test, on day 40.

Behavioural Apparatus:

The open-field consisted of a circular arena 27.85 inches in diameter surrounded by an 11 inch wall. The floor was marked (light green colour) with three concentric circles divided into segments by lines radiating from the centre. The divisions were arranged to give the best approximation to equal distance no matter in what direction the animal moved. Both the floor and

the wall were covered by an adhesive plastic covering which gave an overall neutral-grey background. The field was enclosed inside a 36 x 36 x 36 inches metal framework covered by white muslin. White noise was supplied by a Grason Stadler (Model No. 901A) noise generator connected to 3 x 3 inch speakers, hung at a height of 26 inches above the floor of the arena. Two levels of sound were provided by appropriate resistors: low level 80 - 80 dB; high level 92 - 94 dB (ref. 0.0002 dyn/cm^2). Lighting was provided by 4 x 150 W flood lamps. Three outer lamps were suspended at a height of 20 inches above the floor and a central lamp at a height of 24 inches above the floor of the arena. Two levels of light intensity were used: low level (central lamp) 63 ft-c; high level (four lamps) 500 ft-c. Sound level and light intensity were measured at 1 inch above the floor of the arena.

Behavioural Measures:

Individual animals were removed from their litter, placed in the carrying case and taken into the experimental room. After confinement in the carrying case for 2 minutes, the animal was then removed from the box and placed into the centre of the arena. The 2 minute confinement was used to reduce any effects

TABLE 1

BEHAVIOURAL AND PHYSIOLOGICAL MEASURES SHOWING MEANS, STANDARD DEVIATIONS, AND LEVELS OF SIGNIFICANCE FOR EACH TEST

		BEHAVIOURAL MEASURES												PHYSIOLOGICAL MEASURES			
		Distance Travelled (ins)		Rearing		Defecation (Boluses) Carrying Box		Open Field		Time (Centre to Side) (sec)		Inner Circle Approaches		Ganglia (mgs)		Adrenals (mgs)	
	N	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
MALE																	
I.S.	15	449	197	8.3	8.3	1.0	0.89	0.4	0.49	6.6	6.8	2.5	1.8	0.44	0.18	4.9	1.12
N.H.S.	13	275	144	3.1	3.4	0.38	0.54	0.38	0.43	3.8	3.4	0.8	1.8	1.15	0.28	5.0	1.50
M.H.	10	192	92	2.9	1.8	0.10	0.28	0.90	0.60	4.4	5.3	0.4	0.4	1.08	0.02	5.1	0.43
Overall p		<0.01		<0.05		NS		NS		NS		<0.02		<0.001		NS	
FEMALE																	
I.S.	7	480	321	6.8	6.3			2.8	0.3	2.34	0.7	3.2	1.2	0.42	0.18	6.25	1.19
N.H.S.	7	336	184	4.7	3.5			1.5	0.5	3.1	1.9	2.0	1.7	0.93	0.30	5.44	1.34
M.H.	13	264	206	5.5	6.2			1.0	0.95	3.4	3.1	2.1		1.17	1.3	7.34	1.42

of variation in the time taken to pick any animal out from its litter. During the 2 minute confinement and the initial part of the open-field test, both the sound and light intensity were at the low levels. A record was made of the time taken for the animal to cross from the centre spot to the innermost line of the outer ring. On reaching the outer ring, or if the animal failed to leave the central area within 20 seconds, a foot button was operated which turned on the high level intensities of the light and sound for an automatic period of 2 minutes.

During the 2 minute testing period the following behavioural measures were recorded: (a) activity; number of lines crossed by the animal, (b) centre approaches; the number of excursions made from the outer-most concentric ring into the central areas, (c) rearing; defined as an upward movement in which the animal raised its head and forepaws above the midpoint of its body, (d) grooming; defined as face washing or other behaviour directed towards the animal's body, (e) defecation; number of boluses voided in (1) the carrying case and (2) the open-field, (f) urination. After each test the floor and wall of the open-field were washed with a diluted soap solution, containing hexachlorophene, and dried.

Physiological Measures:

Immediately following the open-field test the animal was killed over a mixture of ether and chloroform. Total body weight was then recorded and the animal dissected to remove the pairs of cervical and thoracic ganglia together with the adrenals. The ganglia and adrenals were cleaned of connective tissue and fat before storing them in 10% formolsaline. After all the litters had been tested and dissected, separate total weights of each individual animal's sympathetic ganglia and adrenals were recorded. Histological sections were made of ganglia from randomly selected animals from each group. There was no significant difference between the relative weights obtained from the dissections made by the experimenter and those made by a technician who lacked detailed knowledge of the experiment.

RESULTS.

Table 1. presents the means and standard deviations for those measures that occurred sufficiently frequently for meaningful analysis. The following data: ganglia size, adrenals, total body weight, distance travelled in the open-field, and time taken to cross from the centre to side of the arena were transformed to log scores to reduce heterogeneity of group

variation, and subjected to analyses of variance for unequal sample size and the Newman-Keuls test for differences between group means (Winer, 1962).

The sympathetic ganglia from the IS animals were much smaller than those from animals in the other two groups and an overall difference was found ($F = 34.35$; $\underline{df}$ 2/35, $\underline{p} < 0.001$). Comparison of group means revealed that the NHS and MH groups did not differ significantly, but that both differed ($\underline{p} < 0.01$) from the IS group. No overall difference was found between the adrenal weights of the three groups. Analysis of the total body weight data was precluded by the initial random selection, which resulted in a very low variance for the weights of animals in the NHS group; however, the total body weights of all the animals were very similar throughout the course of the study.

IS animals ran further in the open-field than those of the other two groups. Analysis revealed an overall difference ($F = 6.32$; $\underline{df}$ 2/35, $\underline{p} < 0.01$), and comparison of the group means for this measure showed that the NHS and MH means were not significantly different from each other, though both differed significantly ($\underline{p} < 0.01$) from the IS group. No significant difference was found between the times taken for the groups to cross from the centre of the field to the outer circle.

The following results were analysed using the Kruskal-Wallis one-way analysis of variance by ranks. Rearing occurred most frequently in the IS animals and a difference ($p < 0.05$) was found between the three groups. Comparison of pairs of groups showed that no significant difference existed between the incidence of rearing recorded for the NHS and MH groups, but that both differed ($p < 0.01$) from the IS group. Likewise, the IS animals made most approaches into the central areas of the arena, the overall difference between groups being significant ($p < 0.02$). Separate comparisons of pairs of groups for this measure showed that the NHS and MH groups were not significantly different, but that both differed significantly ($p < 0.005$) from the IS group. Systematic analysis of the number of boluses voided did not reveal any significant differences between the three groups. However, the percentage of animals in each group defecating in the open-field were: MH 60 per cent., NHS 38 per cent., IS 33 per cent. This trend was reversed for the percentage recorded in the carrying case: MH 10 per cent, NHS 30 per cent., IS 47 per cent.

To test a raw data suggestion that a correlation existed between ganglia size and ambulation, an overall correlation coefficient was computed, $r = -0.55$, $p < 0.001$, Fig. 3. This was further supported by an analysis of covariance in which adjustment for the effects of ganglia size on ambulation gave a non-significant result (Winer, 1962). In order to remove any bias

introduced by the possible interaction of the animal's body weight on distance travelled in the open-field, a further analysis of covariance was carried out on this data. Adjustment for the possible effects of body weight on activity resulted in an increased $p < 0.001$.

DISCUSSION.

In this experiment IS male mice showed significant increases in level of ambulation, reared more and made more excursions into the central areas of the arena when tested in an open field. A negative correlation between ganglia size and distance travelled in the open field suggests that size, or functional level of the sympathetic nervous system might be an important factor in mediating certain types of behaviour. Klingman and Klingman (1965), using an *in vitro* test on ganglia taken from IS rats, have reported a negative correlation between ganglia size and functional integrity using several criteria.

One measure which was not clear-cut was defecation. IS animals showed increased defecation in the carrying box which had previously been shown, using a heart rate index, to have less disturbing effects than occurred from other methods of transport. Recent work has suggested that defecation in mice

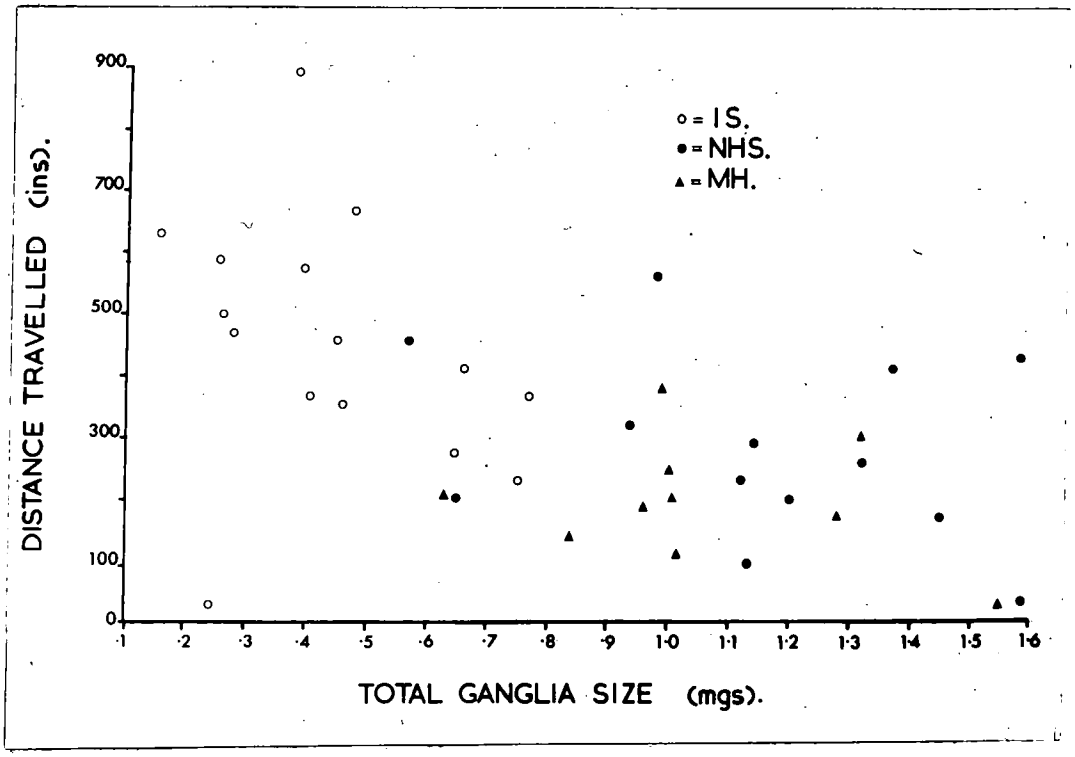


FIG. 3 Scattergram showing a negative correlation between sympathetic ganglia size and distance travelled in the open field.

is related to territory marking. Collins (1966) has indicated that defecation of mice placed into novel environments is determined by multiple factors. The findings of Collins together with the observations of Iverson, Glowinski and Axelrod (1965), and Vogt (1964) that neonatal nerve-growth-factor antiserum injections do not appear to result in any considerable reduction of the mesenteric and coeliac ganglia of rats, would indicate that at this point it would be premature to draw any conclusions from this particular measure.

Since the above experiment was carried out, Wenzel and Jeffrey (1967) have reported that using an inbred strain of mice they found decreased activity in IS animals compared with their control animals. As Wenzel and Jeffrey point out, the strain used in their study could have been an important factor in their failure to find increased activity in IS mice. It is of interest to note that they found that the incidence of defecation was too low for meaningful analysis. High levels of sound and light intensity were not used in the earlier experiment of Wenzel and Jeffrey. In the present study the only measure recorded at the low level intensities, the time taken to cross from the centre to the edge of the arena, was not significant. This suggests that the high levels of illumination and sound might have served to increase group differences.

Whimbey and Denenberg (1967) using rats have recently indicated that for a single short exposure in an open-field, ambulation was not a reliable index of emotionality. For the purposes of this exploratory study it was felt to be more important to give a single test, supporting any conclusions with several measures, in order to reduce the possibility of an alternative bodily system interfering with the main hypothesis. Carpi and Oliverio (1964) have reported large increases of urinary noradrenaline from IS rats subjected to physiological stress.

In the present study the female data has not been analysed owing to a number of deaths resulting in reduced numbers for two of the groups. Group differences for the female data, although the trends were in the same direction, see Table 1, were not so clear-cut as the group differences of the males.

CHAPTER IV.

ACQUISITION OF PASSIVE AVOIDANCE.

The experiment reported in Chapter III had shown that neonatal injections of NGF antiserum for three consecutive days post partum had produced permanent hypotrophy in the sympathetic nervous systems of the mice used. Also, using an open-field test behavioural differences had been shown between IS and NHS littermates. The open-field test is difficult to control, and to specify exactly what is happening in the situation is impossible. The experimental data, although showing differences between the two groups, is open to diverse interpretations of equal validity. Attention was then switched to more rigorous and controllable avoidance situations in which it was possible to make more precise operational definitions.

Wenzel and Nagle, 1965, and Wenzel, 1967, had indicated that only small behavioural differences could be found between IS and NHS mice placed in active avoidance situations. Preliminary studies of active avoidance were made, using a shelf-box apparatus. A jumping response was chosen as being a more natural response for a mouse to make when subjected to an electric shock. The box was designed so that at the beginning of a trial a shelf

was swung into the box at the same time as a tone (CS) sounded. If the animal jumped onto the shelf within the three second CS period he avoided a foot shock delivered from a stainless steel grid floor. Shock was delivered for 10 seconds unless an escape response was made. At the end of a trial the shelf was slowly drawn out where it remained until the next trial. There were several features of the apparatus, designed for use with both mice and rats, which proved to be unsatisfactory. For example, occasionally mice were trapped underneath the shelf as it swung back into the box and in consequence gave very long escape latencies. However, despite these faults, the overall differences between IS and NHS mice did not seem to warrant further development of the apparatus. McKean and Pearl, 1968, have since reported a much more satisfactory shelf-box for use with mice.

In view of Wenzel's apparent lack of clear cut behavioural differences using an active avoidance task requiring a running response and the preliminary studies reported above, it was decided to use a passive avoidance task. This view was further supported by the slight increases found in overall activity level of IS mice when tested in the activity cage. It was thought that as a result of the hypotrophy of the sympathetic nervous system, IS mice might require to maintain a continuous increased

muscular tonus, requiring small increases in activity. Consideration was given to the type of passive avoidance technique which measures the latency of an animal stepping down from a shelf onto a floor area where it had previously been shocked for making that response. The step-down technique appeared to lack some sensitivity in that it did not allow for differences between animals having different levels of activity. For example, an animal with a high level of activity would, presumably, have a higher probability of making a step down response having a shorter latency than an animal with a low activity level. With the evidence from IS mice indicating higher activity levels it was thought that the technique was inappropriate and an alternative technique was sought, Slotnik and Jarvik, 1966, reported a passive avoidance technique which allowed initial activity levels to be measured before the animals were shocked for movements. It was decided to construct a similar piece of apparatus to that reported by Slotnick and Jarvik but designed for use with mice.

METHOD.

Subjects:

Litters from twenty multiparous females were treated by the injection procedure laid down in Chapter II. The young mice were tested in the passive avoidance situation at approximately 30 days. Four unhandled litters were also used as controls.

Apparatus:

The passive avoidance field consisted of a floor 25 x 25 cm sq. covered by 16 rectangular stainless steel places, each being 6 x 6 cm sq. A removable surround 28 cm high and covered in an adhesive plastic material enclosed the floor area. The wall covering gave a neutral grey background and also served to insulate the surround from the metal floor. A photograph of the apparatus is shown in Fig. 4. During tests a Perspex lid covered the top of the activity box. Whenever an animal stepped from one plate to another it completed a circuit that allowed a current of up to 200 μa (200 μa was the absolute maximum value; threshold current between plates was 5 μa) to activate circuitry that finally closed a relay which operated a counter. The circuit diagram of the control panel is shown in Fig. 20. of the Apparatus

appendix. As shown in Fig. 20 the circuit was arranged so that completion of a circuit between two adjacent plates by the mice could result in an electric shock being delivered from a Grason-Stadler (type E 106 4GS) shock generator. The shock level used in this experiment was 0.3 m.a. and of 0.5 sec. duration. Timers and stoppers allowed for an automatic total session time of two minutes and switched the activity counts between counters every 30 seconds.

Bowman Mouse Division Box.

After removal from the litter and during the period of testing, individual mice were kept in separate compartments of a Bowman mouse division box. The complete Bowman unit is 69 x 30 x 12.5 cm deep and consists of 12 individual compartments each 14 x 15 x 12.5 cm deep.

Experimental Procedure.

Sessions were carried out in subdued lighting against a background of continuous white noise (80 - 82 dB, ref. 0.0002 dyn/cm^2.) designed to mask any mechanical noise from the controlling relay racks and the experimenter. Individual mice were carried into the experimental room and placed in the centre of

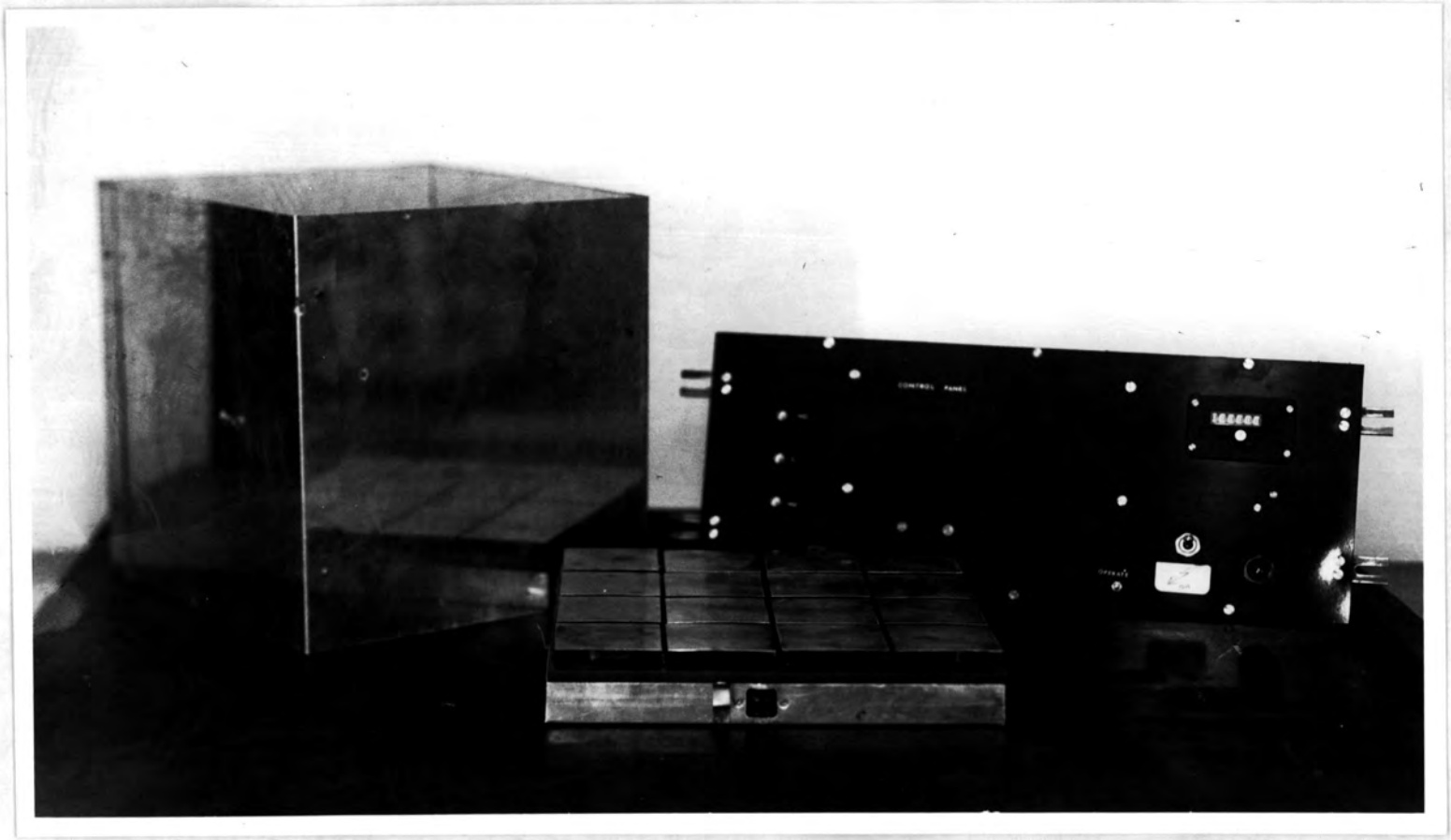


FIG. 4 Passive avoidance field and control panel. The side walls of the field have been removed to show the stainless steel floor plates.

the passive-avoidance field. The initial two minute activity count was started immediately by depressing a handswitch. At the end of any two minute period a timer switched off the activity count and reset the stepper which distributed the counts between the four 30 second counters used. A single switch enabled the experimenter to arrange for an electric shock to be delivered to the mouse for making contacts between adjacent plates. A new 2 minute period was started via the handswitch. During the second shock contingent upon movement(SCM), two minute period, the shock/activity counts were again distributed between the four counters. Following the two minute SCM period, the mouse was either retested immediately with no shock being given, or removed from the passive avoidance field and placed in a separate compartment of the Bowman mouse box. If the mouse was placed in the Bowman box an intervening period of time elapsed until the third and final two minute test period was given.

RESULTS.

Normal mice placed in the field for three consecutive two minute periods without shock showed a decline in the activity count occurring in the final period to approximately 70% of the count obtained during the initial two minute period. If the

shock contingent upon movement (SCM) was given during the second two minute period, activity levels in the third period declined to 10% or less of the values obtained during the first period. Two groups of eight normal mice were tested to observe the effects of an intervening period of 24 hours following the second two minute period. One group received a two minute SCM period while the other group did not receive shock during their second two minute period. During the two minute test given 24 hours later the group that had been shocked in the passive field showed an average overall decline to 45% of the activity level found during the first period. The unshocked group showed an average increase to 118% of the average activity count made during the first period.

The main experimental results are shown in Fig. 5 and Fig. 6. No significant differences were obtained between the IS and NHS female mice for any phase of the experiment. As can be seen from Fig. 5, the females after being shocked for movements in the field made very few movements when replaced in the field, regardless of the length of the intervening period of time. The male IS and NHS mice did not differ significantly in the number of movements made during the initial two minute period or in the number of shocks received. An analysis of variance made on the data obtained from the retesting showed a significant

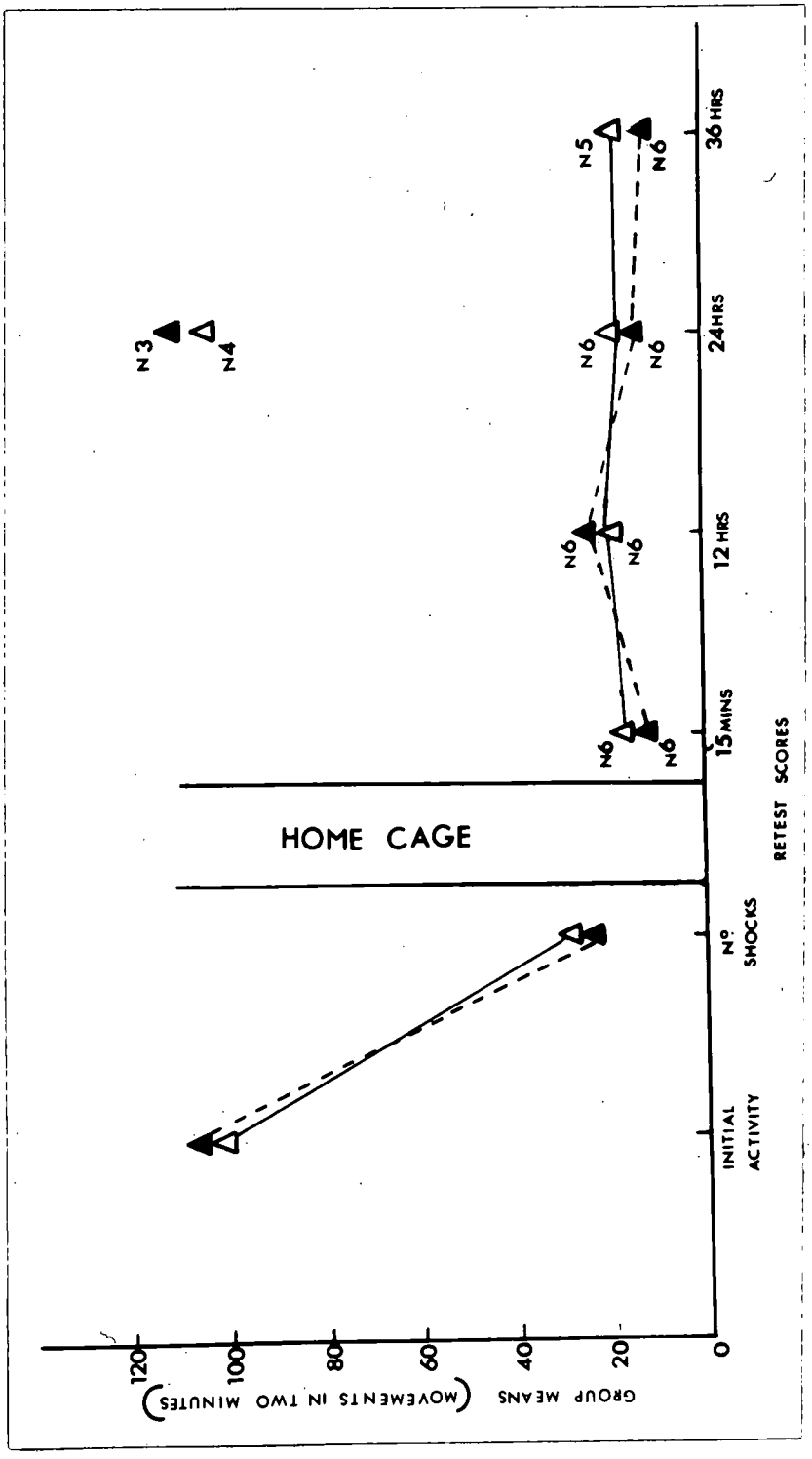


FIG. 5

Mean activity for female mice during the three phases of the passive avoidance test. (▲ = IS; △ = NHS). Numbers of separate animals tested from each group are shown at each retest point. Above the 24 hour retest point are shown the values obtained from mice that did not receive shock in the field.

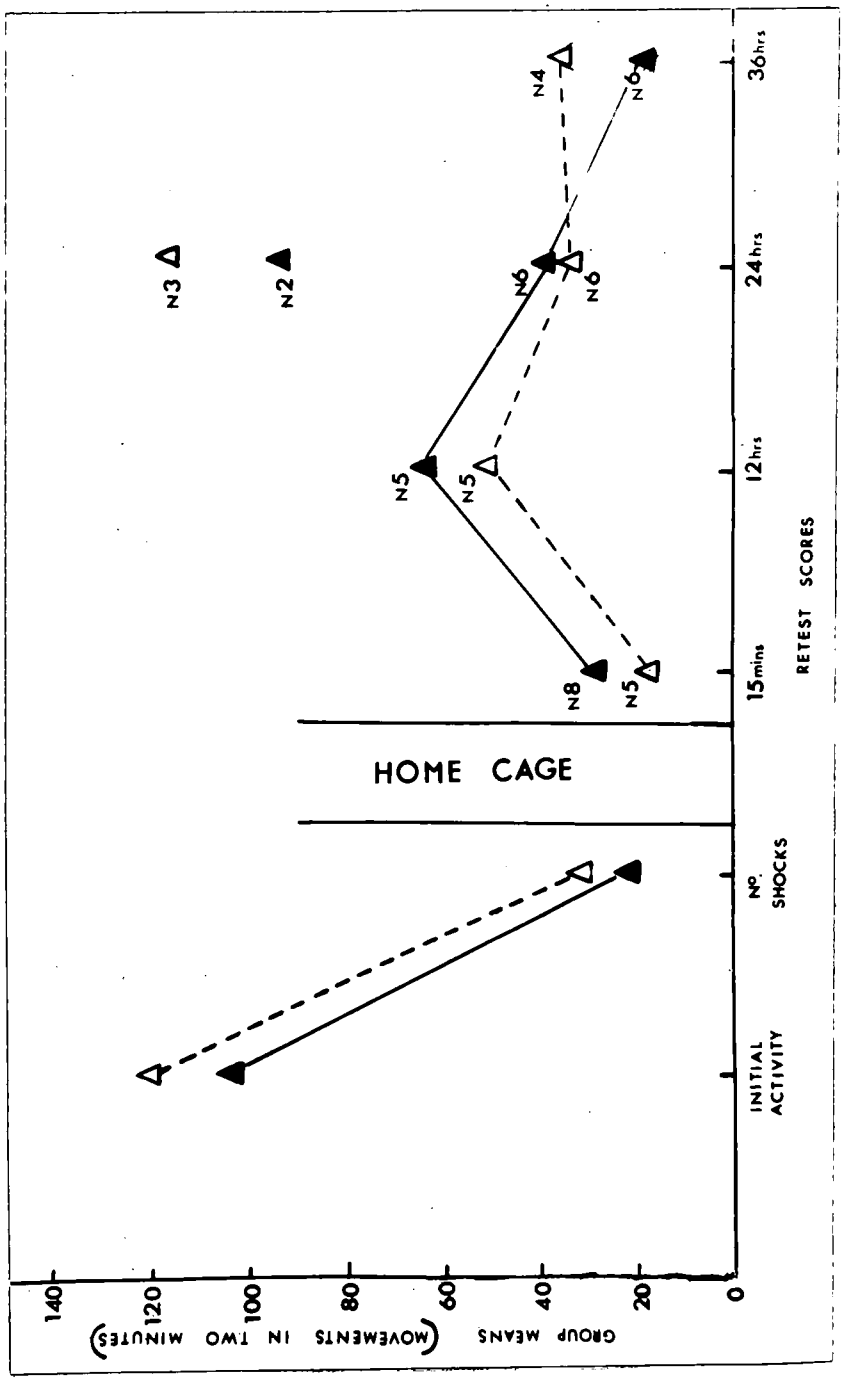


FIG. 6

Mean activity for male mice during the three phases of the passive avoidance test (▲ = IS; Δ = NHS). Numbers of separate animals tested from each group are shown at each retest point. Above the 24 hour retest point are shown the values obtained from mice that did not receive shock in the field.

difference due to time ($p < 0.01$). No significant differences were obtained for the treatment factor, or the treatment x time interaction.

A rank correlation test was computed for the number of shocks received and the retest score. The IS mice did not give a significant result, $r = + 0.11$, $p < 0.50$. However, the data from the NHS mice did reveal a significant correlation $r = + 0.45$, $p < 0.05$. Attempts were made to elucidate this difference and a ratio was computed for each mouse in the two groups which would correct for overall activity level. The ratio computed was $\frac{\text{(2nd period - 3rd period)}}{\text{1st period}} \times 100$. An analysis of variance made on the transformed data did not reveal any significant results.

No significant differences were obtained between mice from the four non-handled litters and the sera injected mice.

DISCUSSION.

The passive avoidance test used did not reveal any essential behavioural differences between IS mice and NHS mice who were littermate controls of the experimental animals. The single significance difference obtained in the experiment, due to time, was the result of animals who were tested following an intervening period of 12 hours giving increased activity scores.

This increase can be seen in Fig. 6. No explanation is offered for this finding.

Following the initial third two minute test period, the animals were tested daily for eight days. These daily retests showed a tendency for the activity levels to increase gradually over days, but no significant differences were obtained between the IS and NHS mice.

At the outset of this experiment it was hoped that, because the avoidance task being used could retest the conditioned animal in a relatively short time period, ~~that~~ it would serve to reduce any confounding effects due to internal compensation. However, even when the animals were tested immediately after the shock contingent upon movement period, no essential differences were obtained.

Carpi and Olverio, 1964, had reported that although urinary excretion of noradrenaline from IS rats (sympathetic nervous system's transmitter substance) was found to be below the level of normal rats, injection of amphetamine caused a marked increase in their urinary noradrenaline levels. This increase of noradrenaline was found even after adrenal demedullation. The authors concluded that, "the results obtained indicate that in the rat, there exists a noradrenergic system which enters in action (sic) when the sympathetic system is eliminated". Carpi

and Oliverio's findings of a reserve adrenergic system might explain the apparently unimpaired ability of IS mice to learn an avoidance task despite the massive morphological reduction in their sympathetic nervous systems.

It was then decided to use one remaining litter of injected animals from this experiment in an attempt to reduce their level of noradrenaline. If IS mice were compensating for their reduced sympathetic nervous system by increased levels of noradrenaline then reduction of this substance should affect them more than mice having a normal sympathetic neural network.

Carlsson, Rosengren, Bertler and Nilsson, 1957; Vogt, 1960; Smith, 1965; Iversen, 1967, had shown that injections of the drug reserpine caused a considerable depletion of noradrenaline stores in the peripheral adrenergic nerves. Iversen, Glowinski and Axelrod, 1965, had also shown reduction in the uptake and storage of labelled exogenous noradrenaline H^3 in reserpine pretreated rats' hearts. Injection of the drug reserpine, apart from its effects of reducing noradrenaline, also depletes other transmitter substances, produces hypothermia and reduced sensori-motor activity; however, the depletion of noradrenaline is very severe and appears to have a longer time course than the other effects found. Following preliminary tests in normal mice, it was decided to inject fairly low dose levels of 1 mg/kg body

weight (the solution used for dissolving the reserpine has been described by Pletscher, Shore and Brodie, 1955) and to allow the mice a 24 hour recovery period before the passive avoidance test was given.

A litter of mice, consisting of six NHS and three IS male mice, was injected and allowed to remain in the Bowman mouse division box for 24 hours. The results obtained from the mice are shown below:-

	1st period average activity.	2nd period average shock/activity.	3rd period average activity.
NHS N = 6	95	20	8
IS N = 3	66	17	16

(The two minute periods were presented consecutively.)

Although it was not possible to base any firm conclusions, in view of the very limited number of mice, on the above results it was the first time that IS mice showed marked differences from their NHS littermate controls. The results were also held to lend some support to the idea of a compensatory process which was occurring in IS mice due to the induced denervation.

A final problem concerned the relatively macroscopic nature of the learning situations being used in the attempt to obtain

behavioural differences between IS and NHS mice. In any avoidance learning task that takes place over many days, clearly compensatory processes have ample time to develop. It had been hoped that the passive avoidance field, which allowed for retesting to take place within minutes, would reduce the confounding influences from any such compensatory process. However, the question arose of whether the few minutes that occurred in the passive avoidance field between acquisition and retest were sufficient for compensatory changes. It certainly seemed as if this might be the case.

At this time the apparatus required to measure noradrenaline was not available within the University of Durham. It was, therefore, decided to use a behavioural avoidance task that would enable the mice to be retested within very short time periods from the acquisition of an avoidance response.

CHAPTER V.

STARTLE RESPONSE.

Extending the earlier works of Prosser and Hunter, 1936; Brown, Kalish, and Farber, 1951, reported that, in rats, the magnitude of a startle response occurring to an explosive sound was increased if the noise coincided with the presence of a non-startling stimulus (originally neutral, CS) that had previously been paired with an electric shock (UnCS). Appley, 1965, used this startle reaction technique to measure a conditioned fear response, taking as his index of the induced affective state the magnitude of the startle responses. During a series of experiments, undertaken to investigate the role of the pituitary-adrenal axis in avoidance learning, Appley found significant differences between hypophysectomized and sham operated animals. Using a 't' test he examined the gains made by each group. He showed significant increases in amplitude of the startle responses made by the control group animals. However, the change in response amplitudes of the hypophysectomized animals failed to reach significant levels. As no significant differences were found between the hypophysectomized and his surgical procedure control animals to the UnCS, Appley concluded that the latter finding indicated that the inferior conditioning found in the hypophysec-

tomized group was not a function of their inability to respond vigorously but of reduced "emotional appreciation". Detailed analysis of the group data was not presented, so that it was not possible to examine Appley's dismissal of the debilitation hypothesis.

The major advantage of the startle response technique for this series of investigations was that it allowed the testing of a conditioned fear response within a relatively short time period of it being established. Another advantage of the technique was that it would allow comparison of the response profiles of both the IS and NHS mice to electric shock per se. Differences occurring to the shock presentations might reveal disparities between the experimental and control mice, due to the considerable reduction in the sympathetic neural network found in IS animals.

METHOD.

Subjects:

Litters from twenty-four multiparous females were treated by the injection procedure laid down in Chapter II. The young mice were tested in the startle chamber from 28 - 32 days.

Startle Chamber.

The startle chamber used in this experiment was developed by the author and A. M. Perry, Senior Technician within the Department of Psychology. A full description and evaluation of the chamber is contained in the appendix, but the design, after careful consideration, was based upon three main principles: The chamber volume was approximately matched to the size of a 30 day mouse; damping of unwanted chamber movements was achieved by placing it on a block of foam rubber; movements of the chamber were detected via a pressure contact linkage held between the two crossed arms of a phonograph crystal. The crystal was contained within a stereo-crystal cartridge taken from the pick-up arm of a record player. A readout of pressure changes from the crystal was displayed on an oscilloscope and a permanent record made of the trace by using photo sensitive recording paper in an oscilloscope camera.

Stimuli.

The conditioned stimulus (CS) was a 3 watt, 100 v AC light bulb, 1.75 sec. duration, with a 3K resistance placed in series with it to reduce the light intensity to approximately 1.6 watts. The bulb was suspended 6 cm above the centre of the chamber. Electric shocks (UnCS), 0.4 sec duration, were delivered from a Grason Stadler type E 106 4GS shock generator via the stainless steel grid floor of the chamber.

The explosive stimulus sound or plop was produced from an oiled paper condensor (120 v D.C., 16 mfd) discharging through a 3 ohm, 7.5 cm speaker suspended obliquely 10 cm above the centre of the chamber. A thick cardboard tube, 7.5 cm x 5.25 cm, was mounted behind the speaker to increase the resonance of the sound. See Fig. 7 for the arrangement of the stimuli sources and a photograph of the chamber. During testing the startle chamber was placed inside a sound-damped and light proofed box.

Procedure:

On the first day of the experiment mice were removed singly from their litter, taken into the experimental room and placed directly into the startle chamber. Trials were begun immediately. Following the conditioning and testing sequence shown in Table 2, the mice were placed (one IS and one NHS of similar sex) into separate cages in the main animal room. Previously, the mice had been kept in the isolated mouse-hut which when the experiment began was not large enough to allow separation of the litters. required by the experimental design used. Except for the daily conditioning and testing trials the mice remained in these cages with ad. lib. food and water for the duration of the experiment. The experiment was run at night to give the necessary uninterrupted eight hours required to run all the animals. After the final session the animals

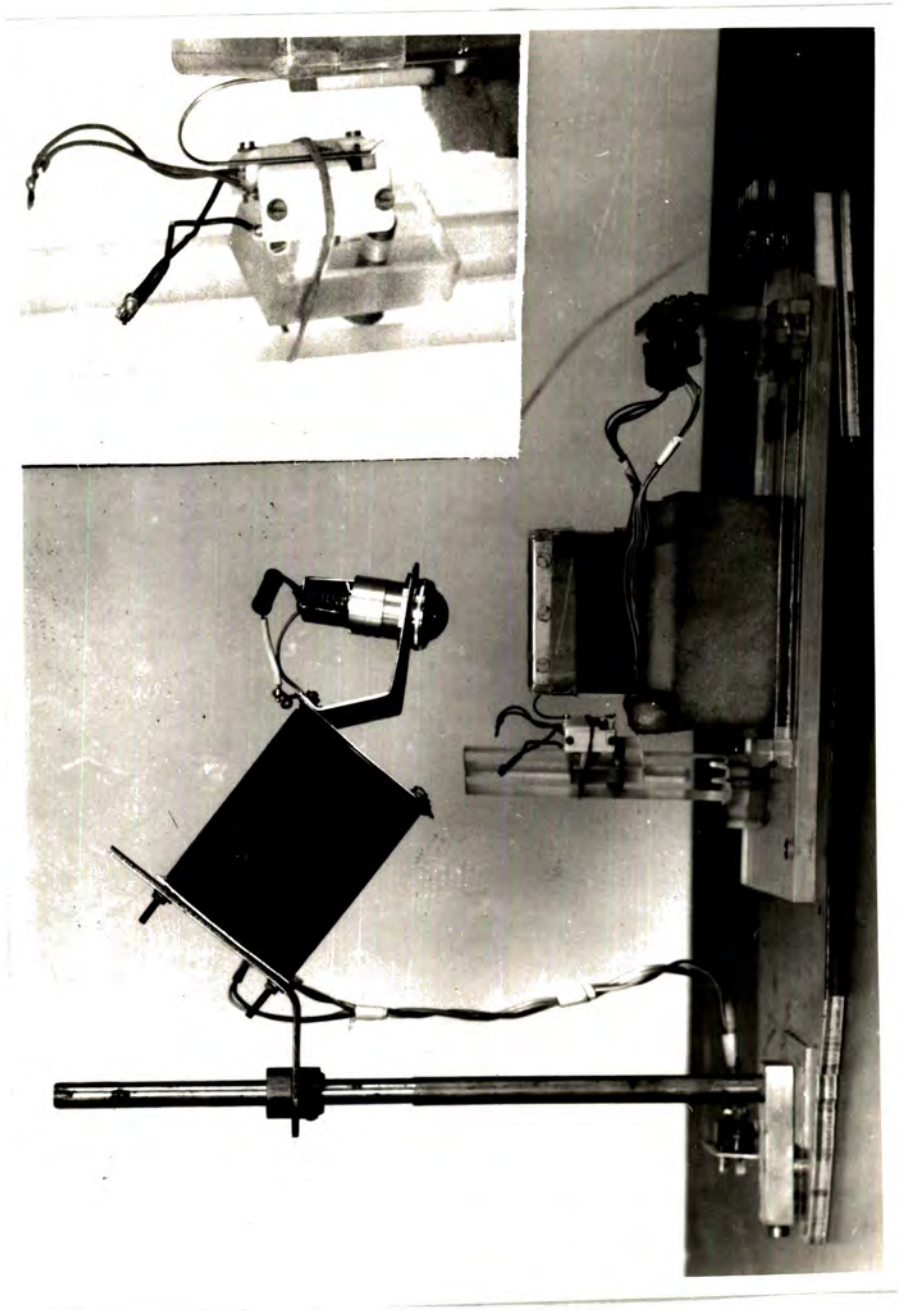


FIG. 7 Startle Chamber and arrangement of stimuli sources. Inset shows an elastic band retaining the U shaped linkage arm from the chamber in firm contact with the crossed arms projecting from the crystal.

were sacrificed and their thoracic and cervical ganglia dissected and weighed.

Experimental Design:

Pilot studies had shown that a maximum startle response was obtained with a UnCS of 1 ma. It was decided to use 1 ma as a low shock intensity and also, in an effort to reduce any internal compensatory mechanism, to include another group receiving a shock intensity of 2 ma. It was realized that problems of tetanizing and other side effects might arise from the use of such high levels, but it was thought that by "flooding the system" behavioural separation between IS and NHS might be obtained (Wynne and Solomon, 1955, used a UnCS of 12 ma to obtain their partial behavioural separation between normal and sympathectomized dogs).

Forty eight sera injected mice were placed into the following groups:-

1.	Male IS	UnCS 1 ma.
2.	Male IS	UnCS 2 ma.
3.	Male NHS	UnCS 1 ma.
4.	Male NHS	UnCS 2 ma.
5.	Female IS	UnCS 1 ma.
6.	Female IS	UnCS 2 ma.
7.	Female NHS	UnCS 1 ma.
8.	Female NHS	UnCS 2 ma.

TABLE 2

Daily sequence of conditioning and testing trials. Trials marked 'record' were recorded onto photographic paper using an oscilloscope camera. Values in brackets refer to paper speed and voltage magnitude displayed on the oscilloscope screen. Intertrial intervals were 20 sec.

TRIAL	1.	CS - PLOP - RECORD (6.35cm/sec. 0.1v/div)
	2.	1ST SHOCK - RECORD (2.54cm/sec. 0.5v/div)
	3.	2ND SHOCK
	4.	3RD SHOCK
	5.	4TH SHOCK
	6.	CS - PLOP - RECORD (6.35cm/sec. 0.1v/div)
	7.	5TH SHOCK
	8.	CS ALONE - RECORD (6.35cm/sec. 0.1v/div)
	9.	6TH SHOCK
	10.	PLOP ALONE - RECORD (6.35cm/sec. 0.1v/div)
	11.	7TH SHOCK
	12.	8TH SHOCK - RECORD (2.54cm/sec. 0.5v/div)
	13.	CS - PLOP - RECORD (6.35cm/sec. 0.1v/div)

See Table 2 for the sequence of the conditioning and testing trials, and also the trials that were recorded each day. Order of testing for the groups was made by random selection on each day.

RESULTS.

Data tabulated in tables was drawn up for individual mice for the nine trials that were recorded from each session over the four days. The recorded traces were used to draw up the tables and the following measurements taken:- (all measurements were made to the nearest 0.5 mm)

1. Magnitude of response; this was taken as the largest amplitude above base line recorded for each trial. If the largest amplitude occurred below the baseline, it was measured as the magnitude of response.
2. Latency of response; this was the distance between the onset of the stimulus and the first movement of the trace away from the base-line. Fig. 22 in the appendix, shows typical traces recorded during the experiment. Measurements of the records were carried out by technicians having no detailed knowledge of the purpose of the experiment.

The tabulated data was analysed using a split-plot analysis of variance. The design used is shown in Table 12 which can also be found in the appendix. Initial analyses were made on untransformed data which was shown to be homogeneous overall.

Magnitude and Latency of Startle Response.

Daily mean values of the startle magnitude and latency from the eight independent experimental groups are shown in Fig. 8. Analysis of the treatment x sex x shock half of the design revealed a significant difference between the sexes, $p < 0.025$. The lower half of the table testing over days revealed the following significant differences:-

Between days	$p < 0.01$
Days x shock	$p < 0.01$
Days x sex x shock	$p < 0.001$
Days x sex x shock x treatment	$p < 0.001$

In seeking an explanation for the significant differences found in the second, third and fourth order interactions it was decided to transform the raw data. Winer, 1962, has pointed out that the original measure used can produce significant interactions. From inspection of the data it was obvious that an animal who only gave one startle response over the total eight CS - plop trials given contributed more to the total variance than an animal who had given a startle response on every occasion

the CS - plop was tested. Accordingly, the data was transformed using a log + 1 transformation in an attempt to reduce this type of interfering factor.

The transformed data resulted in the significant sex difference, found when the raw data was analysed, becoming non-significant. In the lower half of the table the following interactions were still found to be significantly different:

Days x shock	$p < 0.01$
Days x shock x sex	$p < 0.01$
Days x shock x sex x treatment	$p < 0.05$

Thus, the transformed data reduced the level of significance for the interactions but did not eliminate them. This point will be examined in more detail in the discussion section.

The main hypothesis of this experiment was to examine the possibility that a rapid compensation was taking place in the hypotrophic SNS of IS mice, and that this compensation was masking essential behavioural differences between the experimental and control animals. The split-plot design used to analyse the data resulted in the four daily means being reduced to a single score and thus eliminating differences between days. It was, therefore, decided to carry out a separate day by day analysis of variance to examine for possible daily differences. The analysis of variance computed from the separate daily results

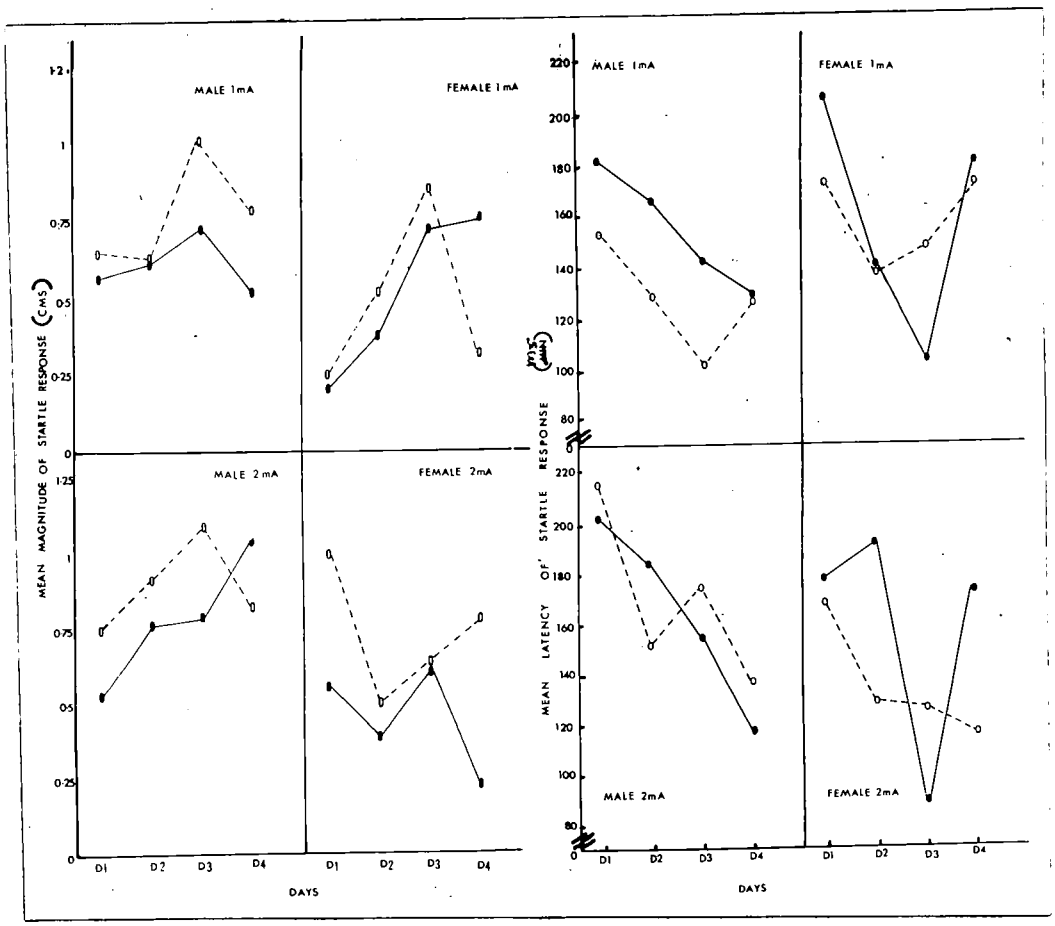


FIG. 8 Mean magnitudes and latencies of startle response recorded from the eight independent groups on each day (O = IS; ● = NHS).

revealed the following significant differences:-

Day 1.	treatment	$p < 0.05$
	shock	$p < 0.01$
	shock x sex	$p < 0.01$
Day 2.	sex	$p < 0.05$
Day 3.	sex	$p < 0.05$
Day 4.	sex	$p < 0.025$
	sex x treatment	$p < 0.025$

The separate day by day analysis revealed a treatment difference on day 1, but at a low level of significance. Inspection of Fig. 8 reveals that the greatest differences occurred in mice receiving the higher shock levels. A correlation coefficient was computed for ganglia size and the magnitude of the startle response. On day 1 a significant positive correlation was found between total ganglia size and the magnitude of the startle response, $r = + 0.55$, $p < 0.01$, for the 2 ma shock level see scattergram shown in Fig. 10. The correlation coefficient computed between the ganglia size and the magnitude of the startle response for the 1 ma level was not significant. Significant correlations between total ganglia size and magnitude of the startle response were not obtained after the first day.

From inspection of the graphs shown in Fig. 8 there appears to be a suggestion that the shock levels used were not comparable between the two sexes. The diagonal graphs, male 1 ma, female 2 ma,

and vice versa, appear to have more common features than the true comparison of male 1 ma, female 1 ma, etc. A more meaningful result might have arisen if the shock levels between the sexes had been adjusted to different levels. To check this an analysis of variance (using a log + 1 transformation) was carried out on the male data alone. This analysis did not reveal any significant differences.

Analysis of the startle response latencies using the split-plot design failed to reveal any significant differences apart from ~~day 3~~ between days where $p < 0.01$. The day by day analysis of variance also failed to show any significant differences, as did analysis using transformed (log + 1) data in the split-plot design.

Magnitude and Latency of Shocked Response.

Daily mean values for the magnitude and latencies of shocked responses are shown in Fig. 9. Analysis of the data using the split-plot design revealed significant differences between sex, $p < 0.01$, and shock, $p < 0.001$, variables in the treatment x sex x shock half of the table. Testing over days revealed significant differences between days, $p < 0.001$ and days x shock, $p < 0.025$. Analysis of variance computed on the day by day results revealed significant differences between the

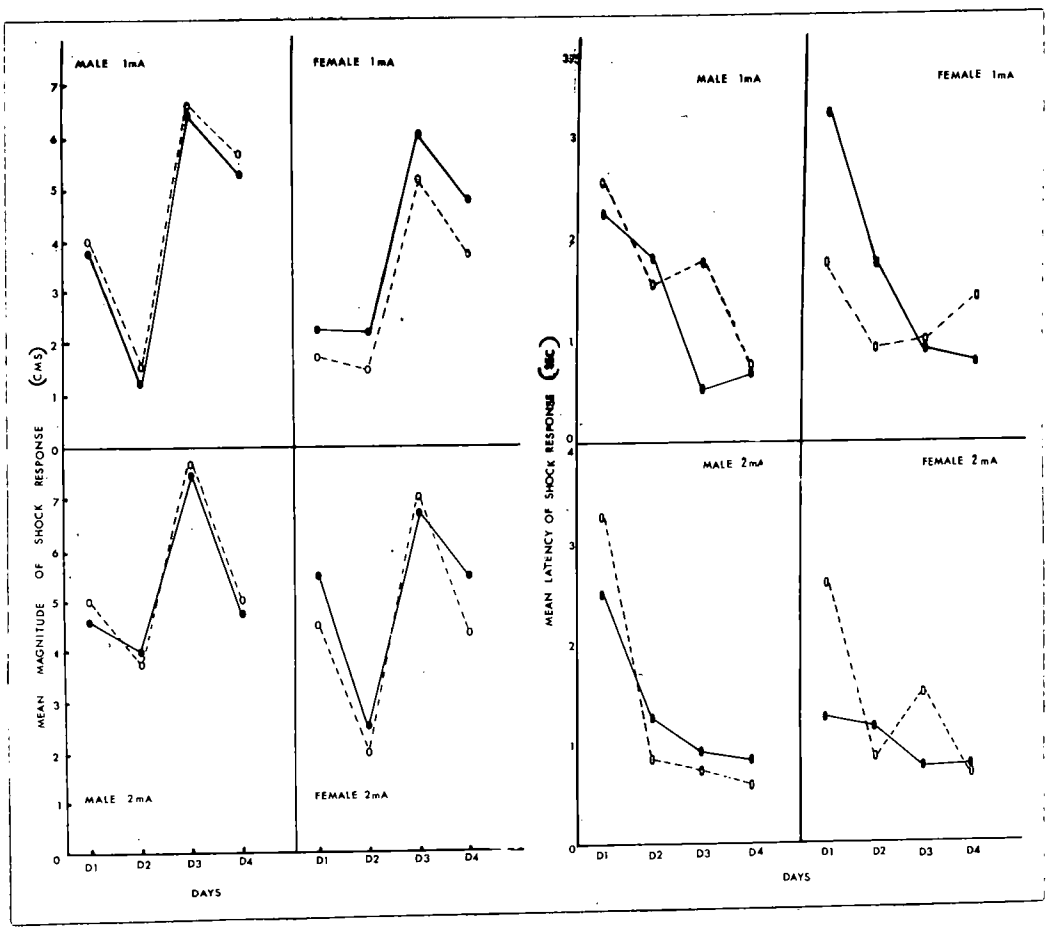


FIG. 9 Mean magnitudes and latencies of shock response recorded from the eight independent groups on each day (O = IS; ● = NHS).

shock levels during the first three days:

Day 1. $p < 0.01$.

Day 2. $p < 0.25$.

Day 3. $p < 0.01$.

The data for magnitude of shocked responses was transformed (log + 1) and re-analysed using the split-plot design. The transformed data revealed the following significant differences:

Shock	$p < 0.05$.
Days	$p < 0.001$.
Days x shock	$p < 0.01$.

Correlation coefficients computed between ganglia size and magnitude of the shocked response for the 2 ma and 1 ma shock levels were not found to be significant. Apart from the between days significance ($p < 0.001$), differences obtained for the latencies of the shocked responses were not significant.

Other Measurements Recorded during the Startle Experiment.

As shown in Table 2, during the thirteen daily trials, traces were recorded of the CS presented alone (trial 8) and the explosive plop presented alone (trial 10). The former measure was included as a check for the possible temporal conditioning to the CS. The latter measure was included to

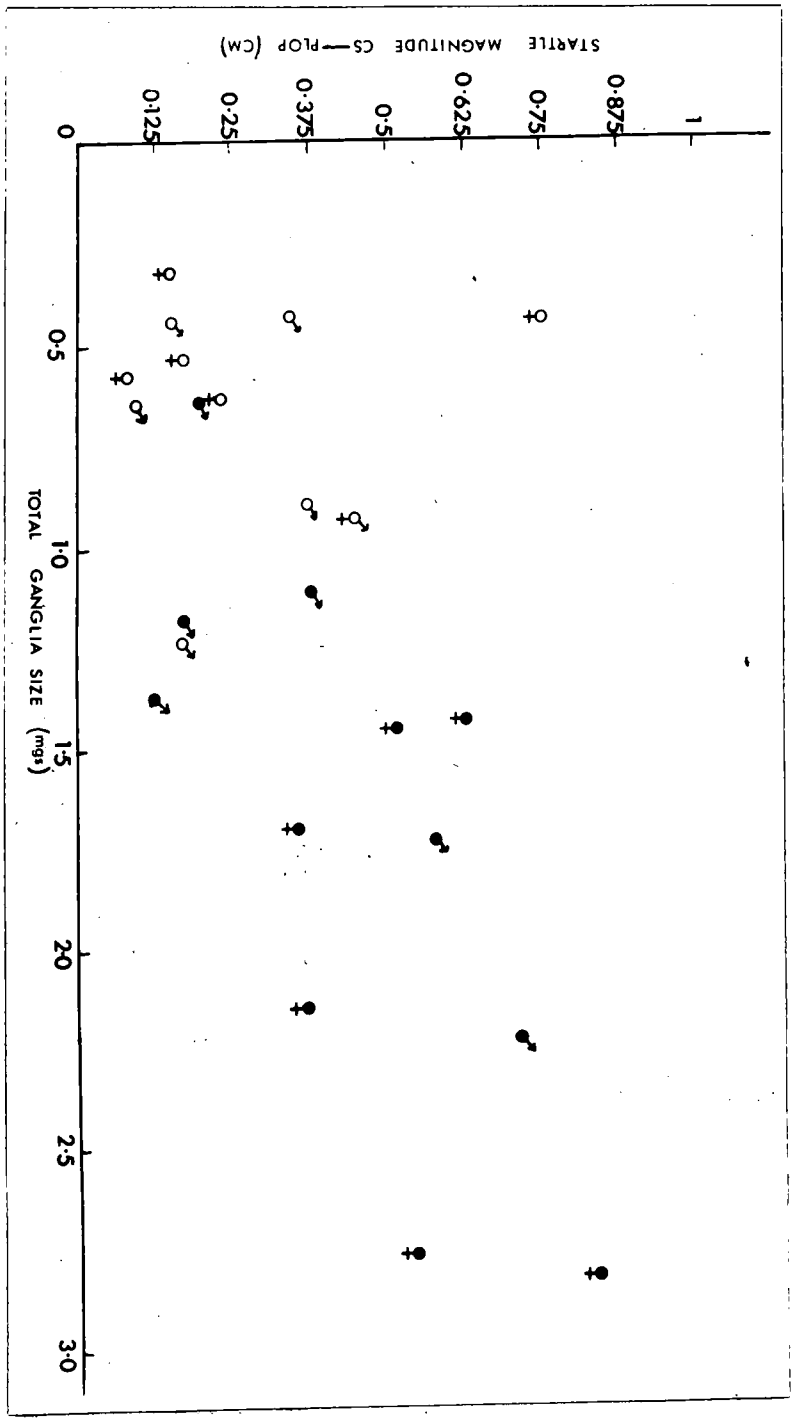


FIG. 10 Scattergram showing a positive correlation between sympathetic ganglia size and startle magnitude on day 1 from the mice who received the 2ma shock level (O = IS; ● = NHS).

guard against phenomena such as disinhibition being confused with what were the main variables being tested during the experiment. However, the explosive plop sound when it was presented in isolation produced movements on only a very low number of occasions. Movement to the CS when it was presented alone was recorded on about two thirds of the trials. In both cases the movements occured throughout the eight independent groups. Movements produced to the CS alone presentations had very different characteristics to the CS - plop startle response. The average amplitude to the former was less than half of those recorded for the startle response; also, the CS alone trials had longer latencies.

Following the final session the mice were killed by a cervical break and the SNS thoracic and cervical ganglia removed. The photographs of histological sections shown in Fig. 2 were prepared from the ganglia of mice selected at random from this experiment.

DISCUSSION.

The main conclusions arising from this experiment are that apart from consistent and significant differences between the sexes and the two shock levels used, no clear-cut and

unequivocal behavioural separation was shown between the IS and NHS mice.

An expected difference was found between the IS and NHS mice on day 1 but it was at a low level of acceptability and very small changes in the recorded measurements would be required for the variable to become nonsignificant. Therefore, it is doubtful whether one would be justified in relying too heavily upon this result. It might be argued that during the few minutes it took to present the first four conditioning trials an internal compensatory (neural or hormonal) mechanism could have occurred in the IS animals. If this is so then it is difficult to conceive of a behavioural test of emotion with a shorter time course. Certainly, nothing like the clear-cut separation found by Appley, 1965, for hypophysectomized and normal rats, was shown.

Kurtz and Siegel, 1966, using a startle response to examine emotionality in rats confirmed the findings of Brown, Kalish and Farber, 1951, but introduced a major modification. Kurtz and Siegel failed to obtain similar results from their rats when the shock was applied to the feet or to the backs of their animals. They found that the magnitude of a startle response was increased when foot shock was used, and suggested that a major componant in the startle response was postural

adjustment. Whereas Brown, Farber and Kalish favoured a drive energization hypothesis, Kurtz and Siegel favoured a postural facilitation hypothesis. In a recent study Horlington, 1968, concluded that the crouching posture adopted during the 'freezing' response to shock was very favourable for a maximum startle response, but that the observed magnitude of the startle was definitely related to previous treatment. Observations made during this experiment also tends to favour this interpretation. Clearly the mice did adopt certain postures; for example, the relatively long latencies before movement occurred to the shock appeared to be due to the mice, when shocked, gripping the bars for as long as they could. However, the CS alone, plop alone and other test trials indicate that the combination of CS - plop was required to produce a maximum response. As indicated earlier the responses occurring to the temporal conditioning aspect of the CS were quite distinct from the startle response.

Hoffman and Searle, 1965, measuring acoustical parameters that modified the startle reaction in the rat reported that mechanisms involved in a startle response are more or less continuously active, even though overt reactions seldom occur. Stewart, Abplanalp, and Warren, 1965, reported a species difference between their study using cats and an earlier report

(Hoffman, Fleshler, and Abplanalp, 1964) using rats in the startle response to electric shock. But both studies revealed qualitative agreement that the initial response in both species was a generalized flinch followed by limb movement. At high intensities jumping occurred in both species. These studies indicate that some species generalization occurs for the response.

Clearly, the reactions observed in the startle response, both to applied shock and the CS - plop situation, are very complex and any complete statement about the system would require parametric studies. Such a study would require competent analysis of the various stresses involved in measuring the force components produced by an animal enclosed in a small space. It would also require electromyographic recordings taken from various groups of muscles in the animal, and some evaluation (perhaps using spreading depression or localised cooling of brain areas) of the central nervous system's role. Also, the chamber used would require careful design, perhaps by a competent engineer. For example, from the experience gained in this study the optimal place for the transducer used to measure the movement of the animal within the chamber would appear to be at a central point in floor of the chamber. This would result in all movements giving comparable response amplitudes.

Analysing and evaluating the various designs used to measure startle reactions (see Hoŕlington, 1968, for the main references) indicates that the production, over time, of more sophisticated devices have revealed the essential complexity of the startle response. The apparatus reported in this experiment had sufficient sensitivity to allow different movements to be distinguished; in fact, it was probably too sensitive.

A great deal of discussion has centred around the possible meaning of the significant interactions found for the startle response and much advice, both from professional and amateur statisticians, sought. Although the results could have hardly arisen by chance alone the experimenters are positive that they do not convey any meaningful information about the animal's emotional state. In a sentence, the interactions while being statistically significant are not relevantly significant for the behavioural system being tested. As indicated in the last sentence of the preceding paragraph the most likely explanation lies in the "sensitivity" of the device.

By the fourth day the mice were very difficult to handle and it might be argued that by the fourth day stress was beginning to show, especially as the separation was greatest at the higher shock intensities. Both the experimenters felt that

the change was occurring in the mice rather than due to changes on their part. As indicated in the Procedure Section, the actual experimental running time was eight hours; during this period concentrated attention was required to alter oscilloscope and camera settings, and reset the control panels between trials. However, it was due to exhaustion of the photosensitive recording paper rather than the experimenters that finally precluded the continuation of the experiment for a longer period. The experimenters alternated, every 30 minutes, between testing animals, and carrying the mice from the home cages and developing the rolls of photosensitive paper.

Fifteen mice, (seven male IS, and eight male NHS) were tested in the startle chamber when they were 90 days old. The mice were surplus to the requirements of the experiment design and were directly comparable with the mice tested at 30 days. The results obtained from these adult mice were very similar to the results reported in this chapter for their younger siblings. Again, no significant differences were found between the IS and NHS groups in these older mice.

Pilot studies on a limited number of mice revealed no essential differences between sera injected and unhandled control animals. Ironically during the pilot study 3/4 IS mice gave no startle response while 4/4 NHS mice showed a clear and marked startle response.

Horlington, 1968, reported startle response latencies of 11 - 12 ms for rats as opposed to an overall mean of 19 ms found for the strain of mice used in this study. These appear to indicate a species difference; certainly the apparatus used in this study gave sharp rise times and there appeared to be no reason to suspect an artifact arising from the device. But this point would need to be checked by using both rats and mice in the same startle response device. Horlington reported that despite the narrow age range of his rats they showed a large response range. In this study, a large variation of response range was also found for mice matched for age and litter.

CHAPTER VI.

PASSIVE AVOIDANCE AND THE METABOLISM OF CATECHOLAMINES.

Using the technique of startle response that was held to be behaviourally microscopic, at least in comparison with the normal active and passive avoidance techniques, the experiment reported in the last chapter had failed to show unequivocal behavioural separation between IS and NHS mice. At this point it was decided to examine the evidence for a possible compensatory process occurring in the reduced sympathetic nervous system of immunosympathectomized animals.

There are a number of reports demonstrating increased sensitivity of the autonomic nervous system following sympathectomy. The Cannon and Rosenblueth, 1949, monograph has been mentioned in Chapter II. More recently Murray and Thompson, 1957, using electronmicrographic techniques have reported minute neurone sprouts occurring in the sympathetic nervous system within five days of sympathectomy. Boyd, 1957, has claimed that small intermediate sympathetic ganglia exist in the sympathetic nervous system. Emmelin, 1961, has published a paper called "Supersensitivity following 'pharmacological denervation'" and Trendelenburg, 1966, reviewing the physiological evidence

for denervation supersensitivity in the automomic nervous system, has reported compensatory changes. Thus, the autonomic nervous system appears to have mechanisms to overcome any imposed reduction of its influence. There does not appear to have been very many published studies relating to possible compensatory mechanisms in the autonomic nervous system of immunosympathectomized animals; however, two studies have been reported which have a bearing on this problem.

Carpi and Oliverio, 1964, have shown that although the levels of urinary noradrenaline from IS rats were below the sensitivity limits of their assay technique, an injection of amphetamine produced a large and dramatic increase in noradrenaline levels of IS rats when compared with control animals. The authors concluded that the large increases in IS rats indicated a noradrenergic system which entered into action following the reduction of the sympathetic system. Schönbaum, Johnson and Seller, 1966, showed that IS rats had a considerable noradrenergic reserves when subjected to cold stress; only when the cold stress was made very severe, by clipping the fur, did IS animals fail to maintain adequate functioning of their hypotrophic sympathetic nervous systems. There would appear to be, therefore, a case for looking at noradrenaline levels to see if a hormonal compensation occurs in the depleted neural networks of IS animals.

As recorded by Burn, 1965, the discovery that the adrenal medulla contained a substance which was active in raising the blood pressure was made independently by at least three workers during the latter half of the last century. Cannon during the 1920's and 30's carried out a number of experimental studies on this substance which he called sympathin, thinking that it was adrenaline (Cannon and his collaborators later held that sympathin had two major components, one having a motor effect, the other an inhibitory effect; see Cannon and Bacq, 1931). Sympathin was shown by later workers to be similar to adrenaline chemically but pharmacological evidence was accumulating which showed that the adrenal medulla substance responsible for increasing sympathetic activity was not adrenaline. In 1946 von Euler showed that extracts from the splenic nerve of the ox and horse behaved like a solution of noradrenaline. Confirmation of von Euler's discovery was provided by Peart, 1949, who demonstrated that stimulation of the splenic nerve liberated noradrenaline. Reviews of the evidence for noradrenaline as the neurochemical transmitter substance for the sympathetic nervous system have been published by von Euler, 1956 and 1966. In 1953 Hillarp and Hokfelt produced evidence that adrenaline and noradrenaline was stored in separate adrenal medullary cells, and

Douglas, 1966, pointed out that the medullary cells are the main repositories of catecholamine in the body (from an embryological point of view the secretory cells of the adrenal medullae are comparable to postganglionic neurones of the thoracolumbar sympathetic outflow. In addition, the secretory activities of these medullary cells are primarily governed by preganglionic SNS nerves running, mainly, in the splanchnic nerves).

Since the discovery of adrenaline and noradrenaline having separate functions many workers have examined their respective roles, mostly in humans. It is not proposed here to review the human studies apart from stating that basically it is maintained that alterations between fear and anxiety are reflected in changes of the bodily adrenaline/noradrenaline ratio. Anger directed outwards appears to be correlated with adrenaline while anxiety, or fear, appears to be correlated with increases in noradrenaline (major reviews in this area are: Elmadjian, Hope and Lamson, 1957; von Euler, Genzell, Levi and Strom, 1959; Silverman, Cohen, Shavonian and Kirshner, 1961; von Euler, 1964; Kety, 1966).

Infrahuman studies analysing concurrent levels of the catecholamines and emotional states are not nearly so numerous as

the human studies, but some work has been reported. Mason, Mangan, Brady, Conrad and MckRioch, 1961 measured concurrent plasma levels during the induction of conditional emotional responses in monkeys. Sigg, Day and Colombo, 1966, measured the spleen and adrenal noradrenaline levels in isolated and communal mice. Moore, 1968, studied the urinary levels of catecholamines in chronically isolated rats. All of these studies reported increases in the catecholamine levels of animals subjected to stress.

For the purposes of this experiment it was decided that the time course required to obtain urinary samples was too long; this method would have also involved the use of a large number of metabolic chambers which were not available. The taking of blood samples was thought to be too traumatic if the use of anaesthetics was to be avoided. It was, therefore, decided to assay the catecholamine levels of various organs taken from mice that had been killed immediately after a behavioural test. In particular, for reasons given above, to concentrate upon the catecholamine metabolism occurring in the adrenal gland. It was realized that extra adrenal chromaffin tissue would be an important factor (Lempinen, 1964, recorded, in a review of the extra adrenal chromaffin tissue in the rat, that Vincent, 1910, had reported the formation of such tissue

in the mouse) and that these formations of extra adrenal chromaffin tissue would serve as additional repositories for the metabolism of catecholamines. Carpi and Oliverio, 1964, had shown the importance of such sources in the rat following adrenal demedullation; also Hamberger, Levi-Montalcini, Nörberg and Sjövist, 1965, had reported the proliferation of extra adrenal chromoffin tissue around the abdominal organs of IS rats. (One additional qualification should be made. Vogt, 1964; Zaimis, Berk and Callingham, 1965, have reported that, following immunosympathectomy the degree of hypotrophy produced in the coeliac and mesenteric ganglia are slight compared to the thoracic and cervical ganglia changes. Iversen, Glowinski and Axelrod, 1965, have reported that organs innervated by the coeliac and mesenteric ganglia retain almost unimpaired ability to accumulate labelled exogenous noradrenaline.) However, it was felt that the adrenal medullary cells, under the direct inervation of the splanchnics, would show the initial increases in the rate of catecholamine metabolism.

Catecholamine levels in the organs of IS mice have been reported by Visscher, Lee and Azuma, 1965, and Klingman, 1966. Visscher and his coworkers found a 60% increase in the mean

noradrenaline content of the adrenal glands of 28 day mice who had received seven daily NGF antiserum injections post-natally. Visscher also reported a marked reduction in the noradrenaline content of heart, spleen, and kidneys from his IS mice. Brain tissue levels were identical with control mice injected with saline post-natally. Klingman found smaller increases in the noradrenaline content of the adrenals from mice injected in utero. The latter study involved mice which were killed from four weeks to seven months after birth. Lagerspetz and Hissa, 1968, have described the post-natal development of the catecholamines stores in the adrenals of mice. By thirty days the adrenal glands of their mice contained very nearly the adult levels. In adulthood the adrenal content of adrenaline had increased 200 times over that of the neonate, while the adrenal content of noradrenaline had increased thirty times over that of the neonate.

It was decided to use the passive avoidance field to test the mice. This allowed for initial activity levels to be tested. Also, the passive avoidance situation required that the mouse remain passive, this might serve to reduce noradrenaline increases due to muscular activity that would occur in an active avoidance situation.

METHOD.

Subjects:

The total number of mice used in this experiment was 176 males and 37 females. The first group of mice tested was comprised of both males and females but following the initial experiment the females were discarded from the litters and not injected (at this time the Wellcome NGF antiserum was not available for sale and in short supply; also the difficulty in assaying large numbers made it expedient to reduce the numbers of mice being tested). The male mice were comprised of the following groups: Nor, 40 mice; IS, 69 mice; NHS, 67 mice. For the purposes of this experiment the normal mice were unhandled until the behavioural test at 30 days. The litters were from multiparous females treated by the injection procedure laid down in Chapter II. As indicated in Chapter II, neonates injected in this, and subsequent experiments reported, ~~reported~~ received twofold concentrated injections of NGF antiserum. Unless otherwise stated, all tables and figures shown in this chapter are for male mice.

Assay Procedure.

Following the behavioural test the mice were killed immediately using a cervical break and the following organs

dissected and weighed: adrenals, hearts, and brains. Following rapid dissection and cleaning, if required, of fat and connective tissue, the tissues were frozen on the side of a beaker immersed in a mixture of alcohol and Cardice. Tissue weights were made on the frozen tissues. Initial assays of brain tissue were of the whole brain but later the brains were divided into a telencephalic, diencephalic/mesencephalic, and medulla/hindbrain areas; the cerebellum was discarded. The dissection of the lissencephalic mouse brain followed the procedure first described for the rat brain by Glowinski and Iversen, 1966.

The adrenals were stored at -15°C O/N and assayed the following day. Other organs were bulked into batches of tissue from 3 - 4 mice from the same treatment condition, and stored at -15°C until assayed 1 - 2 weeks later.

The catecholamine assay technique used for the adrenaline and noradrenaline estimation of the adrenals was a differential pH trihydroxyindole fluorometric method, using potassium ferricyanide as the oxidant. Noradrenaline content estimations were made on the other tissues by precipitating the protein material and making a chromatographic separation of the noradrenaline using small resin columns. Following

the resin separation a trihydroxyindole fluorometric estimation of the noradrenaline was made. Each separate assay contained an equal number of randomly distributed mice from each group being tested on that occasion. For full details of the assay method used see the biological technique appendix.

Experimental design:

The passive avoidance behavioural test used in this study employed a similar technique as that employed in Chapter IV. The following groups were used:

1. Home cage: under this condition mice were removed from their litters and killed immediately, using the cervical break technique.

2. Passive field + 2 minutes: activity counts were taken from mice who were placed into the passive avoidance field for two minutes without shock before being killed.

3. Passive field immediate shock + 2 minutes: mice were placed into the passive field, given an immediate shock lasting for three seconds, and then left in the field for two minutes before being killed.

8. Confrontation by a natural predator.

Following the earlier conditions which used electric schock as the stressor it was decided to assay adrenals taken from mice which had been protectively placed in the presence of a food deprived ferret for a ten minute period.

4. Passive field, 2 minute shocks contingent upon movement (SCM) + 1 minute: mice were placed in the passive field, a free activity count taken for the first two minutes and then two minutes of shocks that were contingent upon movement. Following the second and shocked two minute period the mice were left, without shock, in the field for a further one minute before being killed.

5. Passive field 2 minute shocks contingent upon movement (SCM) + 2 minutes: procedure as above in Group 4, but following the second and shocked two minutes the mice were left, without shock, in the field for a further two minutes before being killed.

6. Passive field 2 minute shocks contingent upon movement (SCM) + 8 minutes: procedure as above in Groups 4 and 5 except the final period following shock was eight minutes, i.e., 4 x 2 minute periods.

7. Stress: the mice were placed in the passive avoidance field and the activity count recorded during the initial two minute period. Following this two minute period the mice were subjected to twenty-eight minutes of shocks contingent upon movement (SCM).

RESULTS.

Passive Avoidance:

Activity counts taken during the initial two minute period and the two minute shock contingent upon movement period are shown in Fig. 11. Counts for IS, and NHS mice were similar to those reported in Chapter IV. All groups recorded 5 - 10 movements/minutes lower than those recorded during the first series of passive avoidance experiments; this was due to an altered sensitivity setting between the first and second series of experiments.

During the first two minute period normal mice tended to make more movements than the other two groups. A Kruskal-Wallis one-way analysis of variance by ranks on the median scores recorded during the first two minute period failed to show significant differences between the three groups. Normal mice also tended to maintain low level activity throughout the two minute shock contingent upon movement period. They did not follow the pattern found in the IS and NHS groups of "freezing" or becoming passive within a short time following the onset of the shocks for movement. A Kruskal-Wallis test revealed that the normal group were significantly different from the IS and NHS groups, $p < 0.01$. Total movements recorded during the

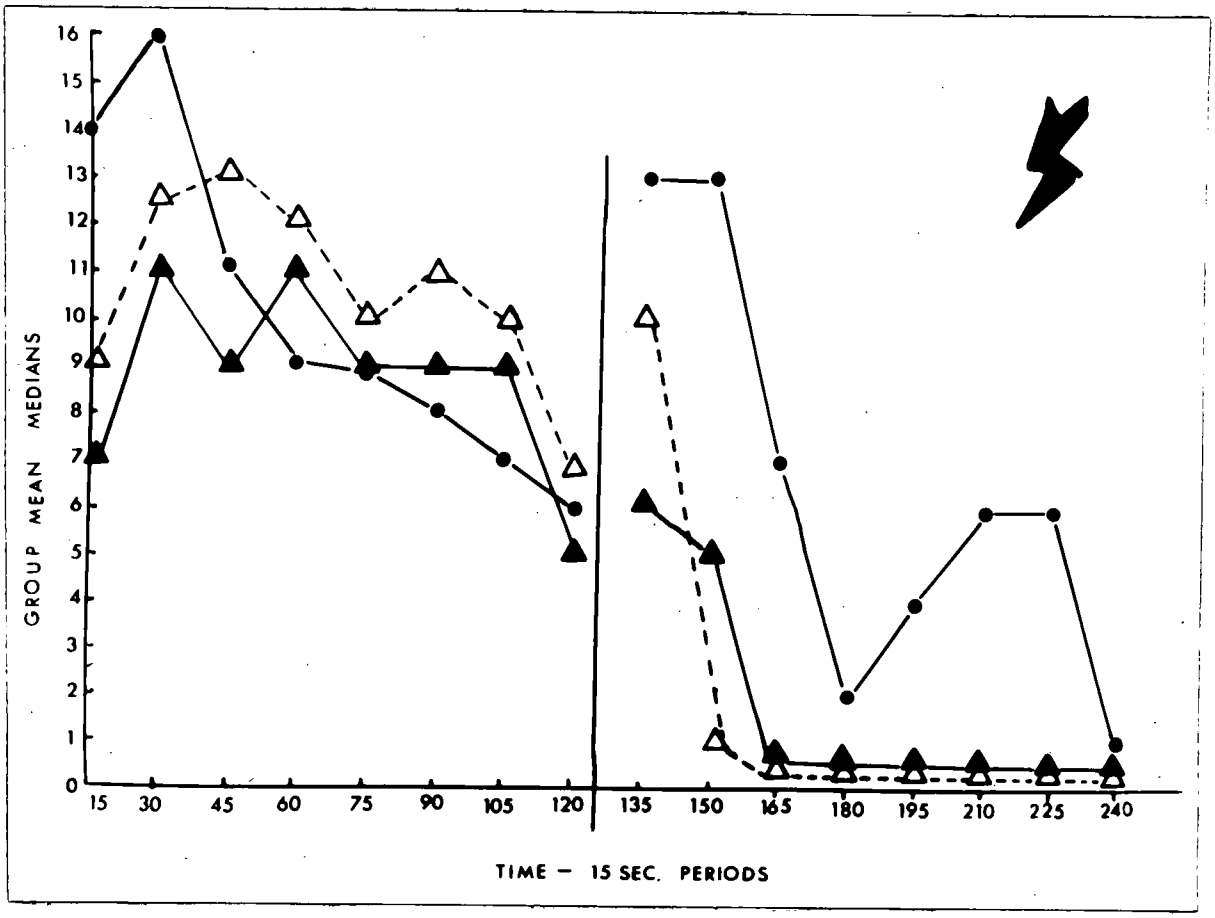


FIG. 11 Passive avoidance test prior to catecholamine assays. Median activity recorded at 15 sec. intervals during the initial 2 mins. of free movement and the 2 min. shock contingent upon movement period (● = normal; ▲ = IS; △ = NHS).

variable intervening time following shock and removal from the field were not significant between the groups, neither was the time period that elapsed before a movement was made following shock.

Female activity counts were similar to the values reported in Chapter IV; although, as indicated for the male data, the overall values were slightly lower than those recorded during the first series of passive avoidance experiments. No significant differences were found for the female data.

Tissue, total body weights, and organ weights for male and female are shown in Table 5 and Table 6 respectively. Although the IS animals tended to be lighter this difference in body weight was not found to be significant. IS mice tended to have larger adrenals which failed to show the large increases found in the NHS mice following the prolonged stress session. Following the stress condition the adrenals of all the animals showed increases in weight and pronounced hyperemia. Critical ratios computed for the means of the body and organ weights failed to reveal any significant differences.

Adrenal Catecholamine Assays:

Results of the catecholamine assays on the adrenal tissue are shown in Table 3 and Table 4. The tabulated values are

TABLE 3

ADRENALIN CONTENT OF ADRENALS FROM NORMAL, N.H.S., AND I.S. MICE FOLLOWING PASSIVE AVOIDANCE SITUATION.

Values are means and standard errors.
Adrenalin is expressed as μg/kg body weight; μg/pair adrenals.

	HOME CAGE			PASSIVE FIELD: NO SHOCK		
	Normal	N.H.S.	I.S.	Normal	N.H.S.	I.S.
μg/kg b.wht.	62.7±5.3	82.6±8.3	79.8±8.0	67.5±3.9	61.12±5.5	86.8±9.0
μg/pr.adrenals	1.46±0.12	1.95±0.23	1.68±0.18	1.47±0.09	1.46±0.09	1.62±0.11
N	10	12	12	8	6	6
	PASSIVE FIELD: 2 SEC. SHOCK + 2 MINS.			PASSIVE FIELD: 2 MIN. *S.C.M. + 1 MIN.		
	Normal	N.H.S.	I.S.	Normal	N.H.S.	I.S.
μg/kg b.wht.	—	83.3±4.8	83.6±6.8	72.3±8.14	64.2±2.5	77.3±1.9
μg/pr.adrenals	—	1.49±0.15	1.28±0.12	1.73±0.19	1.48±0.07	1.48±0.06
N		9	9	12	12	12
	PASSIVE FIELD: 2 MIN. S.C.M. + 2 MIN.			PASSIVE FIELD: 2 MIN. S.C.M. + 8 MIN.		
	Normal	N.H.S.	I.S.	Normal	N.H.S.	I.S.
μg/kg b.wht.	50.1±6.2	91.2±7.7	92.2±7.7	—	84.8±5.8	83.1±5.9
μg/pr.adrenals	1.12±0.09	2.02±0.21	1.90±0.20	—	1.48±0.09	1.24±0.09
N	8	11	13		12	12
	PASSIVE FIELD: 30 MIN. S.C.M.			MOUSE CONFINED IN THE PRESENCE OF A FERRET.		
	Normal	N.H.S.	I.S.	Normal	N.H.S.	I.S.
μg/kg b.wht.	68.8±6.16	55.3±5.53	68.2±9.17	71	77	68
μg/pr.adrenals	1.49±0.11	1.05±0.09	1.07±0.14	1.47	1.59	1.32
N	8	11	11	4	4	4

*(S.C.M. = shock contingent upon movement)

TABLE 4

NORADRENALIN CONTENT OF ADRENALS FROM NORMAL, N.H.S., AND I.S. MICE FOLLOWING PASSIVE AVOIDANCE SITUATION.

Values are means and standard errors.
Noradrenaline is expressed as µg/kg body weight; µg/pair adrenals.

	HOME CAGE			PASSIVE FIELD: NO SHOCK		
	Normal	N.H.S.	I.S.	Normal	N.H.S.	I.S.
µg/kg b.wht.	43.7±4.8	52.6±9.2	52.5±7.6	57.9±6.2	49.2±8.1	68.5±7.5
µg/pr.adrenals	0.99±0.07	1.17±0.19	1.14±0.18	1.27±0.13	1.15±0.13	1.29±0.14
N	10	12	12	8	6	6
	PASSIVE FIELD: 2 SEC. SHOCK + 2 MIN.			PASSIVE FIELD: 2 MIN. * S.C.M. + 1 MIN.		
	Normal	N.H.S.	I.S.	Normal	N.H.S.	I.S.
µg/kg b.wht.	—	72.2±3.8	87.9±5.9	35.5±2.1	43.0±3.4	48.7±5.4
µg/pr.adrenals	—	1.25±0.03	1.34±0.09	0.83±0.94	0.99±0.17	0.93±0.08
N		9	9	6	6	6
	PASSIVE FIELD: 2 MIN. S.C.M. + 2 MIN.			PASSIVE FIELD: 2 MIN. S.C.M. + 8 MIN.		
	Normal	N.H.S.	I.S.	Normal	N.H.S.	I.S.
µg/kg b.wht.	56.0±8.2	55.9±9.9	74±9.9	—	73.5±6.3	82.0±6.5
µg/pr.adrenals	1.23±0.12	1.22±0.19	1.53±0.23	—	1.28±0.11	1.26±0.11
N	8	11	13		12	12
	PASSIVE FIELD: 30 MIN. S.C.M.			MOUSE CONFINED IN THE PRESENCE OF A FERRET		
	Normal	N.H.S.	I.S.	Normal	N.H.S.	I.S.
µg/kg b.wht.	36.0± 7.4	68.0±5.9	74.±5.3	36	45	59
µg/pr.adrenals	0.79±0.06	1.30±0.11	1.19±0.08	0.76	0.93	1.04
N	8	11	11	4	4	4

* (S.C.M. = shock contingent upon movement)

means and standard errors expressed as μg/pair adrenals, and μg/kg body weight. Adrenaline content of the adrenals are shown in Fig. 12 and the noradrenaline content of the adrenals in Fig. 13.

Results obtained from the assays were subjected to an analysis of variance following a transformation ($\sqrt{\ }$) to reduce the heterogeneity of the group variances. Where the assay result of an experimental condition reached a significant level a Newman-Keul's test was carried out to test the individual group means. If the overall result within an experimental condition failed to reach a significant level, Dunnett's test was computed comparing the treatment values with the home cage values (Winer, 1962). For both the adrenaline and noradrenaline values shown in Fig. 12 and Fig. 13 an analysis of variance was carried out on each separate treatment condition, the three intra group results, and comparable groups within an experimental condition. Apart from the three intra group results only results reaching a significance level of $p < 0.05$ are reported.

Adrenaline Content of the Adrenals.

An analysis of variance computed for the overall NHS mice results revealed a significant difference ($F = 4.32$; df 6/66,

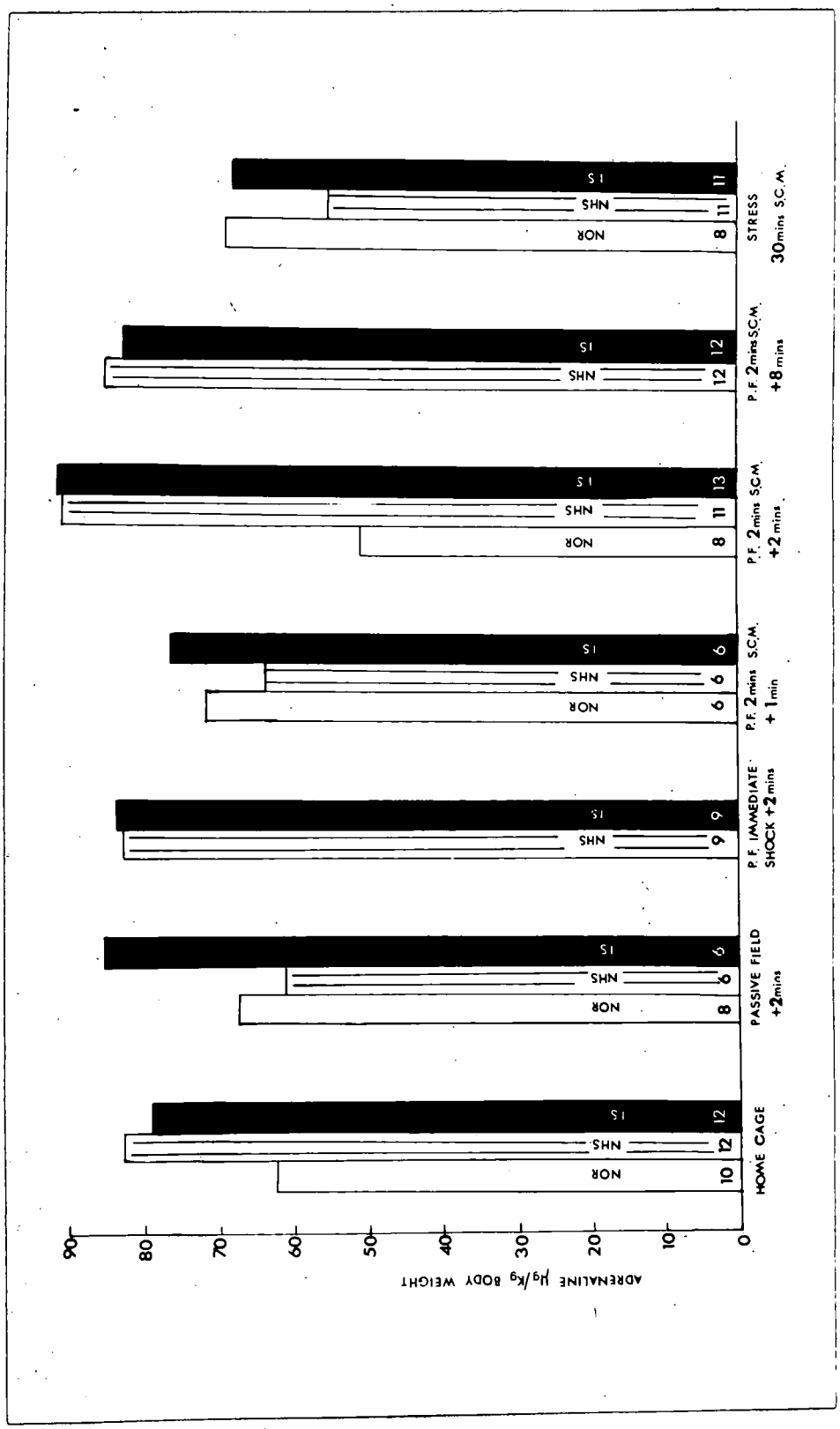


FIG. 12 Histogram showing mean adrenal adrenaline, µg/kg body weight, assayed from each group under the seven different conditions tested. Values shown at the bottom of the blocks are the number of mice assayed (S.C.M. = shock contingent upon movement).

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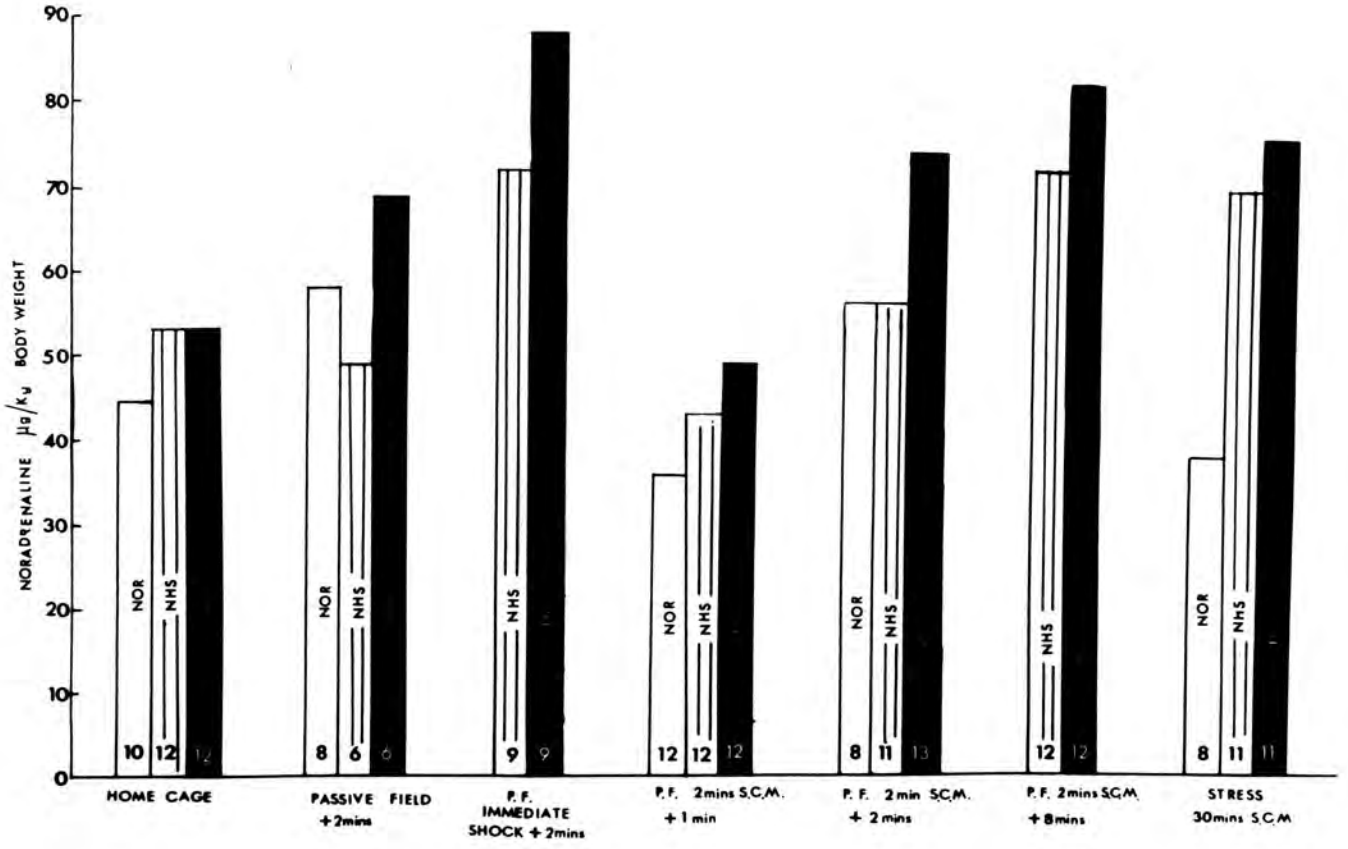

FIG. 13 Histogram showing mean adrenal noradrenaline, µg/kg body weight, assayed from each group under the seven different conditions tested. Values shown at the bottom of the blocks are the number of mice assayed. (S.C.M. = shock contingent upon movement).

TABLE 5

MEANS AND STANDARD ERRORS OF BODY AND ORGAN WEIGHTS
FROM MALE NORMAL, N.H.S., AND I.S. MICE

	I.S.	N.H.S.	NORMAL
BODY WEIGHTS (gms)	18.37 ± 0.479 N = 76	20.57 ± 0.436 N = 76	22.75 ± 0.558 N = 44
ADRENALS (mgs)	3.49 ± 0.152 N = 64	3.43 ± 0.097 N = 60	3.38 ± 0.118 N = 36
Following 30 min. stress (mgs)	3.58 ± 0.200 N = 12	3.96 ± 0.450 N = 11	3.46 ± 0.212 N = 8
HEARTS (mgs)	1030.6 ± 28.4 N = 64	1176.9 ± 29.4 N = 60	1180.0 ± 35.5 N = 36
Following 30 min. stress (mgs)	943.0 ± 49.8 N = 12	1149.0 ± 70.2 N = 11	1121.3 ± 64.3 N = 8
BRAINS: Total (mgs)	2961.2 ± 41.5 N = 38	3046.9 ± 37.7 N = 43	———
DIVIDED:			
Telencephalon (mgs)	1104.8 ± 39.6 N = 26	1199.4 ± 50.6 N = 27	1236 N = 4
Mesencephalon (mgs)	1577.6 ± 68.1 N = 26	1678.4 ± 52.8 N = 27	1566.0 N = 4
Medulla/hindbrain (mgs)	565.6 ± 17.8 N = 26	558.6 ± 21.6 N = 27	507.6 N = 4

TABLE 6

MEANS AND STANDARD ERRORS OF BODY AND ORGAN WEIGHTS FROM FEMALE N.H.S. AND I.S. MICE

	I.S. (N = 21)	N.H.S. (N = 16)
BODY WEIGHT (gms)	18.99 ± 0.607	19.96 ± 0.627
ADRENALS (mgs)	5.48 ± 0.36	5.21 ± 0.27
HEARTS (mgs)	1011.75 ± 46.2	1027.34 ± 62.9
BRAINS (mgs)	2894 ± 65.8	2994.7 ± 62.9

$p < 0.001$). Comparison of the various experimental conditions for the NHS mice revealed the following significant levels:

NHS Stress (30 minutes SCM) mean compared with NHS (2 minutes SCM + 2 minutes) mean $p < 0.01$.

NHS Stress (30 minutes SCM) mean compared with NHS (2 minutes SCM + 8 minutes) mean $p < 0.05$.

NHS Stress (30 minutes SCM) mean compared with NHS (home cage) mean $p < 0.05$.

NHS Stress (30 minutes SCM) mean compared with NHS (P.F. + immediate shock) mean $p < 0.05$.

NHS (Passive field alone) mean compared with NHS (2 minutes SCM + 2 minutes) mean $p < 0.05$.

Overall differences between the various experimental conditions for the IS mice were not found to reach a significant level. A Dunnett's test comparing the IS home cage adrenaline values with the other experimental conditions also failed to reveal any significant differences. Differences in the adrenaline values obtained from mice in the Normal groups also failed to show significant differences, both in the overall analysis of variance and Dunnett's test.

Adrenaline values obtained from the IS, NHS and Normal mice who were tested under the standard procedure of an initial two minute activity level followed by two minutes of shock contingent upon movement (+1, +2, +8 minutes) were analysed, and

the overall difference reached found to have a significant level ($F = 6.04$; df 5/55, $p < 0.01$). Owing to the absence of Normal mice for the +8 minute experimental condition group comparisons, it was not possible to make comparison for each group under each condition. Computation of a Newman Keul's test revealed the following significant differences between the means:

Normal mice (2 minutes SCM + 2 minutes) means compared with IS (2 minutes SCM + 2 minutes) means $p < 0.01$.

Normal mice (2 minutes SCM + 2 minutes) means compared with NHS (2 minutes SCM + 2 minutes) mean $p < 0.01$.

Normal mice (2 minutes SCM + 2 minutes) means compared with IS (2 minutes SCM + 1 minute) mean $p < 0.05$.

NHS mice (2 minutes SCM + 1 minute) means compared with IS (2 minutes SCM + 2 minutes) means $p < 0.05$.

NHS mice (2 minutes SCM + 1 minute) means compared with NHS (2 minutes SCM + 2 minutes) means $p < 0.05$.

Normal mice (2 minutes SCM + 1 minute) means compared with IS (2 minutes SCM + 2 minutes) means $p < 0.05$.

Normal mice (2 minutes SCM + 1 minute) means compared with NHS (2 minutes SCM + 2 minutes) means $p < 0.05$.

Adrenaline values obtained from the female groups failed to show, either between or within group, significant differences.

Noradrenaline Content of the Adrenals.

Seventeen separate assays were made to collect the results shown in Fig. 13 and apart from the home cage condition the mean noradrenaline values obtained from IS mice were always higher than the mean values obtained from the NHS and Normal mice. However, the large variances obtained throughout all the assays prevented statistically significant levels being reached for any of the separate treatment conditions shown in the histogram.

An analysis of variance computed for the overall results from IS mice revealed a significant difference ($F = 3.36$; df 6/71, $p < 0.01$). Comparison of the various experimental conditions revealed the following significant levels:

IS home cage mean compared with IS (P.F + immediate shock)
$p < 0.01$.

IS home cage mean compared with IS (2 minutes SCM + 1 minute)
$p < 0.05$.

IS home cage mean compared with IS (2 minutes SCM + 8 minutes)
$p < 0.05$.

Further analysis using the Dunnett 't' test to compare the mean values obtained under the home cage condition with the mean values obtained from the other experimental condition revealed that the home cage value differed from the passive field +

immediate shock, and the two minutes SCM + eight minute means ($p < 0.01$).

The overall results from the NHS mice and the various experimental conditions did not reach significant levels. Testing differences between the mean values obtained from the home cage condition and the other experimental conditions using Dunnett's test also failed to reveal significant differences.

Computation of the overall data from the Normal mice did not reveal any significant differences. Dunnett's test on the Normal mice data revealed that the home cage mean differed significantly from the passive field without shock ($p < 0.05$) in the Normal mice.

The finding of increased levels of noradrenaline in the adrenals from merely placing the mice in the passive avoidance field for two minutes was quite unexpected; moreover, further analysis revealed no correlation between the noradrenaline level found and the activity count of the animal. This finding appeared to indicate no excessive contribution from muscular activity. It was therefore decided to subject the mice to a brief shock, lasting approximately two seconds, immediately the animal was placed in the field. As shown in Fig. 13 the effect

of this brief shock was to evoke the highest levels of noradrenaline metabolism found throughout the series of experiments. Analysis of variance computed on the assay values obtained from the passive field without shock and the passive field with an immediate shock (unfortunately it was not possible to assay Normal mice for the immediate shock condition) revealed an overall level of significance (F 6.37; df 4/33, $p < 0.01$). Testing the means using a Newman-Keul's test revealed the following significant differences:

NHS (P.F + 2 minutes) mean compared with IS (P.F + immediate shock) $p < 0.01$.

NHS (P.F + 2 minutes) mean compared with NHS (P.F + immediate shock) $p < 0.05$.

NHS (P.F + 2 minutes) mean compared with IS (P.F + 2 minutes) $p < 0.05$.

NHS (P.F + immediate shock) mean compared with IS (P.F + immediate shock) $p < 0.05$.

Normal (P.F + 2 minutes) mean compared with IS (P.F + immediate shock ($p < 0.05$.

Noradrenaline values were computed for the IS, NHS and Normal mice who were tested under the standard procedure of an initial two minute activity level followed by two minutes shock contingent upon movement (+1 + 2 +8 minutes) and found to reach

an overall significance level (F 5.09; df 7/81, $p < 0.001$). Subjecting the eight treatment means under this set of experimental condition to the Newman-Keul's test (again the absence of Normal mice for the +8 minute condition prevents group comparison under each condition) revealed the following significant results:

NHS (2 minutes SCM + 1 minute) mean compared with IS (2 minutes SCM + 6 minutes) $p < 0.01$.

NHS (2 minutes SCM + 1 minute) mean compared with NHS (2 minutes SCM + 6 minutes) $p < 0.05$.

NHS (2 minutes SCM + 1 minute) mean compared with IS (2 minutes SCM + 1 minute) $p < 0.05$.

Placing the mice in the passive avoidance field and subjecting them to 30 minutes of shocks contingent upon movement resulted in the noradrenaline values for this condition reaching an overall significance level ($F=14.42$; df 2/27, $p < 0.001$). The IS and NHS groups while not differing significantly between themselves differed significantly ($p < 0.01$) from the values obtained from the Normal mice.

To test for a possible relationship between the number of shocks received and the catecholamine metabolism of the adrenal glands a number of analyses of co-variance were carried out (Winer, 1962). Similarly, analyses of co-variance testing for

a possible relationship between total body weight and the catecholamine metabolism were computed. All of these analyses of co-variance failed to reveal significant results. Finally, a small negative correlation was found between activity level during the first two minutes and noradrenaline metabolism; however, this result was not significant.

Noradrenaline Content of Heart Tissue:

Noradrenaline values from heart tissue are shown in Fig. 16. The values shown in the histogram are from 3 - 4 separate readings, each reading being composed of 4 - 5 bulked hearts. The E.I.L. Fluorometer used to read the final relative fluorescence required, because of the relatively limited amount of noradrenaline found in heart tissue, that the reading to be made on a non-linear part of the instrument's range. Also, good recovery rates from the internal standards of noradrenaline run through the resin columns were never obtained. In view of this the histogram shown in Fig. 16 should be regarded as a qualitative record rather than a quantitative record.

Noradrenaline Content of Brain Tissue.

Noradrenaline values from total brain tissue are shown in Fig. 14. Values from brain tissue divided into the telencephalic,

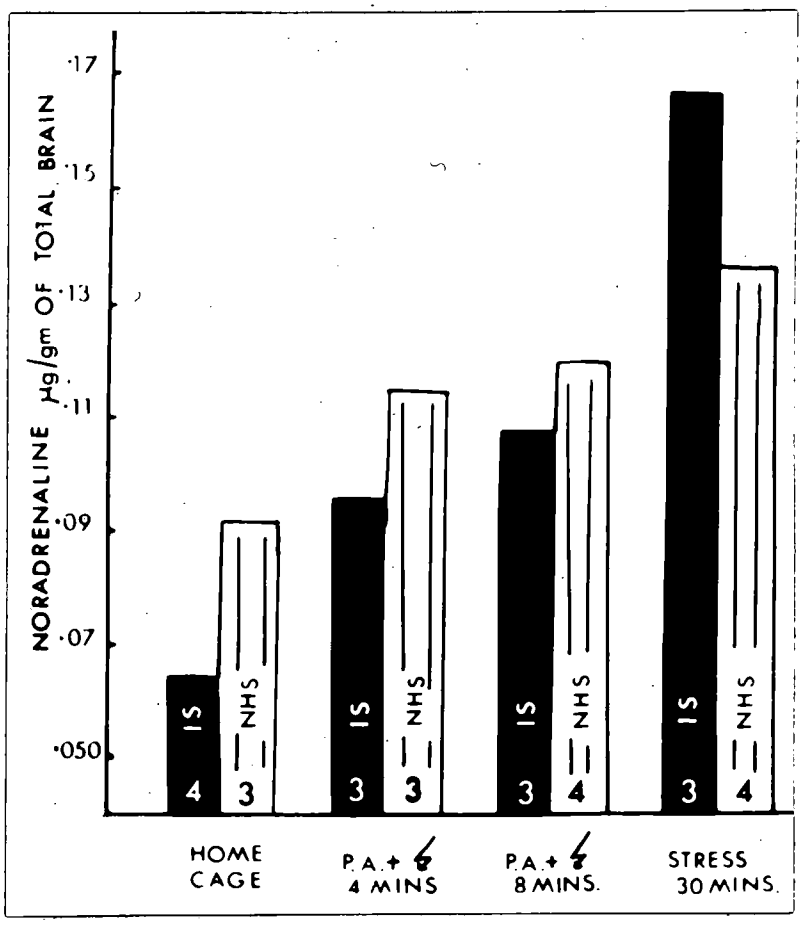

FIG. 14 Histogram showing mean noradrenaline, µg/gm of brain tissue, content of total brain assays recorded for IS and NHS mice. Values shown at the bottom of blocks are the number of assays made.

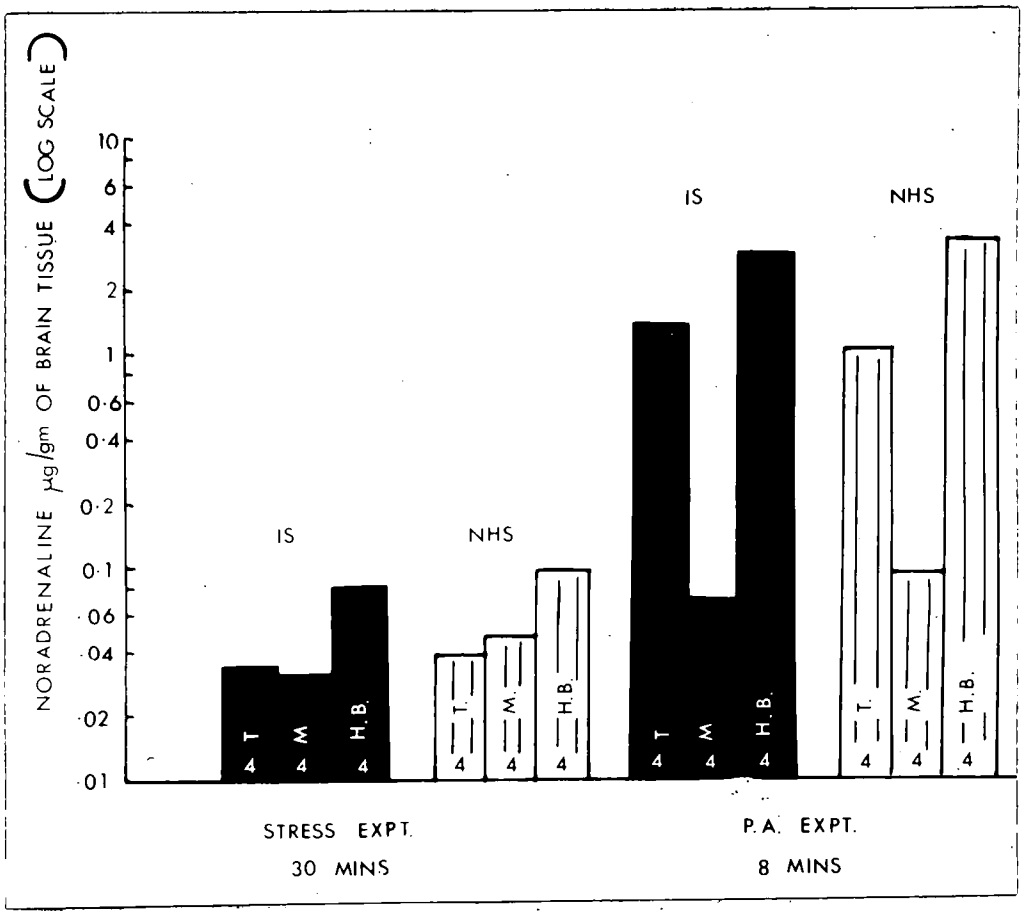

FIG. 15 Histogram showing mean noradrenaline, µg/gm of brain tissue, content in three areas of the brain.
(T = telencephalon, M = mesencephalon, HB = hindbrain).
Values shown at the bottom of the blocks are the number of assays made.

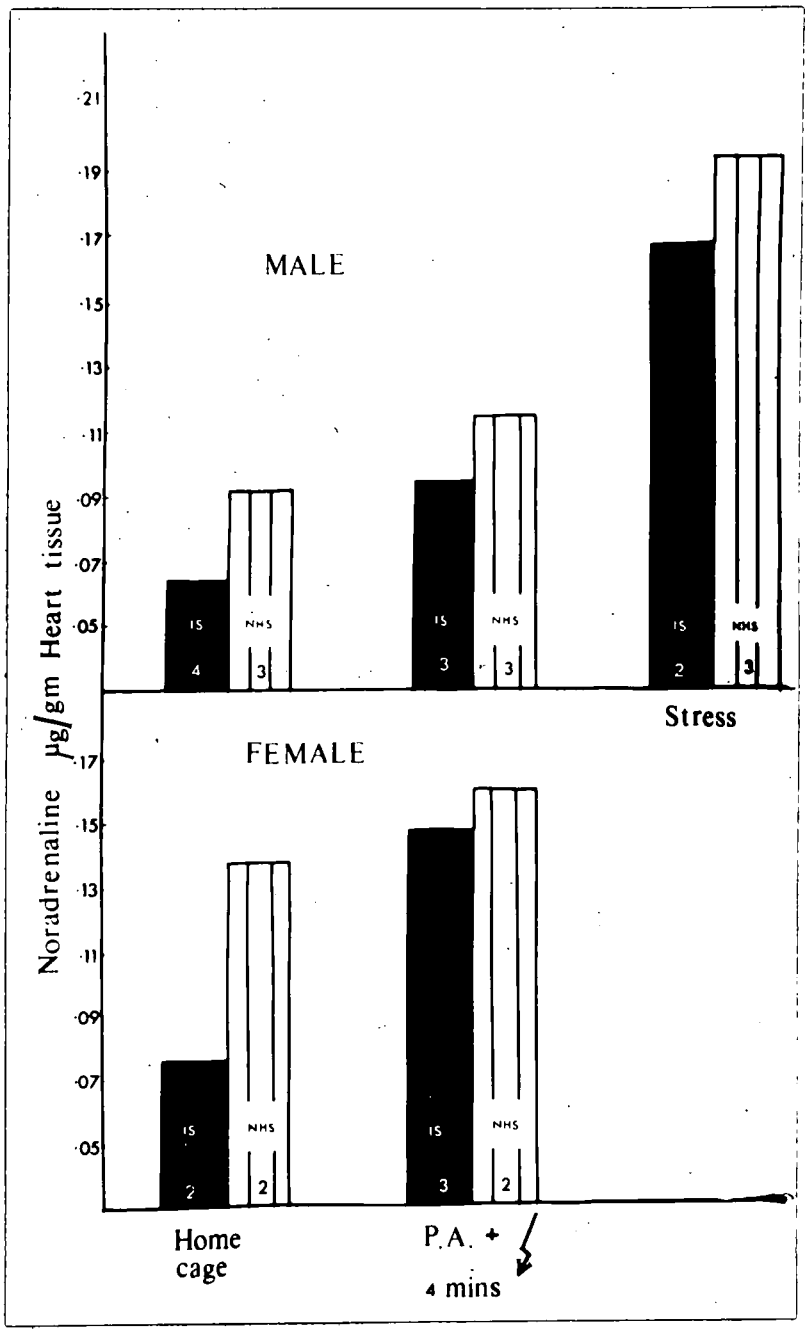

FIG. 16 Histogram showing mean noradrenaline, µg/gm of tissue, content of hearts. Values shown at the bottom of the blocks are the number of assays made.

mesencephalic, and hindbrain, are shown in Fig. 15. The reservations concerning the assay technique pointed out for heart tissue apply equally to the brain tissue assays. In view of the qualitative nature of these results no statistical analyses was attempted on either the heart or brain tissue.

Catecholamine Levels of Mice Protectively Placed in the Presence of a Ferret.

In Table 3 and Table 4 can be found catecholamine values assayed from mice placed in the presence of a food deprived ferret for a ten minute period. No significant differences were obtained when using a ferret, a natural predator, in an attempt to induce emotion in mice. Further discussion of this point will be raised later.

Dissection of Ganglia.

Mice were selected at random throughout the experiment and their cervical and thoracic ganglia dissected out to enable a comparison to be made of the relative sizes between the IS and NHS mice. The technique of injecting concentrated NGF antiserum resulted in ganglia from IS animals being too small to weigh. The top right photograph shown in Fig. 2 shows

the thoracic and cervical ganglia dissected from male IS and NHS mice, of approximately equal weight, taken from the same litter.

DISCUSSION.

In general the results of this series of experiments lend some support to the suggestion that IS mice, under the conditions and treatments used in this experiment, appear to provide some compensation for their hypotrophic SNS by metabolizing increased amounts of noradrenaline in their adrenals. The hypothesis of hormonal compensation however is not proved conclusively.

Under the prolonged stress condition the author noticed that IS mice exhibited a marked ptosis sympathica after 15 - 20 minutes which was not observed in mice from the other two groups. Zaimis, 1967, has apparently used ptosis sympathica as a feature to distinguish between IS and normal rats. In the long series of experimental studies reported in this thesis, using both IS mice and rats, no systematic degree of ptosis had ever been recorded until the use of the 30 minute stress condition. At this point it is not possible to do more than speculate as to whether or not this observed eye-lid droop was due to the depletion of essential reserves of noradrenaline.

The values of the catecholamines found in this study are similar to those reported previously by Visscher et al, 1965, and Klingman, 1966. Previous studies have reported that adrenals assayed from home cage IS mice have slightly higher values than the control animals. The values obtained in the present study are slightly lower than those obtained for the NHS mice. It is felt that the low values recorded here for IS mice might be due to the care taken to reduce litter disturbances to a minimum. Mice were carefully taken from their litters and killed, usually, in one movement. The time taken for this rarely exceeded 2 - 3 seconds. It is possible that the earlier studies have not paid sufficient attention to the need to reduce disturbances to a minimum.

A decision to base the main analyses of the adrenal catecholamine metabolism on the relationship to the total body weight was taken after consideration of the sympathetic nervous system's function in the situation used. The experimental situation consisted of two components; physiological and psychological stress. The SNS role in aversive or novel situations is the mobilization of internal systems that facilitate muscular activity. At the outset of this experiment it was thought that the use of a passive avoidance situation would reduce the influence of the physiological component, i.e., muscular

activity. However, as the testing proceeded it was observed that in, at least, some mice, marked muscular tension or 'freezing' was produced, and it is doubtful if the hoped for reduction in muscular activity was fully achieved. The failure to obtain significant differences between the adrenals of IS and control mice might reflect the organ's ability to cope with a wide range of deficiencies. To use an analogy: the difference between a small and a large man carrying a heavy suitcase up an incline might be very small or neglible if behavioural indices are taken. However, physiological measurements of catabolic or anabolic reactions would reveal the internal and compensating differences that were responsible for the failure to obtain behavioural separation. With these considerations in mind it was thought that the most reliable measure would be to relate the adrenal's activity to total bodily requirements.

Tables 3 and 4 contain catecholamine estimations from a small number of mice from each group who were protectively placed in the presence of a food deprived ferret. The purpose of this experiment was to see if stressful situations other than ones using electric shock would produce adrenal mobilization of the catecholamines. The technique used was to place individual mice inside a small well ventilated Perspex box (15 x 10 x 8 cm).

The Perspex box was placed inside a large wire cage (36 x 18 x 18 cm) in which the ferret was free to roam. The effect on the ferret was quite marked and produced violent attempts to reach the mouse; however, the values of adrenaline and noradrenaline obtained are very little different from the values obtained from animals taken from the home cage and placed in the open-field without shock for two minutes. No behavioural differences were observed in the mice exposed to the ferret. A ferret was used only after the author had found that three cats displayed watchful but unaggressive or harmful behaviour towards the mice. In fact, on three occasions, the three separate mice used, finally climbed onto the "indigant looking" cats' paws and apparently went to sleep. Attempts to elicite an inherent fear response to a natural predator have proved to be very difficult. It is thought that selective breeding may have resulted in the strain of mouse used having high emotional thresholds which are, in consequence, very hard to trigger. Separate tests in which the mice were allowed to roam the wire cage together with the ferret appeared to indicate that mice had no awareness of the ferret. It is hoped in the future to obtain a wild strain of mouse and breed it for several generations in an attempt to produce a strain with a controllable emotional level. That is,

being easily handled, but nevertheless having readily elicitable emotion to aversive stimuli.

The experimental condition that elicited the maximum adrenal metabolism of noradrenaline was the treatment condition where a brief shock was given to the mouse immediately it was placed in the passive avoidance field. Using evidence from a series of experiments using rats and mice in a free operant avoidance situation the author (Van-Toller and Thornton, 1969) has argued that unpredictability tends to maximize emotion in laboratory animals. Caul and Miller, 1968, have also reported experimental studies in which animals were subjected to unpredictable shocks. Mason, Mangan, Brady, Conrad and MckRioch, 1961, in the experiment reported in the introduction, studied the concurrent plasma levels of adrenaline, noradrenaline, and 17 hydroxy corticosteroid in monkeys under conditions of emotional disturbances. They noted that unexpected shocks produced catecholamine elevations. It might be argued that the placing of a mouse in the novel environment plus the immediate shock elicited a greater compensatory process than the normal technique which allowed the animal to explore and reduce the novelty of the field for two minutes before the first shock was received.

As can be seen from the adrenaline histogram shown in Fig. 12 the overall difference for the separate experimental conditions are not so clearly marked as is found for the noradrenaline values shown in Fig. 13. Discounting the immediate shock condition, the general pattern for the IS and NHS groups is of increasing adrenaline metabolism until the +8 minutes delay when the values drop. The stress condition produced a further depletion of the amount of adrenaline found in the adrenals; the depletion occurring in the adrenals of NHS mice being the most marked. Normal mice produce the most variable amounts of adrenaline with the largest amount produced under the stress condition. This finding will be commented upon later. No explanation is offered for the very low mean value found for the +2 minute condition in Normal mice.

As mentioned earlier, in all assays, excepting for the home cage conditions, the noradrenaline means from the IS mice were always higher than the values obtained for the other two groups. Also, the IS noradrenaline values were the only intra-group results to give an overall significant difference. The general pattern for the IS and NHS mice shows an increase of noradrenaline metabolism after being placed in the open-field. This metabolism shows a decline following shock occurring after

the initial two minute exploratory period, then a slow build up of noradrenaline occurs over time. This finding suggests a secondary system coming into action, for despite the 30 minute stress period the noradrenaline values for these two groups are still relatively high. The Normal mice, again, show a varied pattern with a large depletion occurring under the 30 minute stress period.

Could the differences shown between the IS and NHS mice, and the Normal mice be due to the neonatal handling procedures? As indicated in Chapter II attempts were made to reduce any effects due to handling the neonates, or hypothermia during the injection periods. But clearly the sera injected mice experienced a markedly different environment compared to the unhandled animals. Up to this experiment no behavioural differences had been found between sera injected and unhandled mice; however, in this experiment a behavioural difference was found in the number of shocks received by the Normal animals and also a different pattern of catecholamine metabolism from the IS and NHS mice.

From Fig. 12 and Fig. 13 it might be argued that the group showing the greatest facilitation of catecholamine metabolism, especially noradrenaline, are the IS mice. NHS mice show some

facilitation of catecholamine metabolism, but certainly not to the extent found in IS animals The Normal group show a lack of facilitation of catecholamine metabolism. In fact, when stressed the Normal mice show a considerable reduction in the metabolic rate of noradrenaline indicating lack of reserves or appropriate metabolic precursors (Normal mice appear to be more dependent upon the adrenaline metabolism when stressed). Clearly, if the handling procedures undergone by the IS and NHS served to facilitate adrenal metabolism then they should be different from unhandled mice who lack the appropriate stimulation to obtain this effect. Bovard, 1954, held that differences arising from earlier handling procedures was due to a reduction in the sympathetic nervous system. However, from the results obtained for the handling procedures and injection procedures used in this experiment the converse appears to be the case; handling results in hyperactivity, at least of the adrenal medullary cells. Noirot and Pye, 1969, have recently indicated what might be an important and confounding variable that has been overlooked in previous handling studies. They claim that mouse pups when isolated from the mother emit ultrasonic distress calls. Further studies are required on this phenomenon.

One final major question remains to be answered, how valid is the assumption that the noradrenaline metabolism in the adrenals is used by the SNS as a compensatory mechanism? Celander, 1955, in a monograph analysing the range of control exercised by the sympathico-adrenal system has indicated that in his experiments the adrenal medulla plays a limited role in facilitating evoked responses in the SNS. Cannon, Lewis and Britton, 1926, had, using their denervated heart technique, earlier indicated a more positive role for circulating catecholamines. In this experiment the noradrenaline estimates for heart tissue from the IS mice are lower than the NHS group until the stressed condition when an increase occurs in the amount of noradrenaline found in the hearts of NHS and IS mice.

Bliss and Zwanziger, 1966, using three different species under a variety of "emotional stress" conditions have claimed that the stress is associated with a 10 - 30% drop in the noradrenaline levels found in brain tissue. This conclusion has also been supported by Welch and Welch, 1968, using mice. Fig. 14 and Fig. 15 shows the noradrenaline assay values obtained for some of the conditions tested during this experiment. Assays of the total brain revealed an overall increase

and in order to elucidate this the brain was divided into the three major areas. In this second study an overall decrease was found in all areas. Implications of brain noradrenaline levels will be discussed in the concluding chapter, but the reservations about the limitations of the fluorimeter used should be borne in mind when looking at the histograms.

CHAPTER VII.

THE WORK OF B. WENZEL USING IMMUNOSYMPATHECTOMIZED MICE.

This chapter is intended to give a brief summary of published behavioural studies using IS rodents and an attempt to interpret them in more detail than is found elsewhere in this thesis. In particular, to consider the work of Professor B. Wenzel and her colleagues who have reported a series of behavioural studies using the immunosympathectomy technique with the Swiss Webster strain of mice. Without the work of Professor Wenzel, the task of providing possible interpretations of the results reported here would have, at the very least, been considerably more difficult.

During the period of time in which the experiments reported in this thesis were being carried out, Wenzel and her colleagues have published a series of experiments (Wenzel and Nagle, 1965; Wenzel, Carson and Chase, 1966; Wenzel and Jeffrey, 1967; Wenzel, 1967; Wenzel, 1968a; Wenzel, 1968b). The experimental approach used by Wenzel in her published series of experiments has been very different from the approach reported in this thesis. Wenzel's technique is to subject her IS and NHS mice (earlier

saline was used as the injection material for the control mice) to a battery of tests designed to measure avoidance and emotional behaviours, these tests usually extend over a period of some months. Wenzel also did not, due to "identification difficulties", use the split-litter injections technique. In later papers Wenzel uses four groups of mice: IS, NHS, Handled and Unhandled. The summary in this chapter will deal with the papers in chronological order of publication.

Wenzel and Nagle, 1965, using histological techniques demonstrated (using NGF antiserum from the Abbott Laboratories, U.S.A.) hypotrophy in the paravertebral sympathetic ganglia of a strain of Webster Swiss mice. They also reported two behavioural techniques. The first involved an active avoidance situation using a shuttle box (CS, light onset 3.5 sec; UnCS, 0.2 ma shock). The second situation used non-shocked passive avoidance with food rewarded lever pressing involving a DRL, 10 sec; VR, 2.5 sec reinforcement schedule. The rewarded component of the chain was signalled by light onset. No significant correlation was found by the authors between the number of remaining sympathetic cells and the various performance measures recorded. Chemical assays were made on a small number of mice from each group of the adrenal concentration of corti-

costeroids and ascorbic acid. No significant differences were shown by these assays; however, the authors report that they were highly variable. Conclusions drawn about the behavioural measures were, that the IS mice displayed larger variabilities in escape times for the shuttle-box situation. Other than this measure, the mice reacted normally in virtually all aspects of the various learning situations tested.

In the Wenzel, Carson and Chase paper, the cardiac responses of IS and control mice under normal and aversive conditions were examined. As the authors' point out, there were a number of studies which had shown catecholamine depletion in heart tissue of IS animals (see appendix for references of work on IS animals). It was therefore important to test the cardiovascular concomitants of IS mice in aversive or emotional situations. The study found that IS mice compared with the control animals showed a significant decrease in their heart-rates during a 30 minute adaptation period when placed in a strange environment. Administration of a light foot shock resulted in an increase of heart rate for both the IS and control mice, but the increases shown by the IS mice were only to their initial elevated levels when first placed in the chamber. Control mice showed heart rate increases above their initial elevated levels. This paper also reports the conclusion that the

difficulty shown by IS mice in improving their escape and avoidance performances in aversive situations was due to their experiencing a reduced internal reaction to aversive stimuli. Wenzel used the mediational hypothesis to explain the slower acquisition times of her IS mice, i.e, they were receiving proportionately less reinforcement from escape and avoidance behaviours.

Wenzel and Jeffrey, 1967, reported a battery of behavioural tests given to IS and control (saline injected) mice over a period of four months. Apart from the sera injected mice they included handled and non-handled control animals. Each treatment group were composed of complete litters and no specific attempt was made to control for intra litter variability. The timing of treatment or behavioural tests are shown below:

Day.	Treatment or Behavioural Test.
0-6.	daily injection programme.
21.	weaned and ear punched.
29.	open-field and water maze.
43.	water maze.
50-53.	active avoidance using an alley-way.
64-(?).	operant behaviour.
106.	activity test in a jiggle cage.
113.	open-field.

The conclusions were that IS mice failed to increase their escape speeds from aversive situations as rapidly as normal mice, even

though the initial escape speeds were the same for all groups on the first trial. No significant differences were found for level of activity, or food and water intake. The most striking feature of the results was the inferior performance of IS mice in the two water tests.

Wenzel, 1968(a), essentially repeated the above battery test using NHS injections to eliminate possible confounding variables due to gamma globulin. The most interesting and relevant part of the second paper was Wenzel's use of massed water trials in an attempt to obtain a greater behavioural separation in a test which had revealed differences between the IS and control mice when space trials had been given. The finding was that the differences found during spaced trials disappeared when massed trials were given.

Cannon and his associates (1929) wished to place less emphasis on the role of the thoracolumbar autonomic outflow in mediating emotional reactions and instead place emphasis upon the sympathetic nervous system's homeostatic role in temperature regulation. This important function of the SNS might be used to explain Wenzel's findings of differences between massed and space water trials. Making the animals swim in water, would result in drops of the body temperature (Wenzel used a water temperature of 23°C). Spacing the trials

would result in intense sympathetic activity to maintain body temperature between trials. The water saturated condition of the mice would result in the animals shivering intensely (a casual observation made in this laboratory by a student appeared to indicate that IS mice shiver more than NHS mice when subjected to drops of temperature). Wenzel did not report in her paper if this was the case. But the need to maintain bodily temperature during the intertrial interval would mean a considerable depletion of noradrenaline. Thus, when these IS mice were required to swim again 15 minutes later, they were at a disadvantage compared to the control mice in that they had depleted reserves of the essential neurotransmitters required to aid the muscular work involved in swimming. Massed trials given 15 seconds apart meant that the increased SNS activity invoked to maintain the body temperature could be maintained over the relatively short time period required for a single experimental session. Massed trials given over a much longer time period should result in the eventual separation of the IS and control mice.

In other words, the IS mice because of the hypotrophy induced in their SNS neural network do have a reduced range of adaptive responses but the animals are able to maintain the

system adequately under moderately demanding conditions. But it is interesting to note that requiring an animal to swim would bring the SNS role of temperature maintenance very much to the fore. As reported earlier, Schönbaum, Johnson and Seller, 1966, reported on the effects of acclimatization to cold stress in IS rats. It was concluded that the residual SNS activity in IS rats was adequate to maintain life at 4°C. However, under conditions of severe cold stress significant impairment of sympathetic function was observed.

Wenzel, 1968(a), used the work of Brody (Brody, 1963; Brody, 1964; Brody, 1966, these papers deal with a series of experiments carried out on the electrical activity and cardiovascular responses in IS rats) as evidence for functionally reduced activity in IS animals, and again favoured the mediation hypothesis. The essence of her conclusion appears to be contained in the following passage:

> "Thus, the lowered responsiveness of specific effectors to sympathetic stimulation could account for poorer avoidance performance by reducing, or possibly eliminating, the mediational cue".

In this paper Wenzel also points to the possible role of the adrenal medullae as the primary source of circulating

catecholamines following hypotrophy induced by the immunosympathectomy technique.

The final paper in the series (Wenzel, 1968) concerns the finding of significant differences between control and IS mice in an avoidance situation which was described as non-discriminated avoidance. However, the experimental situation would be better described as non-signalled, free operant avoidance (sometimes called a Sidman schedule, after the originator, Sidman, 1953). Wenzel's schedule used an R-S cycle of 20 seconds followed by continuous shock until an escape response was made. Any response made during the R-S period delayed the shock for a further 20 seconds from the time of the lever press. From the use of the term nondiscriminated and from her discussion, Wenzel appears to accept that the predominant feature in the acquisition of avoidance in the free operant situation was an internal timing mechanism.

Mowrer and Keehn, 1958, originally proposed an internal timing of responses to explain how free operant avoidance was maintained in the absence of external signals. Anger, 1963, has reviewed and discussed the internal trace hypothesis and other possible explanations of how reinforcement occurs in the Sidman avoidance situation. It is now accepted by many workers

that even within the sterile environment of the operant box, animals might be using features other than internal traces or mediational mechanisms. This appears to be especially true if one considers that the predominant behaviour found in the free operant avoidance situation is holding the bar down (Hurwitz and Millenson, 1961; Keehn, 1967).

Wenzel's paper does not discuss any of the above points and no report is given of different forms of behaviour between the IS and control mice. Measures such as bar holding or inter response times (IRTs) are also not given. However, the use of a lever consisting of a large Plexiglass ball on the end of a rod suggests that unrecorded precautions were taken to reduce bar holding behaviour. The paper reported that of the eight sessions run, on session 1, 7 and 8 the IS, NHS, Handled and ~~and~~ Non-handled mice differed significantly in the mean number of responses made; the IS mice making the least responses. Also, during session 7 the mean number of shocks received by the groups was significantly different with the IS mice receiving more shocks than the other groups. The data from the other sessions was said to be too variable to allow an overall significance level to be reached. Dunnett's 't' test was applied to the data for session 1 and 8 in order to compare the IS mice with each of the three control groups.

For the first session no significant group differences were found. During session 7 it was found that the IS mice differed from the Handled and Non-handled animals in number of responses made, and from all three control groups in the number of shocks received. For the eighth and final session the IS group were significantly different from the Handled and Non-handled group in number of responses made and the number of shocks received. The figures for the mean number of responses made and shocks received are very orderly, the IS group always made the least number of responses and received more shocks. A trend analysis over sessions was not reported.

Wenzel concluded that the behavioural separation found in this free operant task was due to its less demanding nature compared to other avoidance tasks, (the height of the bar was not stated and it is possible that the mice were required to rear upwards in order to press the bar). The earlier failure to find differences in a shuttle box situation was explained as being due to the Webster Swiss strain of mouse used, Bovet, Bovet-Nitti and Oliverio, 1966, reported that this strain of mouse gave poor learning scores in a shuttle-box avoidance situation. Finally, it was suggested that the IS mice might have a higher skin impedance with a consequent reduction in the subjective intensity of shock.

Why this final point was made is not too clear. Assuming the electrical source used was a constant current (the source is not stated in the paper) of 700v DC across a 700k resistance, this would be a typical value for such studies. If the extreme differences for an IS mouse was 25k and a perspiring NHS mouse 5k, then the differences between the mice, compared with the total 700k resistance in the circuitry, would be very small and unlikely to be a major factor in the situation. Also, if we consider the problem of a high skin impedance we know that the current reaching the body through cutaneous skin having a high impedance would be through relatively small areas such as cracks or blemishes in the skin. This subjectively would result in a more painful sensation. It might be argued that the reason why IS mice tend to have longer escape latencies is that they have to overcome partial tetanizing effects arising from the increased current intensity that they experience due to their high skin impedance.

In conclusion it would appear that the major difference in the series of reports by Wenzel and those carried out by the author are probably due to the different strains of mice used. Wenzel has used a pure strain of Webster Swiss while the work reported in this thesis has been carried out on a genetically

heterogeneous strain. For example the strain used in these experiments has given a consistent and significant sex difference of such an order that the author is inclined to agree with Sampson's (1966) conclusion that the innervation to the pineal gland from the superior cervical ganglia of the sympathetic nervous system, may play a role in producing these sex differences (the pineal gland has recently been held to have a regulatory or 'biological clock' role in behaviour; Wurtman and Axelrod, 1965). Wenzel's strain of mouse does not appear to produce a clear-cut difference between the sexes. This lack of a sex difference is probably due to the inbred nature of her mice. Clearly the problem of strain differences requires examination in later studies.

CHAPTER VIII.

FREE OPERANT AVOIDANCE.

The novel use by Wenzel of mice in the free operant avoidance situation was interesting in view of the series of experiments that were being carried out by the author and undergraduate students using rats. These studies initially involved the use of IS and NHS rats on a C.E.R. schedule. However, finding no essential or reliable behavioural differences a non-signalled free operant avoidance was then used as a means of checking for possible defects of internal mediational systems in a situation that contain no external signals. Again, the findings were essentially negative. Wertheim, Conner and Levine, 1967, reported that the number of shocks received by rats working on an operant avoidance schedule were negatively correlated with injections of exogenous ACTH over the range 8-12 I.U. Following this paper the author examined the pituitary-adrenal axis in normal and IS rats. Our conclusions are, that not only have we been unable to confirm the Wertheim, Conner and Levine paper, but we have also found that hypophysectomized IS rats are able to acquire and maintain free operant avoidance behaviour. These experiments with rats are

currently being continued. It should be borne in mind that the degree of hypotrophy produced in rats by injection of NGF antiserum is considerably lower than that produced in mice. Injections made by Mr. Thornton into rats of 0.5 ml over a period of 5-6 days consecutive days post partum have been shown to produce decreases in the order of only 40 - 50%.

It was decided to attempt to replicate Wenzel's results with the Durham strain of mice. Initial experiments carried out at Durham and using mice in a free operant avoidance situation were performed by Brown, 1969, in an undergraduate thesis, carried out under the direction of the author. Subsequently the use of free operant avoidance schedules have been repeated by the author himself and two separate groups of undergraduate students.

METHOD.

Subjects:

Brown's study used 19 male mice taken from eight litters. The litters had been injected and treated in accordance with the standard procedure laid down in Chapter II.

Apparatus:

Two special operant boxes were constructed, see Fig. 17. Internal measurements of the boxes were 10 cm x 6 cm x 6 cm. The floor of the boxes consisted of a stainless steel grid, constructed from hypodermic tubing 0.2 cm in diameter spaced 0.5 cm apart. In each box the response bar consisted of a stainless steel 1 cm sphere (the mice were unable to grip a sphere of this dimension in their mouths nor were they able to hold the bar down using their paws). The sphere was welded onto the end of a length of stainless steel wire. Adjustments to the bar assembly prevented the mice from gaining access to behind the metal sphere. Passing through a slit in the wall of the box, the bar was attached to the pivot of a low torque microswitch (obtained from Radio Spares, London, E.C.2). A Grason Stadler shock generator (model 106495) delivered a scrambled constant current shock to the floor grids. Inter-response times (IRTs) were recorded on banks of counters connected to a Grason Stadler Multiple Class Time Analyzer (model E 3950A). The remaining parts of the programmes were arranged via electro-mechanical circuitry. During sessions the mouse operant boxes were placed inside a rat shuttle box which served to provide lighting, sound damping, and ventilation. The noise

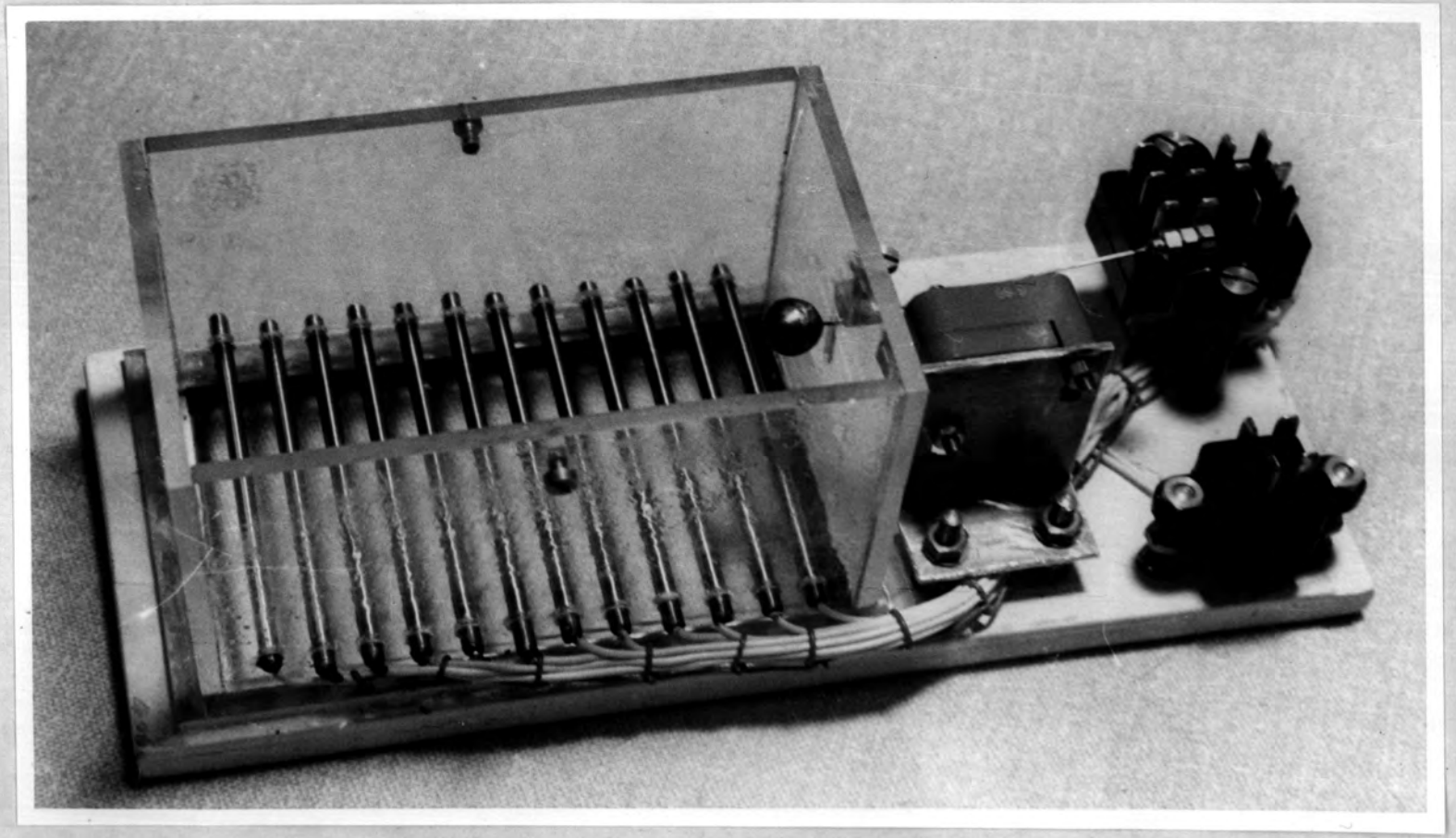

FIG. 17 Free operant avoidance box designed for use with mice.

level inside the boxes, produced by the ventilation fans was approximately 72 db (0.0002 dyne cm^2). The sound proofing encasing the rat shuttle boxes provided sound insulation of the operant boxes from one another and also from noises coming from the operant control room next door.

Shaping and Schedule:

The mice were carefully shaped to press the bar. The technique used was one of successive approximations towards the actual behaviour of pushing the bar down, but care was taken to ensure that initially the animals were never shocked whilst actually making a bar response. Continuous shock was used as earlier studies had shown that it produced a more uniform response profile between animals; it also reduced the failure rate to a very low level. During the initial stages of shaping the shocks were delivered via a hand switch but later the controlling and timing circuits were switched on and the sessions controlled automatically. The final avoidance schedule consisted of a R-S cycle of 15 seconds with a continuous shock, 0.6 ma, until an escape response was made. Any lever press during the 15 second R-S period reset the cycle, with the next shock being programmed to occur 15 seconds later.

Behavioural Measures Recorded:

The following measures were taken from each animal at every session: total number and amount of shock received, total number of bar responses and amount of bar holdings, and inter response times (owing to a misunderstanding during the first study total bar holding and total shock duration were not recorded). Cumulative records were also taken from each animal.

Brown also carried out adrenal assays and tissue for catecholamines. In accordance with the standard procedure, two of his mice, one IS and one NHS, were selected at random and the thoracic and cervical ganglia dissected out for examination.

RESULTS.

It is only proposed to discuss the parts of the initial study that are relevant for the following chapter. In his experiment Brown failed to find significant differences between his IS and NHS mice for: mean number of shocks received; mean number of responses made, and median I.R.Ts. Analyses of linear trends for these three measures also failed to reach significant levels. As indicated earlier, the amount of shock received by the animals was not recorded and it was felt that the actual

number of shocks received was not an accurate measure of escape behaviour. A low total number of shocks could indicate either good avoidance behaviour or very poor avoidance behaviour. Therefore, it was decided to select an arbitrary latency of 5 seconds, measure all the cumulative records and record the number of escape latencies greater or equal to 5 seconds for each animal. These high latency scores were subtracted from the total number of shocks received and a Chi square test computed on the group data. The Chi square test showed that the IS animals received a significantly greater proportion of long latency shocks ($p < 0.02$).

Using the standard laboratory procedure one IS and one NHS mouse were randomly selected for dissection, to compare the relative sizes of the thoracic and cervical ganglia. Unfortunately the dissection revealed very little difference between the ganglia taken from the two mice. By this time the other animals that had been used in the experiment had been disposed of so that there was no possibility of checking the other mice. The author, dissecting ganglia taken from mice that had been injected at the same time as Brown's mice found the normal marked hypotrophy in his IS mice. It is thought that the most probable explanation for the lack of

differences in ganglia sizes was due to incorrect ear marking. For the purposes of sterility Brown was given his own phial of NGF antiserum which he used up whilst injecting his mice. But it was the first record of failure to find hypotrophy in the SNS of presumed IS mice and it is thought to be very unlikely that the NGF antiserum was at fault.

DISCUSSION.

The failure to observe hypotrophy in so-called IS mice meant that no really firm conclusions could be drawn from this study. But certain general findings reported have been confirmed by subsequent experiments using identified IS mice. The most interesting result was the very high response rates maintained by the mice. The mean hourly response rate throughout the experiment was 919 responses per hour. This is much higher than response rates found in this laboratory for other species. Examination of the cumulative records showed that mice who received a low number of shocks displayed high response rates from the earliest sessions. This finding may indicate a species difference between rats and mice. Previously the criterion used by most workers for successful avoidance was a low number of shocks, and inter response times (IRTs) shorter

than the R-S interval. But in this experiment the successful avoidance by the mice appeared to have been an incidently by-product of the high response rates. Of course, it is possible that the manipulandum used contributed to the high rates. The lever was primarily designed to prevent the mice from holding them down and the paw scrabbling behaviour shown by some mice would tend to produce high rates. However, rats given a ball lever have not been found to produce such high response rates. When shock was received the mice produced longer escape latencies than those usually found in the rat. Indeed, some mice changed gradually after receiving a very few shocks, from a fast rate to much lower response rates.

Wenzel, 1968(b), did not discuss the response profiles found in her mice. But from this limited study it was concluded that with this type of manipulandum mice typically responded at high rates. Also, because of the reduced SNS innervation IS mice tended to be unable to maintain response rates as high as those found in the normal mouse; this could be due either to muscular asthenia, revealed under conditions requiring a high and consistent work rate, or because of an inability to maintain required levels of the transmitter substance noradrenaline.

Wenzel reported that her IS mice made fewer intershock responses and received more shocks. The study carried out by Brown used a session length of 60 minutes as opposed to Wenzel's session length of 20 minutes. Wertheim, 1965, using rats and analysing some sequential aspects of IRTs emitted in the free operant avoidance situation has reported a marked warm-up effect at the beginning of each session. If a warm-up effect is found in mice, Wenzel's sessions length may have resulted in a marked bias. Another major difference was Wenzel achieved separation between her groups on the first day, whereas Brown's groups did not separate until the seventh day. Brown also used a shock of 0.6 ma which was much higher than Wenzel's 0.25 ma.

Despite certain parametric differences, both Wenzel and Brown showed that IS mice were able to acquire and maintain free operant avoidance behaviour. The studies indicated a suggestion that IS mice were slightly inferior to normal animals but no convincing and unequivocal proof had been shown in either study.

Two further studies using a total of 10 IS and 10 NHS male mice essentially confirmed Brown's initial study. One major

difference was that although the mice tended to give high rates the very high rates obtained by Brown were never found. It is presumed that his very high response rates were a direct result of his shaping technique.

CHAPTER IX.

COMBINED FREE OPERANT AND ACTIVE AVOIDANCE SITUATIONS.

At this point a review was made of the known behavioural studies using IS mice. The evidence for the sympathetic nervous system playing an important role in the mediation of fear or emotional states appeared to be in considerable doubt. It is maintained that unambiguous behavioural separation had never been shown between IS mice and control animals. From the results of the catecholamine assays it was possible to argue for some hormonal compensation for the hypotrophic SNS in IS mice; or, at the very least, facilitation of the SNS in these mice. Some evidence was available to show that reduction of the neurotransmitter substance of the SNS, noradrenaline, did appear to increase behavioural separation (the use of reserpine in the passive avoidance experiment reported in Chapter IV). Overall, however, the evidence was relatively weak. But clearly the reduction in the SNS of IS animals must reduce the possible range of the animal's internal adaptive responses to environmental changes. The question that had been asked was; does the large morphological reduction obtained in IS animals affect

the animal's appreciation of aversive, and sometimes subtle, environmental stimuli?

An experimental technique was required that reduced the range of an IS animal's internal adaptive response by depleting the animal's store of essential neurotransmitter substances, and then to make a behavioural test. Moreover, in line with previous policy, a reasonably natural process was required, one that avoided recourse to surgical or pharmacological intervention. The experiment that evolved from these ideas was as follows: IS and NHS mice would be trained on a free operant avoidance schedule. When the animals were producing relatively stable avoidance behaviour, the sessions would be split into two equal periods. In between the two equal daily sessions of operant avoidance, a task would be inserted that involved a reasonable level of sympathetic nervous system activity. The task was required to deplete the animal's reserves of noradrenaline. The task finally selected was an active avoidance task using an alleyway. To ensure adequate levels of muscular activity in the runway it was decided to incline it and make the mice run uphill. Subject to a properly balanced design, it might prove possible to show differences between the first and second sessions of the free operant avoidance due to further reduction of the range of the animal's adaptive response imposed by the alleyway task.

METHOD.

Subjects:

The subjects were six male mice from a single litter. Three were IS and three were NHS control animals. The injections and treatment were as described in Chapter II. At the beginning of the experiment the animals were 40 days old.

Apparatus:

A full description of the free operant avoidance boxes used for the mice is contained in Chapter VIII. The alleyway was made up from three straight 45 cm long covered sections, which were bolted together, and a 6.25 cm start and goal box at either end, having a total length of 1.35 m. Photographs of the alleyway are shown in Fig. 18 and Fig. 19. In cross section the alleyway was 3.44 cm in width and 5.31 cm in depth. The floor of the alleyway consisted of stainless steel bars, 0.125 cm in diameter spaced 0.75 cm apart. At the start of the straightway lengths, in the middle, and at the end were hinged floor sections (7.5 cm long) activating low torque micro switches. Depression of the hinged sections by the mice allow escape latencies, and running times over both halves of the

runway to be recorded. The hinged floor sections, shown in Fig. 18, moved on two lever arms (12.5 cm in length) which rotated about a knife edge at their fulcrums. Two light expanding springs attached to the walls of the alleyway held the floor sections up. The hinged floor sections were designed to be activated by a 10 gms weight. Baffles, see Fig. 19, were attached to the roof of the alleyway at midpoints over the hinged sections to prevent mice from leaping over these sections and failing to activate the microswitches as they passed that point. The baffles required the mice to pass through a gap of 2.75 cm in height; a pilot study failed to show any significant slowing in running speeds caused by insertion of the baffles. Electrical shocks could be delivered to all parts of the alleyway floor with the exception of the goal box which was not wired into the shock circuit. Ceiling to floor grids enable the mice to be confined in either of the two end boxes. For the purposes of this experiment the runway was inclined at 1/8, or 2.5 cm/20 cm.

Holding Box:

The wooden holding or carrying box (22.5 x 10 x 10 cm), mentioned in Chapter III, was used to retain the mice on the occasions when they were not run in the alleyway.

FIG. 18 Side view of inclinded alleyway designed for use with mice.

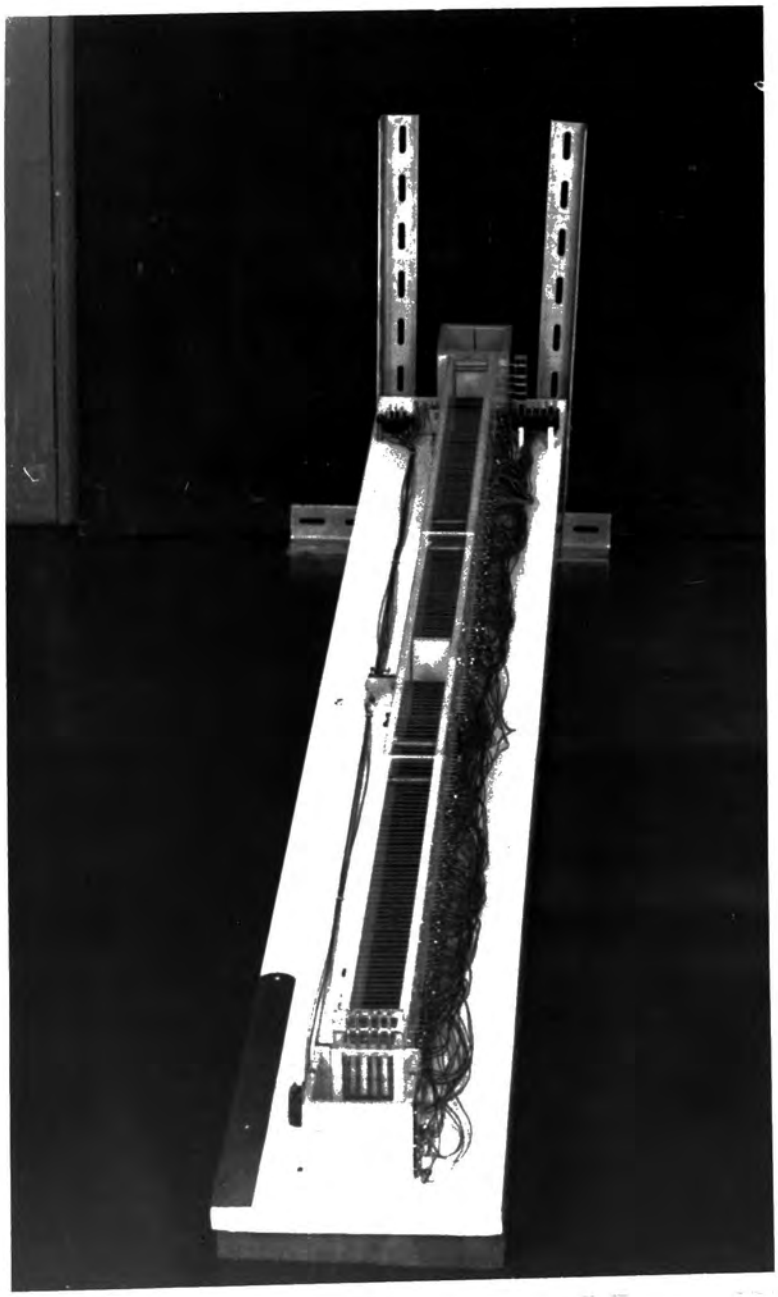

FIG. 19 Overhead view of inclined alleyway designed for use with mice.

Stimuli:

The electric shock was delivered to the alleyway from a Grason Stadler (type E 1064 GS) shock generator at an intensity of 1 ma. White noise, used as the conditioned stimulus (CS) was obtained from a Grason Stadler (model 901 A) noise generator. The CS was delivered for 5 seconds via a 3 inch loudspeaker placed 5 cm above the start box. The unconditioned stimulus (UnCS) was continuous shock until the final hinged section at the end of the alleyway was depressed. The CS was terminated either by UnCS onset or, in the case of an avoidance response, depression of the final hinged section. Escape and running times were recorded on Hengstler's 1/50 second counters.

Free Operant Avoidance Schedule:

The free operant avoidance schedule consisted of an R-S cycle of 15 seconds followed by continuous shock. As in the previous experiments reported in Chapter VIII, responses reset the R-S cycle. Shock intensity was 0.6 ma.

The animals were shaped on the free operant avoidance schedule by hand and then given 12 daily training sessions of 60 minutes duration. The mice were run in pairs and alternated between the two boxes on consecutive days. Following the

acquisition stage the animals were run for three days with the hourly session split into two equal periods of 30 minutes. During the time between the initial split-session periods, the mice were retained in the small holding box for five minutes before being returned to the operant box for the final 30 minute period. The next stage of the experiment involved the random allocation of the mice to an equal number of holding box retentions, or active avoidance acquisition in the runway; this stage lasted for a total of eight days.

Alleyway Procedure:

The mouse was placed in the start box with the grill that blocked access to the alleyway in the closed position. After three seconds the CS was presented, and the grill blocking access to the alleyway was drawn up. If the mouse failed to escape up the alleyway from five seconds of the CS onset, shock was delivered through the grid floor. As the mouse entered the goal box the end grill was lowered behind it to prevent the mouse from re-entering the alleyway. The recorded times were noted and the counters reset, before the animal was removed from the end box and replaced into the start box. The next trial was started three seconds later. Trials were massed and

emphasis was placed upon a speedy, though gentle, transfer of the mice back into the starting box. At the end of the 10 massed trials the mouse was put back into the holding case and returned to its operant box.

Response Measures:

In the free operant avoidance situation the total number of responses, number of shocks, median IRTs, total amount of shock, and cumulative amount of time spent holding the bar down were recorded. Measurement recorded from the alleyway were; ~~escape~~ avoidance latencies following onset of the CS, and running times over the first and second halves of the alleyway.

Reserpine Injections.

Following the eight day holding box v alleyway phase of the experiment the mice were injected with 0.1 µg reserpine/ 10 gms body weight at 12 hourly periods. The mice were run in the free operant avoidance situation and all run in the alleyway. During this final phase the mice were shocked in the alleyway and not allowed to escape. The CS-UnCS were presented as previously, but the end grill was not drawn up until shock had

been given for 15 seconds. The mice were given four massed trials of this stressful situation. If the mouse was not at the end point in the alleyway at the end of the 15 second period, shock was switched off by the experimenter. The reserpine injections were given 12 hours and $\frac{1}{2}$ hour before the daily session began.

The technique of injecting microamounts of reserpine to deplete noradrenaline levels was first seen by the author in the Department of Pharmacology, Royal Free Hospital, London. During a visit to the hospital the author noted that he could detect no obvious sensori-motor deficits in rats that had received injections of micro amounts of reserpine over a period of five days, although the adrenals of these animals were subsequently shown to be considerably depleted of noradrenaline.

RESULTS.

All response measures reported in this experiment were transformed (log + 1) before the various analyses of variance were computed. The type of analysis of variance used on any group of measures varied but in general an appropriate factorial design was selected from Winer, 1963.

The means and standard errors of response measures recorded during the first 30 minute period in the free operant avoidance situation are shown in Table 7. As had been shown in the earlier studies using this technique, both groups of animals showed individual variations. In this particular study the NHS group shown greater variability than the IS mice for the five response measures recorded. The only response for which a significant difference was shown for the first 30 minute periods was escape latencies; IS mice recorded faster mean times for turning the shock off, $p < 0.05$. Because of a slower cumulative recorder speed it was not possible to confirm Brown's finding that IS mice receive a greater proportion of longer shocks. Visual inspection of the daily cumulative records did not suggest that they had received a greater proportion of longer shocks.

The high response rates of NHS mice found during the initial study was, again, not confirmed. In this study the IS mice had the higher response rates and shorter escape latencies, they also spent approximately the same mean time as NHS mice bar holding. It can be said that the results obtained from the first 30 minute periods of free operant avoidance in this experiment are similar to the results obtained for the earlier studies made in this laboratory.

TABLE 7

MEANS AND STANDARD ERRORS OF RESPONSE MEASURES
RECORDED DURING 1ST 30 MIN. PERIOD OF FREE OPERANT AVOIDANCE

	MEDIAN I.R.T. (SEC)	ESCAPE LATENCIES (SEC)	SHOCKS	BAR RESPONSES	BAR HOLDING (MIN)
I.S.					
S1	2.21 ± 0.21	0.46 ± 0.07	54 ± 4.6	359 ± 27	7.88 ± 1.31
S2	2.42 ± 0.36	0.53 ± 0.05	66 ± 4.8	307 ± 15	10.15 ± 1.26
S4	2.22 ± 0.33	0.80 ± 0.22	31 ± 5.4	415 ± 29	16.73 ± 2.41
Group $\bar{x}$	2.28	0.59	50	353	11
N.H.S.					
S3	6.56 ± 1.43	1.24 ± 0.26	57 ± 6.5	245 ± 19	10.81 ± 1.58
S5	5.23 ± 1.86	1.56 ± 0.70	70 ± 6.0	403 ± 107	10.40 ± 1.99
S6	3.97 ± 1.71	1.01 ± 0.19	78 ± 6.4	361 ± 55	11.30 ± 0.11
Group $\bar{x}$	5.25	1.27	68	336	10.5
Overall p.	NS	0.05 (treatment)	NS	NS	NS

It is thought that the large variations found between the animals is explained by subtle differences occurring at early shaping and acquisition stages. The use of continuous shock helps to reduce the total inter-animal variation found, but the free operant procedure and present techniques are still not sufficiently controllable to give more uniformed behavioural patterns.

Free Operant Avoidance Prior to and Following Alleyway Trials or Holding Box Retention.

Ratios of response measure recorded during the first 30 minute period divided by response measure recorded during the second 30 minute period were used to produce a measure that reflected changes of behaviour during the two periods. The ratios computed for the five measures recorded during the first and second 30 minute periods are shown in Table 8. The four ratios obtained under each intervening condition were transformed (log + 1) and analysed using a factorial analysis of variance having treatment, intervening condition, and days as the main factors. A significant difference was found between the number of shocks received by the IS and NHS mice ($p < 0.007$). IS mice received more shocks following the intervening period regardless of whether it was the holding box or the alleyway

situation. A significant difference was found ($p < 0.05$) between the holding box and the alleyway condition. Both groups received more shocks following retention in the holding box. The IS group received 50% more shocks following the holding box, and 20% more shocks following the alleyway trials. The NHS group received 20% more shocks following the holding box, and 20% less shocks following the alleyway.

The differences found for the number of shocks received by the two groups were paralleled by the findings of a significant difference in bar response rate ($p < 0.05$) following the two intervening conditions. Both groups tended to reduce their overall response rate following retention in the holding box and to increase their overall response rate following the alleyway trials. Despite the increase in number of shocks received, the shock escape latencies remained fairly uniform between the first and second periods. The median IRT ratios were significantly different for the two intervening conditions. Retention in the holding box resulted in increases in the IRTs while following the alleyway trials they were slightly shorter.

Alleyway Trials.

The escape latencies were analysed using a factorial analysis of variance with treatment, days, and trials as the

main factors. No significant main or interaction effects were found. The overall mean latencies for the IS group was 3.11 seconds and for the NHS group 3.74 seconds. Total running times were analysed using the same factorial design used for analysing the escape latencies. Significant differences were found between the treatment ($p < 0.05$) and trials ($p < 0.01$). Contrary to expectation the IS mice recorded overall faster running times for the inclined alleyway. IS mice had an overall running speed of 3.29 seconds compared with NHS mice with an overall mean of 3.71 seconds. The significant effect of the factor related to trials was caused by a general speeding up that occurred over the ten daily trials.

In view of the finding that the IS mice, although not reaching a significant level, had an overall slightly shorter escape latencies than NHS mice; moreover, that they were significantly faster over the total runway, it was decided to analyse the amounts of time taken to traverse both halves of the alleyway by the two groups. The running times in the two halves of the alleyway were analyzed in a factorial analysis of variance with treatment, first half/second half, days, and trials as the main factors. The analysis made using this design confirmed the significant treatment ($p < 0.025$) and trials ($p < 0.001$) effects shown in the previous analyses.

TABLE 8

MEAN RATIOS (1ST 30 MIN./2ND 30 MIN.) OF RESPONSE MEASURES RECORDED DURING FREE OPERANT AVOIDANCE

	MEDIAN I.R.T.		ESCAPE LATENCY		SHOCKS		BAR RESPONSES		BAR HOLDING	
	* H.B.	A.W.	H.B.	A.W.	H.B.	A.W.	H.B.	A.W.	H.B.	A.W.
I.S.										
S1	0.89	1.73	0.90	1.22	0.43	0.74	1.20	0.90	1.08	1.25
S2	0.71	0.87	0.90	0.98	0.56	0.56	1.20	1.07	0.85	1.09
S4	1.16	1.55	1.28	0.89	0.34	1.15	1.10	0.91	1.03	0.93
Group $\bar{x}$	0.92	1.38	1.02	1.03	0.44	0.82	1.20	0.96	0.99	1.09
N.H.S.										
S3	0.95	1.06	0.80	0.79	0.86	0.88	1.03	0.98	0.85	1.11
S5	0.93	1.09	1.01	0.91	0.83	1.82	1.26	0.88	0.81	0.93
S6	1.05	1.09	1.04	1.05	0.81	1.17	1.11	0.99	1.39	2.10
Group $\bar{x}$	0.98	1.08	0.95	0.92	0.83	1.29	1.10	0.95	1.02	1.38
Overall p.	0.05 (HB v AW)		NS		0.007 (treatment) 0.05 (HB v AW)		0.05 (HB v AW)		NS	

* (H.B. = Holding Box; A.W. = Alleyway)

Differences between running times for the two halves did not reach significant levels. However, it is interesting to note that whereas the NHS mice speeded up over the second half of the alleyway the IS mice slowed down. The percentage amounts of time spent in the two halves were: NHS, 1st half 53%, 2nd half 47%; IS, 1st half 43%, 2nd half, 57%.

Results Obtained from the Reserpine Phase of the Experiment.

The means and standard errors of response measures recorded during the first 30 minute periods of free operant avoidance following the administration of reserpine are shown in Table 9. The response measures were transformed (log + 1) and analysed using a two-way analysis of variance with treatment and days as the factors. As the final part of the experiment was only run for three days the number of variables analysed for each animal was limited. The only measure, as shown in Table 9, for which a significant difference was found was number of shocks received over days ($p < 0.01$) and number of bar responses made over days ($p < 0.05$). All animals showed a progressive drop in bar responses over the three days. The raw data did not reveal a similarly systematic effect for the number of shocks received.

In general, the number of shocks received increased over days but not in all cases. For example, IS mouse S4, rose from 4 through 18 to 43 shocks received on the three days, whereas IS mouse S1, rose from 40 through 52 and finally dropped to 39 on the third and final day.

Table 10 shows the ratios of the response measures recorded during the initial 30 periods of the three session prior to, and the three session following the administration of reserpine. The only significant difference found was number of bar responses made over days ($p < 0.01$). There was a general tendency for the response rate to drop but certain mice showed large variations. NHS mouse S5, on for the first day gave a ratio of 0.61, dropping to 0.29 for day 2 and finally rising to 1.11 on day 3. The mean ratios first 30 minute/second 30 minute periods following the administration of reserpine are given in Table 11. The IS mice received significantly more shocks following the intervening stress period that was given to all the animals during the reserpine phase of the experiment. Expressed as a percentage of the number received during the first 30 minute period the IS group received an average of 140% shocks during the second period compared with an average 115% received by the NHS group during their second period.

TABLE 9

MEANS AND STANDARD ERRORS OF RESPONSE MEASURES
RECORDED DURING THE FIRST 30 MIN. PERIOD OF FREE OPERANT AVOIDANCE FOLLOWING THE ADMINISTRATION OF RESERPINE

	MEDIAN I.R.T.(SEC)	ESCAPE LATENCIES(SEC)	SHOCKS	BAR RESPONSES	BAR HOLDING(MIN)
I.S.					
S1	2.68 ± 0.59	0.29 ± 0.06	34 ± 10	474 ± 129	13.3 ± 2.5
S2	1.62 ± 0.17	0.69 ± 0.06	58 ± 6	333 ± 32	8.5 ± 1.7
S4	2.16 ± 0.33	0.51 ± 0.04	22 ± 9	470 ± 97	11.2 ± 1.4
Group $\bar{x}$	2.15	0.50	38	426	11
N.H.S.					
S3	3.64 ± 1.10	0.70 ± 0.13	53 ± 8	335 ± 43	10.8 ± 2.7
S5	0.30 ± 0.04	0.35 ± 0.11	62 ± 2	1332 ± 110	7.4 ± 2.4
S6	10.0 ± 2.16	0.71 ± 0.15	71 ± 12	206 ± 22	9.6 ± 0.3
Group $\bar{x}$	4.65	0.59	62	624	9.3
Overall p.	NS	NS	0.01 (days)	0.05 (days)	NS

TABLE 10

MEAN RATIOS OF RESPONSE MEASURES RECORDED DURING THE FIRST 30 MIN. PERIODS OF THE LAST THREE SESSIONS PRIOR TO AND THE FIRST 30 MIN. PERIODS FOLLOWING THE ADMINISTRATION OF RESERPINE

	MEDIAN I.R.T.	ESCAPE LATENCIES	SHOCKS	BAR RESPONSES	BAR HOLDING
I.S.					
S1	1.07	1.05	1.41	0.85	1.67
S2	1.03	0.88	1.38	0.90	3.59
S4	0.72	0.90	1.61	0.93	2.90
Group $\bar{x}$	0.94	0.94	1.47	0.89	2.72
N.H.S.					
S3	0.39	0.87	0.86	0.87	3.73
S5	2.28	2.21	1.12	0.22	4.74
S6	1.01	0.85	0.89	0.89	0.33
Group $\bar{x}$	1.23	1.31	1.00	0.66	2.93
Overall p.	N.S.	N.S.	N.S.	0.01 (days)	N.S.

Physiological Measurements.

Following the final session the mice were killed by cervical separation and their adrenal glands and sympathetic ganglia dissected out. The cervical and thoracic ganglia revealed the normal result with the ganglia from the IS mice being very much smaller than the ganglia from the control NHS mice. Catecholamine assays were carried out on the adrenal glands by the methods reported in Chapter VI. The three IS and three NHS mice were matched with three IS and three NHS mice that had been injected at the same time but left undisturbed until killed for the adrenal assays. The average noradrenaline content of the IS mice that had received the six injections of micro amounts of reserpine was 31% of the noradrenaline content of adrenals taken from previously undisturbed control IS animals. The average noradrenaline content of the adrenals taken from the NHS mice that had received reserpine was 39% of the noradrenaline content of adrenals taken from previously undisturbed NHS animals.

DISCUSSION.

The nature of this experiment meant that relatively small numbers of mice would be in each group, but it was decided to

TABLE 11

MEAN RATIOS (1ST 30 MIN./2ND 30 MIN.) OF RESPONSE MEASURES RECORDED DURING FREE OPERANT AVOIDANCE FOLLOWING THE ADMINISTRATION OF RESERPINE

	MEDIAN I.R.T.	ESCAPE LATENCY	SHOCKS	BAR RESPONSES	BAR HOLDING
I.S.					
S1	2.21	5.89	0.72	0.64	0.61
S2	0.83	0.67	0.92	1.43	0.93
S4	1.21	1.43	0.90	1.31	0.92
Group $\bar{x}$	1.42	2.66	0.85	1.13	0.82
N.H.S.					
S3	1.06	1.45	1.26	1.28	0.90
S5	0.87	3.99	1.14	1.38	1.60
S6	1.17	1.24	1.45	1.49	0.81
Group $\bar{x}$	1.03	2.23	1.28	1.38	1.10
Overall p.	NS	NS	0.025 (treatment)	0.05 (days)	NS

running times over the ten massed trials. One feature of the alleyway running times was the apparently random variability

use small numbers and to subject the data to parametric statistical analyses in the hope that any finding would have a limited generality for further study.

The free operant avoidance task, in common with the three earlier studies, failed to produce any real evidence for the mediational hypothesis of affective states. In this experiment the IS mice had faster escape latencies to shock onset; moreover, they received a lower number of shocks, though not significantly so. This is contrary to Wenzel's finding reported in Chapter VII, although it might be held to be consistent with the hypothesis of increased skin impedance in IS mice causing a more painful shock. Clearly the question of whether a differential shock threshold exists between IS and normal mice requires examination. It might prove to be a confounding variable in studies using immunosympathectomized animals and electrical shock.

No significant differences were found between IS and NHS mice in running speeds over the inclined alleyway. In fact, the IS mice confounded one of the hoped for results by running faster than NHS mice. Neither did the IS show any slowing in running times over the ten massed trials. One feature of the alleyway running times was the apparently random variability

of all the mice. An animal recording a slow time on one trial might run very fast on the next, or equally, run another slow trial, etc. Visual inspection of the data showed no consistent relationship. The running times recorded by the Durham strain of mouse was a little slower than the speeds reported by Wenzel, 1968a. This might have been due to the higher shock level used by the author. The high shock level of 1 ma (Wenzel used 0.25 ma) was used to increase the aversiveness of the situation. Besides giving a slower overall time the higher shock level could have been responsible for some of the variability in the running times. Only a parametric study would reveal this. But the running times showed no tendency to a bimodal distribution that might indicate that when shock was received it always resulted in a much slower running time. The IS mice did tend to be slower over the second half of the alleyway which was consistent with the hypothesis that they would not be able to maintain such high levels of sustained effort as the NHS animals.

During the phase of the experiment when the alleyway trials or holding box retention was interposed between the two equal sessions of free operant avoidance it was found that the alley-way trials produced a facilitating effect when the mice were returned to the operant box. A significant increase in the

number of responses and a significant decrease in the IRTs was found for both groups following alleyway trials. The alleyway was not successful in disrupting the free operant avoidance behaviour of IS mice. In fact, the inclined alleyway appeared to facilitate any internal process that would aid the animal in the free operant avoidance situation.

Following the failure of the interpolated alleyway task to reveal any meaningful change, attempts were made to further reduce the store of adrenal noradrenaline by using a technique of injecting micro amounts of reserpine. Considerable evidence exists (Iversen, 1968) to suggest that there is a form of noradrenaline which is bound in a form so that it is not accessible to reserpine depletion. However, the injection would have acted to reduce large proportions of noradrenaline both in the adrenal and sympathetic nerves. Iversen indicates a percentage of 85% that is rapidly depleted following reserpine injections. Paradelis (personal communication) at the London Royal Free Hospital, indicated that a series of injections giving micro amounts of reserpine over a period of a week, or in some cases less, eventually produced a 'rebound effect', and large amounts of noradrenaline were then found in the animal's tissues. In view of this finding it was decided to inject twice a day and to only inject the reserpine for a limited period of three days.

In conjunction with the reserpine injections it was decided to subject the mice to a more stressful situation in the alleyway in the hope that it would produce additional depletion of noradrenaline.

The results obtained showed that the reserpine injections caused a drop in the bar responses over days and a decrease in number of shocks received. Despite the rather drastic nature of this combined assault upon the IS mice, it was still not possible to obtain clear-cut behavioural separation between IS and NHS mice.

CHAPTER X.

CONCLUSIONS.

The current assessment of experiments using the immunosympathectomy technique is very reminiscent of the position arrived at by the neurophysiologist Karl Lashley during the 1930's. Lashley had shown that very considerable damage could be made to large areas of the brain without any demonstrable qualitative behavioural differences being found between his insulted and normal animals. Similarly, in this series of experiments (supported by the work of Wenzel; although she might not agree) despite the large morphological reduction of the peripheral neural network of the sympathetic nervous system, it has not, as yet, been possible to demonstrate reliable and systematic differences in the acquisition of avoidance behaviour between IS and NHS rodents. What conclusions can be drawn at this point?

Mowrer, 1947, using behavioural studies, proposed an essential role in mediation for the sympathetic nervous system during the acquisition and maintenance of escape and avoidance behaviours. Cannon and his co-workers (1927 and 1929) using physiological studies, purported to have shown the non-essential character

of the thoracolumbar autonomic outflow in emotional rage and fear reactions. Cannon's statements were modified, if not ignored, by later workers who published findings claiming that feedback from the thoracolumbar autonomic outflow played a more important part in the elaboration of emotion than had been supposed by Cannon.

At the outset of this thesis it was argued that the failure of Cannon, and the limited success of the later primary behavioural studies (e.g. Wynne and Solomon, 1955) was due to the use of adult animals. By using adults, it was reasoned a greater probability existed for some form of vicarious functioning to be occurring. It has been suggested that nervous structures which initially are not sufficient by themselves to cause a reaction may come, by conditioning processes, to be sufficient in old animals. But clearly the experiments using young mice reported here, if not excluding, clearly reduce the possibilities of such influences. However, other possibilities exist and the failure to demonstrate unequivocal behavioural separation between IS and NHS animals might be due to one of the following:

1. The peripheral aspects of the sympathetic nervous system do not crucially aid the mediation of aversive situations. (This statement might imply that the brain has considerable autonomy for the mediation of emotion; or that another peripheral system, e.g. pituitary adrenal, is either of greater importance, or more crucial for the processes of emotion).

2. Rodents are sympathetically dominant animals and as such are able to tolerate the hypotrophy induced by the immunosympathectomy technique and still maintain high levels of sympathetic activity.

3. Supersensitivity of denervated tissue or hormonal compensation are serving to counter-balance the hypotrophic sympathetic nervous system found in immunosympathectomized animals.

4. A critical period, involving some initial neonatal stimulation of the sympathetic nervous system, is required for optimal functioning at a later date.

All of the above points require some discussion and before any overall conclusion is attempted it is proposed to enlarge upon the headings given above.

Complementary to the work on the sympathetic nervous system was the work stemming from Selye, 1950, on stress and the adaptation syndrome. Selye showed the importance of the pituitary-adrenal axis and later workers (Mason and Brady, 1956; Mason, Brady and Sidman, 1957; Levine and Soliday, 1960; Levine and Jones, 1965; Appley, 1964) have shown concomitant relationships between the axis and avoidance conditioning. Of the two inter-related and major outflows from the CNS the hormonal pituitary adrenal axis appears, from published work, to be more reliable for the unequivocal demonstration of emotional differences between normal and experimental animals. Why should this be so? Discussion of the two systems' physiological roles may well provide some indication for the reason for the reported relative differences that have been claimed when the systems have been manipulated in attempts to induce emotional differences.

The neural thoracolumbar outflow and the hormonal route from the pituitary (mediated by corticotropin ACTH) share the adrenal gland. Admittedly the adrenal has two separate parts,

the medulla serves as the major repository of the SNS transmitter substances, while the adrenal cortex metabolises important steroids that are released when the organism is subjected to stress states (stress is defined here as meaning any departure from the normal physiological limits) . The relationship between these two major systems are not qualitatively or quantitatively interchangeable but the peripheral nervous system occupies a central role in making adaptive responses and it would be difficult to describe a stressful situation resulting in increased steroid output from the adrenal cortex which would not simultaneously affect the adrenal medulla. Pohorecky and Rust, 1968, have discussed the biochemical interrelationships between the adrenal cortex and medulla.

In general, behavioural studies manipulating the pituitary-adrenal axis have revealed more positive results for an essential role in mediating emotional behaviour than studies manipulating the sympatho-adrenal complex. However, Levine and Soliday, 1962, have reported an effect on a CAR response following adrenal demedullation but the effects, to quote the authors; "depend upon subtle techniques to demonstrate them". Moyer and his colleagues in a series of papers (Moyer and Binnell, 1959; Moyer and Moshein, 1963; Moyer and Bean, 1964;

Moyer, 1966(a); Moyer, 1966(b) describing the effects of adrenalectomy and adrenal demedullation on avoidance responses in the rat, have concluded that the adrenal medulla plays no essential role in mediating emotional states. But there appears to have been no published attempt to analyse the roles of these two important systems and relate the physiological and the psychological findings.

Ramey and Goldstein, 1957, have reviewed the biological literature of the pituitary-adrenal axis and the sympatho-adrenal system. Much of what they have to say appears to be pertinant to behavioural studies that use interference of these systems as the independent variables for studying avoidance learning. Both sympathectomy and adrenalectomy narrow the range of an organism's adaptive responses; of the two surgical procedures, the latter produces the greatest physiological stress sensitivity. Adrenalectomized animals are required to be maintained in very carefully controlled environments. To quote from Ramey and Goldstein's paper; "Severe muscular work represents a fatal stress to the adrenalectomized animal while medullectomy and sympathectomy have been reported to be without effect on work performance". The authors further suggest that the data on sympathectomy when carefully analysed with respect

to work load and completeness of sympathectomy shows that a decided impairment of the work performance is found. Ingle and Baker, 1953, have reported a bioassay for cortical steroids based upon their quantitative ability to support work performance of adrenalectomized rats. Yet, psychologists have persistently manipulated this latter system under behavioural situations requiring active avoidance. A recent paper by Weiss, MeEwen, Silva and Kalkut, 1969, has contained a hypothesis concerning pituitary-adrenal influences on fear responding in the rat. They reported that their adrenalectomized animals had the slowest response times and argue that this was consistent with its normal function being excitatory; however, their results could also be explained by the reduction of the adaptive response range which arises from the adrenalectomy.

The main conclusions from Ramey and Goldstein's paper, from the behaviourists point of view is that some degree of muscular asthenia will be present in all studies that reduce the adrenals' hyperplastic reaction to stress. Sympathectomized animals appear to occupy a position somewhere between the normal and the adrenalectomized animal in terms of adequacy of adaptive responses and survival in a hostile environment. Stress at a level adequate to produce behavioural

separation between normal and those with pituitary-adrenal interference is unlikely to produce behavioural separation in sympathectomized animals. These latter animals would require a higher level of stress to show the same effect. The conclusions of Ramey and Goldstein are quoted in toto: "The extensive literature on the action of the cortical steroids and the epinephrines lends itself to the following generalizations. The adrenal cortical steroids and epinephrines appear to operate largely as a functional unit physiologically. The multiple sites of action and character of tissue and organ responses to the two species of hormones are strikingly similar. Many actions attributed to the steroids may be ascribed, in effect, to action of the epinephrines. Many actions of the epinephrines are not elicited in the absence of steroids. Corticoids and neurohumors are not interchangeable, however. Steroids maintain the integrity and responsiveness of tissues in the process of reacting to the epinephrines. This relationship is best seen on exposure to stress, when the defect of steroid lack may be elicited by heightened sympathetic-medullary activity. In the absence of the corticoids, responses to the neurohumors are progressively lost, while the destructive symptoms of adrenal insufficiency

are progressively exhibited" (author's underlining).

In summary, steroids maintain the integrity and responsiveness of tissues in the process of reacting to the epinephrines. Jenkins, 1968, has discussed the current role attributed to the steroids which are thought to act on cell regulators via genes in the cellular nuclei. Any final understanding of internal mediation of emotion will require integrated knowledge about both of these systems. Mason, 1968, in a collection of fifteen papers has pointed out the need for more integrated studies, the need to look at the "overall" hormonal balance for a complete understanding of emotion and endocrine organization. Teichner, 1968, has also discussed stress as an essential interaction between psychological and physiological aspects.

It is interesting to recall Wenzel's (1967 and 1968a) behavioural separation of IS and control mice using her swimming task. When spaced trials were used the mice needed to maintain their body temperatures by calorigenic action and this calorigenic requirement appears to have reduced the adaptive response of the animals sufficiently to have resulted in behavioural separation. The inclined alleyway experiment,

reported in Chapter IX, was a calculated attempt to reduce the range of adaptive response in IS mice. The attempt failed, presumably because the task was not sufficiently demanding. In fact, it appeared to aid the animals and the effect obtained was similar to Wenzel's use of massed trials in her swimming task. What the inclined alleyway and massed trials swimming task appear to have indicated, is a Yerkes-Dodson effect. The IS animals may be aided by a situation making limited demands. Clearly this problem requires further examination and it is proposed to carry out a series of experiments using the inclined alleyway in a cold chamber. The primary purpose of these experiments will be to attempt separation of the temperature regulation and mediation role of the sympathetic nervous system.

Adolph, 1967, in a paper examining postnatal cardiac control in rats has pointed out, as shown by Clark (1927), that large mammals tend to have adult heart rates that are tonically restrained, whereas small mammals have heart rates that are tonically prodded. This concept might suggest that rodents are heavily over invested with regard to their sympathetic nervous system and that the degree of sympathetic neural network reduction induced by the immunosympathectomy technique is not sufficient to reveal deficits. It will be

recalled that the Wynne and Solomon, 1954, experiments, generally quoted as evidence (see Rescorla and Solomon, 1967) in favour of the autonomic nervous system's mediation of affective states, ~~in~~ used dogs. These, according to the concept advanced by Adolph, being tonically restrained, might be expected to have a lower threshold than the smaller, tonically prodded mammals. Up to the present time attempts to find a small, tonically restrained mammal have not been successful.

A further confounding variable might be that the mice require to experience postnatally, a minimal level of sympathetic nervous system stimulation if the system is to function adequately in later life. This critical period of stimulation or function, if omitted, results in the animals revealing no essential deficits when adult. King, 1962, found that kittens sustaining bilateral amygdalectomy failed to show the behavioural changes seen in adult animals subjected to a similar lesioning procedure. Similarly, Isaacson, Nannerman and Schmaltz, 1968, writing of the behavioural and anatomical ~~sequence~~ sequelae following damage to the infant limbic system in, again, cats, have reported that damage to the limbic system before a critical period resulted in the adult animals failing to show behavioural deficits. The suggestion from both of these papers

is that a certain level of neuroendocrine function or maturation may be necessary for the effects of certain changes to be seen at a later date. Although both these papers concern a specific central nervous system area and were recorded for a different species to those reported here, this type of concept should not be overlooked in techniques using neonatal immunological techniques. No attempt, as yet, has been made to test for the possibilities of a critical period. Once the initial hyperplasic growth period of the sympathetic nervous system is completed, it has been reported that it is difficult to produce any substantial sympathetic nervous system reduction using the immunosympathectomy technique (Levi-Montalcini and Booker, 1960).

Within the last two years, two excellent reviews discussing the theory and methodology of the two factor theory of avoidance-learning have been published by Rescorla and Solomon, 1967, and Herrnstein, 1969. The former, although basically favourably disposed toward the continuing value of the theory, seems to contain more telling arguments against the theory than the latter review which is distinctly unfavourable. Herrnstein argues that the contortions invoked in two-factor theory by current experimental work have imperceptibly pushed the theory over the line into irrefutability. For him, reinforcement of

avoidance behaviour is a reduction in time of aversive stimulation. Herrnstein's main argument rests on a very ingeneous experiment (Herrnstein and Hineline, 1966) in which the authors were able to show that shock frequency reduction could serve as negative reinforcement. Their technique involves a free operant avoidance lever situation with the control of shock frequency via a double channel tape. The two channels differed only in the probability of shock at any point in time. Channel A, giving a higher shock frequency, remained in control until a lever press which switched control of shock delivery to Channel B, giving a lower shock frequency. Channel B remained in operation until delivery of the next shock when Channel A assumed control again. A further lever press switched shock control into Channel B, etc.

Thus, by responding the animal could remain in the low probability shock channel. Herrnstein and Hineline found that the response rates were directly related to the amount by which the response reduced shock frequency, and the authors concluded that their procedure showed that avoidance conditioning can occur without the benefit of either classical or exteroceptive stimuli, as shown by Sidman, 1953, or covert stimuli inferred from the temporal constancies of the procedure, as argued by Anger, 1963. Herrnstein points out that there is within the

free operant situation, sufficient latitude to invoke a two-factor mediational hypothesis; but that such an argument involves stretching the hypothesis beyond the bounds of credulity. He does indeed highlight interesting difficulties in his review paper; but omits to inform us how he came to get his animals to respond on what would be, in the author's experience, a very difficult schedule for rats to acquire. The answer, found in the first paper, is that Herrnstein first trained the animals on a free operant avoidance schedule with a variable R-S cycle and then transferred them to his random shock procedure. Thus, his animals would have developed a temporal discrimination of reduced shock following lever pressing and it might be argued that all Herrnstein showed was that this "press-lever-to-avoid-shock" discrimination is much finer than earlier workers have previously supposed.

From work in this laboratory and from conversations with other workers using the free operant technique, it is felt that the response profile exhibited by any particular animal is acquired in the very early stages of an animal being placed on a free operant avoidance schedule. Also, it has been shown that the response profiles once acquired are stable over many months. The author, while tending to agree with Herrnstein

about the reduced value of the two factor theory, is not so sure that Herrnstein's main example is a particularly good one.

Rescorla and Solomon have written; "two factor process theory has persisted as a systematic influence since 1928, and interest in it has increased rather than decreased. Lacking the elegance and simplicity of a postulated, single learning process, and the parsimony of a single reinforcement principle, as well as the proselytizing influence of a vigorous 'school of thought' nevertheless it has been a major heuristic tool in the stimulation of the new conditioning and training experiments". One of the major points that can be made against the two factor theory is that it has tended to mould experimental design and theoretical arguments.

The two factor theory eliminated the need to attribute foresight to animals by arguing that avoidance of an aversive situation was due to the linking of the subsequent avoidance behaviour to the original escape behaviour. Psychologists, ironically, allowed their experimental animals an automatic, subjective, affective state but denied them any objective appreciation of the avoidance situation. We have perhaps paid a high price for making our avoidance techniques and theories

conform to the dogma of "publically observable". Keehn, during a symposium, held at Sussex University in 1967, pointed out this problem; but, alas, dropped discussion of it for his chapter in the book (Gilbert and Sutherland, 1969) that subsequently appeared concerning papers read at the symposium. As Herrnstein, 1969, and Mowrer, 1960, have shown, to return to a single factor theory of avoidance will not be easy. Mowrer's return to a single factor theory which although it allows for problems such as expectancy, appears to complicate rather than simplify the explanation of avoidance learning.

Rescorla and Solomon, and Weisskrantz, 1968, have pointed out that operant behaviour is mediated by a complex of CRs, both autonomic and skeletal, no one of which may be necessary per se for the operant behaviour but each contributes to that behaviour. Both of these reviews point out that CRs may not themselves be mediators of instrumental behaviour, but rather indices of a central nervous system state which does mediate the behaviour. As Lacey, 1956, has demonstrated, the autonomic nervous system is ubiquitous and this makes the 'sufficient and necessary' aspects difficult to untangle.

Since Mowrer's paper in 1947, psychologists have concluded that affective or emotional states were always brought about

by aversive stimulation, yet clearly this is not always so. Not infrequently when attempting to manipulate emotion by the use of aversive situations, experimenters encounter animals who contradict the normally acceptable principles. The author, for example, has run two rats who were littermates on a free, operant avoidance schedule for a period of twelve months. One of the animals when trained received an average of fifteen shocks during the daily 60 minute session, while the other animal received an average of 185 shocks. The animals alternated daily between the two operant chambers and lived together in the homecage. They were comparable in final body weight and gain in body weight. Apart from squealing during the shock onset, no ill effects were shown by the animal receiving the high level of shock. No generalized fear to the apparatus was shown by the rat receiving the 185 shocks per hour and the animal readily left the experimenter's hand to enter the chamber. It was difficult to see why the constantly shocked animal should not be fearful of the situation. Similarly, his littermate did not appear to maintain his higher response rates because of fear. The different response profiles seen, appear to have arisen from strategies adopted during the very early stages of acquisition of the avoidance behaviour. The infre-

quently shocked rat was held to be successful, while the constantly shocked rat was held to be unsuccessful because his stragegy resulted in a behaviour pattern which produced a large number of shocks. The success, or lack of success, was defined by the criterion set by the experimenter and not the animals. Eventually a drastic alteration of the stimulus conditions (the house light failed in one of the boxes) caused the unsuccessful animal to alter his response pattern and in consequence his shockrate dropped to 50 - 60 per hour.

If, as I have attempted to suggest above, the avoidance procedures used at present are unreliable how are we to isolate emotion in laboratory animals? One method is by the communication of affective states but this technique involves several problems. Mirsky, Miller and Murphy, 1958, reported a method in which a rhesus monkey was placed in an operant chamber and taught to avoid shock by lever pressing. Following several days of extinction trials, the monkeys were given rest days before again being placed into the chamber. After determining that the monkeys did not lever press, they were shown, through a one way screen (according to the experimental report, sound and smell were excluded) another monkey receiving a shock. The effect of seeing this second monkey receiving shock was

to restore bar-pressing behaviour in the first monkey. Mirsky, Miller and Murphy concluded that their experiment demonstrated communication of an affective state; but it might be argued that it merely revived an extinguished conditional response. There need not have been fear on the part of the observing animal.

A more obvious example of a confounding variable may be observed in the Welch and Welch, 1968, experiment reported in Chapter VI. Welch and Welch concluded that their observer mouse, made to observe fighting mice beneath its cage, was subjected to intense sociopsychological stimuli which was revealed by the lowered noradrenaline levels found in the experimental animal's brainstems. In the Welch and Welch study the failure to eliminate olfactory cues might well have introduced a confounding variable. Bowers and Alexander, 1967, have shown that smell is an important dimension in mice and Bruce and Parrott, 1960, have reported that pregnancy blocking occurs in female mice subjected to the smell of a strange male mouse. Gleason and Reyniers, 1969, have recently reviewed the increasing evidence for the behavioural significance of pheromones in vertebrates. Clearly, the Welch and Welch experiment should be repeated with careful controls to see if

a purely psychological affective state can be isolated in mice. As indicated in Chapter VI, the attempts to induce fear by using natural predators was a failure and clearly the triggering of emotion is a very complicated problem. This point raises the query that perhaps in the studies reported here the shock levels used were too high. It is argued that this does not appear to be the case. Wenzel who has used much lower shock levels has failed to reveal behavioural differences. Also, the attempt to use a more natural stressor failed and usually the higher shock levels tended to slightly increase behavioural separation. The combination of high intensity light and sound in the open-field situation produced differences between IS and NHS mice which have not been reported by workers failing to use these additional stressors in the open-field situation. At present no additional comments can be added to the experiment reported in Chapter III except to say that the differences were very clear cut.

Following the observation that unpredictable shock coupled with the novelty of the passive field appeared to invoke "emotion", as defined by catecholamine metabolism, a search of the literature was made and several earlier publications were found reporting this type of phenomenon. Mason, Mangan, Brady, Conrad and McKRioch, 1961, reported marked

elevations of the plasma catecholamines occurred following "free or undeserved shocks". Sawrey and Sawrey, 1963, have also claimed that the incidence of gastric ulceration in rats is increased if the unpredictability of shock is increased. Caul and Miller, 1968, reporting on the effects of shock probability on heart rate of rats during classical conditioning, concluded that intertrial bradycardia was a function of the uncertainty or ambiguity of the situation.

Richter, 1957, reported a peculiar finding that rats deprived of their vibrissae drown very rapidly in a situation where they were required to swim continuously in order to survive. Normal rats were able to sustain their swimming for periods longer than one day. Richter's experimental situation placed the rats in a frightening situation and on top of this they were deprived of an important tactile sense. The combination of the fearful situation and the tactile deprivation produced a high level of unpredictability which resulted in the animals dying very rapidly. Thus, evidence is accumulating that the traditional methods of analyzing affective states require modification and that there is a need for newer and more refined techniques. Future experiments on emotion should be more concerned with confronting the animal with unpredictable

situations, perhaps using both reward and punishment in an unpredictable manner.

Implicit in any discussion of emotion has often appeared the idea that increased peripheral feedback resulted in increased emotionality being experienced. Clearly the experiments reported in this thesis do not support a peripheral activation viewpoint. One ever constant factor when using the technique of immunosympathectomy is the group of minor, overt signs that arise from animals with such massive morphological damage to their sympathetic nervous systems. Initially, it was hoped that a graded reduction in the sympathetic nervous system would result in a similarly graded and correlated behavioural response. However, this result was never achieved unequivocally, but it is interesting to note that significant correlations between the level of hypotrophy and a behavioural response were never obtained after the two-fold, concentrated antiserum injections became the standard technique.

In general, the peripheral feedback theory was not supported and it is interesting to note the results obtained by Taub and his collaborators in a series of experiments using monkeys with totally deafferented limbs. Mott and Sherrington, 1895, found that animals displayed no purposive movement in a limb deprived of all sensory nerves. But Taub and Berman, using a technique

which restrains the monkeys' normal limb, (Knapp, Taub and Berman, 1963; Taub and Berman, 1963; Taub, Bacon and Berman, 1965; Taub, Ellman and Berman, 1966; Taub, Teodorn, Ellman, Bloom and Berman, 1966; Taub and Berman, 1967; Taub and Berman, 1968), have produced experimental evidence that by using a trace conditioning avoidance procedure, monkeys were able to produce a CR that involved a forelimb flexion in a totally deafferented limb. Moreover, the monkeys were able to make the correct response even if they were unable to see their deafferented limb. Taub and Berman (in press) have argued that their results call into doubt stimulus termination theories of avoidance conditioning, or alternatively, theories which attribute the acquisition and maintenance of avoidance behaviour to the termination of secondary negative stimuli that are proprioceptive, interoceptive or exteroceptive in nature.

Weiskrantz, 1968, has written; "mammals have central nervous systems, which can alter without any effects necessarily being reflected in the peripheral nervous system". Perhaps, at a future time, we might need (as Taub has suggested) to extend this idea to include the notion of the brain being autonomous, and although peripheral feedback might facilitate the central nervous system's appreciation of the external world, peripheral feedback is by no means an essential part of the brain's decision-

making role. The peripheral nervous system's importance comes from its executive role in making interpretation of the central nervous system's decisions.

The final question is, what can we conclude from the results collected from immunosympathectomized animals up to the present time? Where shall we look to enable us to pick up an end of the entangled skein that confronts us in the study of emotion? One of the problems facing the psychologist is that, unlike other biological scientists, he has no clearcut in vitro and in vivo tests. Because of the very nature of their discipline they are unable to extract and ~~clearly~~ isolate the systems that they are studying. To do so would destroy that which they were seeking to understand. Yet clearly many published experiments by psychologists have more in common with the in vitro test used by the biological scientist. From the results reported from many experiments, one can doubt if a similar result would have been obtained with the animal in a free behavioural situation. For example, reports are made of experiments using transmitter substances that exceed by many-fold the normal physiological ranges; even when the doses are near to the physiological limits, the time span is usually considerably contracted. At an earlier point, the traditional

experimental methods used when studying emotion were questioned and these doubts are supported by other workers investigating the elusive phenomenon of emotion. McCleary, 1966, studying the limbic system and avoidance behaviour, has reported that cats are unable to acquire an active avoidance task in a shuttle box when they are required to shuttle back and forth. If, however, they are required, by arrangement of the boxes, to always run forward in one direction, they apparently experience no difficulty in learning successful avoidance behaviour.

One area not examined in detail at present, is the brain. There is a case to be made for considering the hypothalamus as the "terminal ganglia" of the sympathetic nervous system. Gellhorn, 1965, has produced a theory in which he attributes a crucial role for the hypothalamus in emotion, and clearly this area should be investigated both in normal and immuno-sympathectomized animals subjected to stress. The technique reported in this thesis has been to reduce the efferent outflow of the sympathetic nervous system at the peripheral level and then to look for differences. Ward and Hester, 1969, have examined intracranial self stimulation rates of cats surgically deprived of autonomic outflows. They were unable to demonstrate any reliable differences following bilateral sympathectomy; but

clearly the method should be applied to immunosympathectomized animals.

Stein, 1969, and Wise and Stein, 1969, have reported experiments in which they have examined the central nervous system's transmitter substances. The authors have claimed evidence indicating separate reward and punishment systems in the midbrain areas. The techniques used by Stein are very interesting and his theoretical position is similar to the attempt made by Bindra, 1969, who has made theoretical and experimental attempts to demonstrate a single brain state for emotion and motivation. Again, the combination of the above author's technique combined with immunosympathectomy could prove to be very valuable in future studies.

There is now a considerable literature on the function of brain monoamines. Vogt, 1959 and 1962, has written two reviews which examine the pharmacological activity of the brain monoamines. Schildkraut, 1965, has discussed the evidence for the catecholamine hypothesis of affective states. The hypothesis links affective states with midbrain catecholamine levels. As stated in Chapter VI, most of the evidence up to the present time concerned humans; but given a behavioural situation that reliably evokes emotion in animals, it would clearly be profitable

to examine the catecholamine hypothesis in animals.

Apart from brain tissue assays, in future studies it is proposed to use a technique reported by Corrodi and Jonsson, 1966, and make histochemical studies of animals exposed to stress situations. This technique will involve stopping neurochemical metabolism immediately following the presentation of an emotional or an avoidance situation. Fuxe and Hanson, 1967, have already reported histochemical difference in mid-brain areas of animals exposed to behavioural avoidance situations.

One technique it is hoped to use to examine the central nervous system and peripheral influences in the body will be to produce unilateral splanchnic neurectomy. This technique will allow, in a single animal, central and peripheral influences to be assessed and it may prove possible to examine more precisely the role of the adrenals in animals exposed to emotional situations.

Although the technique of immunosympathectomy has not produced the originally predicted results, the technique has great potential and in combination with other appropriate techniques should help to further our understanding of emotional behaviour.

APPENDIX.

PREPARATION OF NERVE GROWTH FACTOR.

The original method used to isolate and purify NGF was the one published by Cohen, 1959 (snake venom), and 1960 (mouse salivary glands). In common with other workers the author found that he was unable to obtain very high yields of the active protein using Cohen's method and in 1965 changed to the method used by Edwards and Fenton, Wellcome Research Laboratories, Beckenham, Kent.

PREPARATION OF N.G.F. PROCEDURE.
AFTER EDWARDS AND FENTON, 1966.

Stage.	
(1) Homogenisation.	50 gm (wet weight) of male mouse salivary glands-(homogenised while still frozen) in distilled water + ice-cubes for 3 minutes in Waring blender to give total volume of 200 ml. of homogenate. Homogenate strained through coarse gauze and residue re-homogenised with 50 ml. of (distilled water + ice). Second homogenate strained and pooled with 1st homogenate. Residue (1) discarded.
(2) $(NH_4)_2SO_4$ fractionation.	30 gm $(NH_4)_2SO_4$ added slowly with stirring to each 100 ml. of suspension. 0.25 N NaOH added dropwise at same time to maintain pH between 7.0 - 7.1. Container set aside at + 2°C for about 1 hour to allow suspension to flocculate, then suspension filtered by gravity through Postlip paper in cold room. Clear red filtrate collected overnight.
	28 gms $(NH_4)_2SO_4$ added slowly with stirring to each 100 ml. of clear

		filtrate, with stirring and drop-wise addition of 0.25 N NaOH to maintain between pH 7.0 - 7.1. Collect red ppt. by filtration on vac using a 9 cm. diameter Buchner funnel with double layer of Whatman No. 1 filter paper. Add 0.5 gm. Supercel Hyflo to suspension and prepare a pre-coat on papers with 0.5 gm Hyflo suspended in ammonium sulphate solution, (58 gms. ammonium sulphate + 100 ml. water) - before filtration. Discard clear filtrate.
(3)	Dialysis and removal of toxic fraction.	Suspend ppt. + hyflo in a small volume of distilled water, stir thoroughly and centrifuge to sediment the hyflo. Decant the clear red super-natant into Visking dialysis tubing. Wash hyflo ppt. on centrifuge with several small volumes of water adding supernatants to dialyser. Dialyse against several changes of distilled water in cold room, preferably with stirring, until heavy ppt. has formed in dialyser. Centrifuge to remove (toxic) ppt. and retain supernatant.
(4)	Removal of Haemoglobin.	Prepare a small (10 x 2 cm) column of SE Sephadex C.50 and equilibrate with 0.02 M phosphate buffer, pH 6.6 (see notes). Pass the red supernatant from previous stage through the column and collect the yellow effluent. Wash the column with 2 or 3 column volumes of phosphate buffer, 0.02 M pH 6.6 and add to main effluent. All haemoglobin is retained on the column (for regener-ation of SE Sephadex - see notes).
(5)	Concentration of NGF for gel filtration.	Add 1 N-NaOH to pooled SE Sephadex effluents to pH 8, measure volume of solution and add 70 gms $(NH_4)_2SO_4$ to

		each 100 ml. - with mechanical stirring in cold room. Collect ppt. by filtration on vac, in cold room, using a 9 cm Buchner funnel and double layer of Whatman No. 1 paper. Obtain a brilliantly clean filtrate by re-cycling then discard. Suck the filter cake as dry as possible. Weigh the semi-dry filter cake and dissolve each 10 gms in 40 ml. of 0.02M phosphate buffer, pH 8 (see notes). Centrifuge and wash the small (dark brown) ppt. with 10 ml. of buffer. Collect all supernatants, adjust total volume to 60 ml. with buffer and divide into 30 ml. aliquots. Store frozen for next step.
(6)	Gel-filtration on Sephadex G.75 to separate nucleoproteins, mucleic acids, etc.	Prepare a column (75 x 2.8 cm) of Sephadex G.75, bead form, washed to equilibrium with 0.02 M phosphate buffer pH 8 - in cold room. Layer a 30 ml. load of solution from previous stage on to the column and follow with phosphate buffer. The first fraction excluded is coloured and contains N.G.F., E.G.F. and is still toxic. The second fraction is rich in nucleoproteins but contains negligible N.G.F. activity. When all protein has been excluded, the column is washed with ammonium sulphate and is re-used without re-packing. Up to 8 complete cycles can be run before the column flow rate becomes unduly slow.
(7)	Salt transfer and ion-exchange chromatography (DEAE cellulose)	Dialyse the 1st fraction from G 75 to equilibrium (in cold room) against 0.01M acetate buffer pH 5.9 (see notes). Prepare a DEAE cellulose and equilibrate with buffer (see notes). Run the dialysed fraction through the column at a slow rate, N.G.F. is adsorbed and the first fraction eluted with 0.01 M acetate pH 5.9

contains N.G.F. and is also capable of producing stunting effects in new-born mice. N.G.F. is eluted from the column either (a) by desorption with 0.1 M NaCl, or (b) by application of a linear gradient of NaCl on 0.01 M acetate pH 5.9 and collecting fractions between 0.05 and 0.15 M NaCl. Gradient elution is still experimental but N.G.F. produced by desorption with 0.1 M NaCl has been shown to be non-toxic and although of high specific activity, still impure.

Stages.	Working conditions.
(1)	Room temperature - ice-cold solutions.
(2)	Mainly in cold room. Vacuum filtration can be done at room temperature.
(3)	Cold room.
(4)	Room temperature.
(5) (6) (7)	Cold room.

NOTES.

(2) Residues on filter paper are re-extracted with 0.4 sat^{d} $(NH_4)_2SO_4$ pH 7 and re-worked.

(4) 0.02 M phosphate buffer, pH 6.6 = 1:50 dilution of stock solution. Stock solution = 3.5 gms. KH_2PO_4 + 56 gms. $Na_2HPO_4 2H_2O$ to 1 litre, preserved with $CHCl_3$.

S.E. Sephadex C 50.

Initial treatment.

(1) Swell for 23 hours in water.
(2) Add HCl to 0.5 N - stand $\frac{1}{2}$ hour.
(3) Wash with distilled water by suspension, decantation and filtration.
(4) Suspend in 0.5N - NaOH for $\frac{1}{2}$ hour.
(5) Repeat (3) - wash thoroughly.
(6) Suspend in 0.02 M Phosphate, pH 6.6.

(7) Repeat (3) but use 0.02 M phosphate, pH 6.6.
(8) Pack column and wash with 0.02 M phosphate, pH 6.6.

Subsequent treatment after use.

(a) Elute haemoglobin from column with 0.2 M NaCl.
(b) Unpack column and suspend Sephadex in 0.2 M NaCl.
(c) Wash by decantation and filtration, using distilled water.
(d) Carry out steps 6, 7 and 8 as above.

(5) 0.02 M phosphate buffer pH 8 = 1:25 dilution of stock solution. Stock solution = 3.25 gms. KH_2PO_4 + 83.6 gms Na_2HPO_4 $2H_2O$ to 1 litre - preserved with CHl_3.

(7) 0.01 M acetate buffer pH 5.9 = 32.5 ml. solution (A) + 467.5 ml. solution. (B) diluted to 10 litres with distilled water.

Stock solutions.

(A) = 0.2 M Acetic acid = 11.6 ml. glacial acetic acid to 1 litre.
(B) = 0.2 M Sodium acetate = 27.2 gms. CH_3 COO Na. $3H_2O$ to 1 litre.

Fresh DEAE cellulose is treated initially with dilute HCl, 0.05 M, then washed thoroughly with distilled water by decantation and filtration, followed by treatment with 0.02 M NaOH and thorough washing in similar manner.

The washed cellulose is then suspended in 0.2 M acetate buffer, pH 5.9 (i.e. 32.5 ml. solution A + 467.5 ml. solution B) and the pH of the suspension checked carefully (glass electrode). If necessary, add solutions A or B in excess until the pH remains constant for at least $\frac{1}{2}$ hour.

A column, (55 x 1.5 cm) is packed with the cellulose, then washed to equilibrium with 0.01 M acetate buffer, pH 5.9 for

loading. This size of column has been used for production of N.G.F. by desorption with 0.1 M NaCl. Experiments with gradient elution are in progress using a smaller column (26 x 1.25 cm) and DEAE cellulose prepared in the same way.

Varon, Nomura and Shooter, 1967(a) and 1967(b) have reported a very reliable method which is currently being used by the Wellcome Research Laboratories team at Beckenham to prepare NGF antiserum. Schenkein, Levy, Bueker and Tokarsky, 1968, have also reported a new method claiming high yields and very specific activity. However, the author has no experience or detailed knowledge of their method.

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Hanging drop tissue culture technique used for the bioassaying of NGF.

The original assay technique was reported by Cohen, Levi-Montalcini and Hamberger, 1954, and Cohen, 1959. For general details about tissue culture techniques the author consulted Paul, 1961, and for details about the development of chick embryos, Hamilton and Hamberger, 1952.

The final method used to assay NGF.

Fertile eggs were incubated for 9 days (Lillie's stage 35) and the dorsal root ganglia were sterilely dissected out under a low powered stereo microscope. The ganglia were freed of their surrounding capsules and any attached nerve. If the ganglia were not cleaned it resulted in a failure to obtain the dense halo effect of the fibres seen in Fig. 1. When sufficient ganglia had been collected for an assay, Conway plates (these contain a central concavity) were set up as follows:

1 drop of 0.7% saline containing a serial dilation of NGF.
1 drop of Eagle's medium.
3 to 5 ganglia.
1 drop of unclotted Fowl plasma.

(The drops were measured from 100 microlitre disposable Microcap pipettes).

The plates were then quickly mixed by a swirling action and allowed to clot. After clotting the plates were inverted and placed into a biological incubator 37°C and read at 6 hourly intervals. It was found necessary to add a very small amount of thrombin to the 100 ml stock bottle of Eagle's medium in order to get the fowl plasma to reliably clot quickly.

All plates and instruments used were sterilized and the eggs swabbed with alcohol prior to cracking and removal of the embryos. Sterile precautions were taken at all times during the dissection procedure. Small dissecting instruments were made by hammering flat the end of a piece of nichrome wire and setting it into a glass handle; these instruments had the advantage that they could be sterilized by heating in a bunsen flame.

Fibre outgrowth was recorded on a 0 to +3 scale. The final plate reading technique was to switch the incubator on automatically at 3 a.m. and to read the plates at hourly intervals from 9 a.m. onwards. The plate showing the initial fibre growth was taken as the one giving the maximum response. The biological activity has been defined as the µg of protein/ml required to produce a +3 growth response from the spinal dorsal root ganglia.

Unclotted fowl plasma.

Blood was drawn gently into an iced 5 ml hypodermic syringe from the brachial vein of a young fowl. The blood was then transferred into an iced, plastic (non wetting) centrifuge tube and the blood cells spun down slowly for approximately 5 minutes. At this point a check was made to see that the obtained fluid was plasma, and not serum, by inducing, in a small drop, clotting by the use of thrombin. The plasma was then placed into small waxed Lambert tubes, capped and stored in the -15°C freezing compartment of a refrigerator until used. Each Lambert tube was filled with sufficient plasma to enable one complete assay to be carried out. During the whole procedure it was found necessary to work at low temperatures. Great care was taken to exclude the introduction of froth from

the hypodermic syringe into the centrifuge tube. Failure to exclude froth completely invariably resulted in the plasma clotting while the blood cells were being spun down.

Preparation of NGF antiserum.

A modification of the technique reported by Cohen, 1960, was used to prepare NGF antiserum. Two rabbits were injected into their footpads with purified NGF together with Freud's adjuvent. Injection levels were similar or lower than those reported by Cohen. Blood samples were taken from the rabbit's ears and the titre of the serum determined by assaying it with serial dilutions of a known NGF standard supplied by the Wellcome Research Laboratories; NGF 15/0.3, 5,000 units/mg. As indicated in Chapter II titres of 3,000 anti-units was obtained. Production by the Wellcome Research Laboratories of NGF antiserum from horses ended the need for the author to continue preparation of NGF antiserum.

Publish references refering to the IS technique and not given elsewhere in this thesis.

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Trihydroxyindole methods of catecholamine estimation using potassium ferricyanide as the oxidant.

The method reported below was shown to the author during a one week visit to the Department of Pharmacology, Royal Free Hospital, June, 1968. My warm thanks are due to Professor Eleanor Zaimis and her staff for demonstrating the catecholamine estimation method that they were using. The basic principles of fluorescence assay of catecholamines were gained from Udenfried, 1962. Crout, 1961, contains a very lucid account of the trihydroxyindole differential pH method together with a worked example. Other useful references were; von Euler, 1959, and Häggendal, 1966.

Adrenal Assay.

Dissected adrenals were stored $^{O}/N$ at $-15^{\circ}C$ and the following day ground up in an iced Potter's glass homogenizer with 4 mls of iced 0.01N hydrochloric acid. The homogenate was accurately made up to 25 ml in a volumetric flask using the dilute acid, and stored in a refrigerator until the actual assay procedure was carried out later in that day. Standard solutions of adrenaline and noradrenaline, containing 0.1 µgm/ml in 0.01N hydrochloric acid, were made from stock solutions. The stock solutions of adrenaline bitartrate or noradrenaline bitartrate, containing 1 mg/ml in 0.1N hydrochloric acid, were made up fresh each month. During the month the stock solutions were stored

at 2°C and used for making up standard solutions when required.

The assay technique used was a trihydroxyindole, differential pH reaction with potassium ferricyanide as the oxidant. This technique uses the fact that both monoamines are oxidised to the aminochrome stage at pH 6.0 whereas only adrenaline undergoes any significant oxidation at pH 3.5. Prior to the assay the diluted homogenates were shaken and spun down for 5 minutes. Two mls of the supernatants were pipetted into each of two tubes, one containing 2 mls of acetate buffer pH 3.5, the other containing 2 mls of acetate buffer pH 6.0.

The reading tubes contained the following solutions:

pH 3.5.	pH 6.0.
2 mls sample	2 mls sample
2 mls pH 3.5 buffer	2 mls pH 6.0 buffer
0.1 ml 0.5% zinc sulphate	-
0.1 ml 0.25% potassium ferricyanide	0.1 ml 0.25% potassium ferricyanide
leave 3 minutes.	
2 mls alkaline ascorbic acid	2 mls alkaline ascorbic acid
read after 12 minutes.	

After the addition of each solution the tubes were mixed using a mechanical shaker. The standard solutions of adrenaline and noradrenaline were also set up at pH 3.5 and pH 6.0. The 'blank' tube contained 2 mls of the 0.01N hydrochloric acid, used to dilute the homogenate and the 0.25% potassium ferricyanide was added after addition of the alkaline ascorbic acid. The relative fluorescence obtained from the samples was read in E.I.L. fluorimeter (model 27c).

Tissue Assay.

The tissues other than the adrenals were only assayed for the presence of noradrenaline and used a trihydroxyindole reaction at pH 6.5(levels of adrenaline in tissues other than the adrenals is neglible). After dissection the tissues were bulked

into 4 - 5 organs of similar treatment, etc., and stored at -15°C until required. The tissues were ground up using a pestle and mortar with a little sand added. After grinding the tissue and sand was then mixed and transferred into a small centrifuge tube using a total of 9 ml of 0.4N perchloric acid in three washings. The tubes were then sealed and stored at -15°C O/N. The following day the tubes were thawed, mixed and centrifuged for 5 minutes. The supernatants were decanted into a large clean centrifuge tube and the pellets resuspended with 1 ml of 0.4N perchloric acid. Following a further centrifuging for 5 minutes the washings were added to the original supernatant (during this and the following process the tubes were immersed in ice). The pH of the supernatants were then adjusted to pH 6.0 using a pH meter, a magnetic stirrer and acid and alkaline solutions (solutions of 4N and 0.4N potassium hydroxide and 1N and 0.1N hydrochloric acid were used to make the pH adjustment). The pH adjusted solutions were then left to stand for half an hour before being centrifuged for 5 minutes, to remove the potassium perchlorate precipitate that had formed. The supernatants were then run onto ZEO-KARB resin columns. The resin columns were washed with 20 mls of iced distilled water and finally the noradrenaline was eluted using 10 mls of iced 1N hydrochloric acid. The eluates were collected in small bottles, capped and stored at 2°C until all the samples had been collected. Two 10 ml volumes of noradrenaline containing 1 µg/ml in 0.01N hydrochloric acid were also run through the columns to serve as internal standards.

The glass columns consisted of a stem, narrowed at the bottom, 13.5 cm long having an internal diameter of 0.6 cm attached to an upper glass reservoir which was 9 cm high and had an internal diameter of 3.1 cm. The resin columns were prepared by placing a plug of glass wool at the bottom of the stem and placing a resin column of approximately 2.5 cm on top of the plug. The assay tubes were prepared as follows:

1.5 ml sample
1.5 ml pH 9.9 buffer
) pH of mixture to be 6.5; this was carefully checked.

0.1 ml 0.25% potassium ferricyanide

leave 3 minutes.

2.5 ml alkaline ascorbic acid

read after 25 minutes.

After the addition of each solution the tubes were mixed using a mechanical shaker. The 'blank' tube contained 1.5 mls of the 0.1 N hydrochloric acid, and the 0.25% potassium ferricyanide was added after the addition of the alkaline ascorbic acid. The Electronic Instruments Limited, model 27C Direct Reading Fluorimeter uses a mercury vapour lamp as a light source and limited spectral adaptation is provided by using colour filters with fixed wave lengths. As pointed out by Udenfriend, 1962, this system prevents accurate determination of all tissues and blood catecholamines with the exception of the adrenals, which contain relatively large amounts of adrenaline and noradrenaline.

Solutions for Catecholamine assay.

Sand:

The sand was washed several times with deionized water and finally with a 1% solution of ethylenediaminetetra-acetate (EDTA).

Preparation for the Resin for the Tissue Assay.

Approximately 200 g of resin (Zeo-karb 225 (SRD16): 8% cross linked over 200 mesh) were suspended in deionised water and the cloudy supernant was decanted after approximately 10 minutes. This decantation process was carried out until the supernant remained clear after 10 minutes. The resin was then transferred to a large glass column fitted with a sintered glass filter disc and a stop-cock.

The column was washed with the following solutions:

(a) 2.5 litres 2N sodium hydroxide containing 1% EDTA.
(b) 1 litre deionised water (to remove the alkali).
(c) 2.5 litres 2N hydrochloric acid.
(d) 1 litre deionised water (to remove the acid).
(e) 4-5 litres of 0.1M phosphate buffer (pH6.5) containing 0.1% EDTA.

The phosphate solution was run through until the pH of the column effluent was 6.5 (the resin could be seen in the golden-yellow form). The resin was removed from the column, washed several times with deionised water and stored ready for use.

Alkaline ascorbic acid solution.

This solution consisted of 1 ml of a 2% $^{W}/_{V}$ ascorbic acid and 9 mls of 5N sodium hydroxide to which 0.2 ml of a 97%

ethylenediamine (EDA) solution was added. The alkaline ascorbic acid solution is oxidised very quickly and it is important to make up the volume required immediately prior to using it.

Acetate buffers for adrenal assay.

Acetate buffer pH 3.5: to 47 mls of 1M acetic acid was added 3 mls of 1M sodium acetate ($CH_3COONa \cdot 3H_2O$; mol. wht. 136.08).

Acetate buffer pH 6.0: to 47 volumes of 1M sodium acetate (hydrated, see above) was added 3 volumes of 1M acetic acid.

pH 9.75 - 9.90 buffer for tissue assays.

To 205 mls of 2M acetic acid pH 5.0 was added 45 mls of 5M potassium carbonate (anhydrous).

Acetate buffer pH 5.0: To 2 volumes of 2M sodium acetate (hydrated) was added 1 volume of 2M acetic acid.

Phosphate buffer, pH 6.5, for ZEO-KARB resin column.

Stock solutions: (a) 0.2M monobasic sodium phosphate.
(b) 0.2M dibasic sodium phosphate.

To 342.5 ml of solution (a) was added 157.5 ml of solution (b) and diluted to 1 litre. 0.1% EDTA was then added.

Stock solution of noradrenaline.

99.68 mgs of noradrenaline bitartrate ($C_8 H_{11} NO_3 H6 O6, H_2O$; mol. wht. 337.29) was dissolved in 0.1N hydrachloric acid and made up to 100 mls in a volumetric flask using the dilute acid solution.

Stock solution of adrenaline.

181.92 mgs of adrenaline bitartrate ($C_9H_{13} NO_3 \cdot C_4H_6O_6$; mol. wht. 333.30) was dissolved in 0.1N hydrochloric acid and made up to 100 mls in a volumetric flask using the dilute acid solution.

Standard solutions were prepared by making dilutions in 0.01N hydrochloric acid prior to any assay.

All glassware was immersed O/N in 10% chromic acid and rinsed thoroughly in tap water before use. Deionized distilled water was used as a final rinse for all glassware and also to make up any chemical solution etc. The fluorimeter cuvettes were kept immersed in chromic acid while not being used.

Passive Avoidance Field.

Fig. 20 shows the circuit diagram used for the passive avoidance field. The actual control panel is shown in Fig. 4. A 4-way wire connected the control panel to the field. The 18-way Jones plug was connected to a Grason Stadler (model E1064GS) shock generator. The counter shown in the control panel circuit diagram accumulated the total count but an output was provided to enable the count to be distributed to a bank of counters.

Startle Chamber.

A wide variety of startle chambers and different types of transducers used to record the chambers movements have been reported. These range from modified postage scales (Brown, Kalish and Farber, 1951) to a sophisticated accelerometer transducer (Horlington, 1968). The latter author gives a brief review of the various types of transducers used. Initial studies considered and rejected several published designs on two main grounds; one was that the previous reported designs were capable of measuring startle forces exerted by a 200 - 300 gm rat but were relatively insensitive to the startle response of a mouse which is some 10x lighter. Secondly, in most designs it was difficult to quickly damp down the chamber oscillations without overall loss of sensitivity in detecting the chamber's movement. The final design used, shown in Fig. 7, used a converted photographic slide box 7 cm x 4 cm x 4 cm. A grid floor was constructed using stainless steel (18 gauge) hypodermic tubing to which electric shock could be applied. The total weight of the chamber, including the wires carrying the shock to the grid floor, was 30 gms. A chamber approximately matched in weight to the weight of the mice resulted in a sharp rise time in the evoked startle response. By using a small chamber the animals' movements were restricted; it was just possible for the mouse to turn around. The design incorporated two main principles:

(a) a foam rubber base was used to sit the chamber on and this base controlled the chamber's sensitivity. By varying the thickness of the foam rubber unwanted chamber oscillations could be eliminated. Increased sensitivity was obtained by removing cores from the middle of the foam rubber. By varying the thickness and coring it was found that precise detection could be obtained for any specified movement of the animal. The foam rubber used was Dunlopillo polyether Gold Crown grade; this is a dense foam rubber and as it proved satisfactory no other types were tested. However, there is no reason why the principle could not be applied to any reasonably dense foam rubber.

(b) a crystal in a stereocrystal cartridge taken from a record player pick-up arm (Garrard S.P. 10) was used to detect movements of the chamber. Movements were transmitted via the U shaped linkage attached to one corner of the chamber (18 gauge hypodermic tubing was used). The readout from the crystal was displayed on an oscilloscope. One end of the linkage tubing was set in a Perspex cornerpiece attached to the chamber while the other end was unattached resting with pressure contact, maintained by a light elastic band, between the crossed arms of the crystal. The linkage system used, protected the crystal against accidental overloading and allowed the chamber to be moved back from the front stop in order to facilitate placing the animals into and removing them from the chamber. A permanent record was made of the oscilloscope display by using Ilford NS6 perforated, recording paper in an oscilloscope camera.

Validation tests on the Startle Chamber.

Initial tests were made using a number of normal mice and observing the animals' behaviour while at the same time watching the oscilloscope screen. As shown in Fig. 7 the outputs from the crystal were paralleled producing an algebraic summation of the two movement produce voltages. It was realized that it

would have been better to have summed the absolute values of the produce voltages but this would have considerably increased the complexity of what was a relatively simple system. Also, the experiment designed required a comparison to be made between experimental and normal mice and there was no reason to suspect that one type of mouse would produce a peculiar movement, resulting in the two crystal voltages cancelling each other out, for that group alone. Any artifacts produced from the crystal should have been random over all the animals. As it happened, on no occasion was a movement seen in a mouse that did not produce a voltage change. On several occasions during the actual experiment peculiar results were seen on the oscilloscope screen and checking the trace against the animal's movements always showed that the observed voltage change was reflecting a change of behaviour on the part of the animal. In particular, on the last day the evoked startle responses were clearly larger than on the previous days, but by observing the animals visually, the experimenters confirm that the mice were producing greater startle responses. Fig. 21 shows the results obtained from a series of tests made by either rolling steel balls from varying distances down an inclined groove or dropping them from varying heights above the chamber. Quantitative comparisons were made, using the steel balls and normal mice, ~~for comparisons~~ between tests when the chamber was removed and repositioned before the next test was made. The traces were always directly comparable during these tests. Initial tests were carried out without the linkage being restrained by an elastic band and on no occasion was a mouse found to have altered the position of the chamber. However, for the purposes of the actual experiment it was decided to use an elastic band to restrain the linkage arm rather than risk the possibility of losing the record of an animal's daily session. Fig. 22 shows the typical traces recorded during the experiment; trace C, shows clearly the trace differences that were found between normal body movement and the startle response (irregularities in the actual trace are due to inking over the original trace for photographic purposes). Evidence obtained before and during the experiment indicated that the records obtained accurately reflected the size of the startle responses made by the animals.

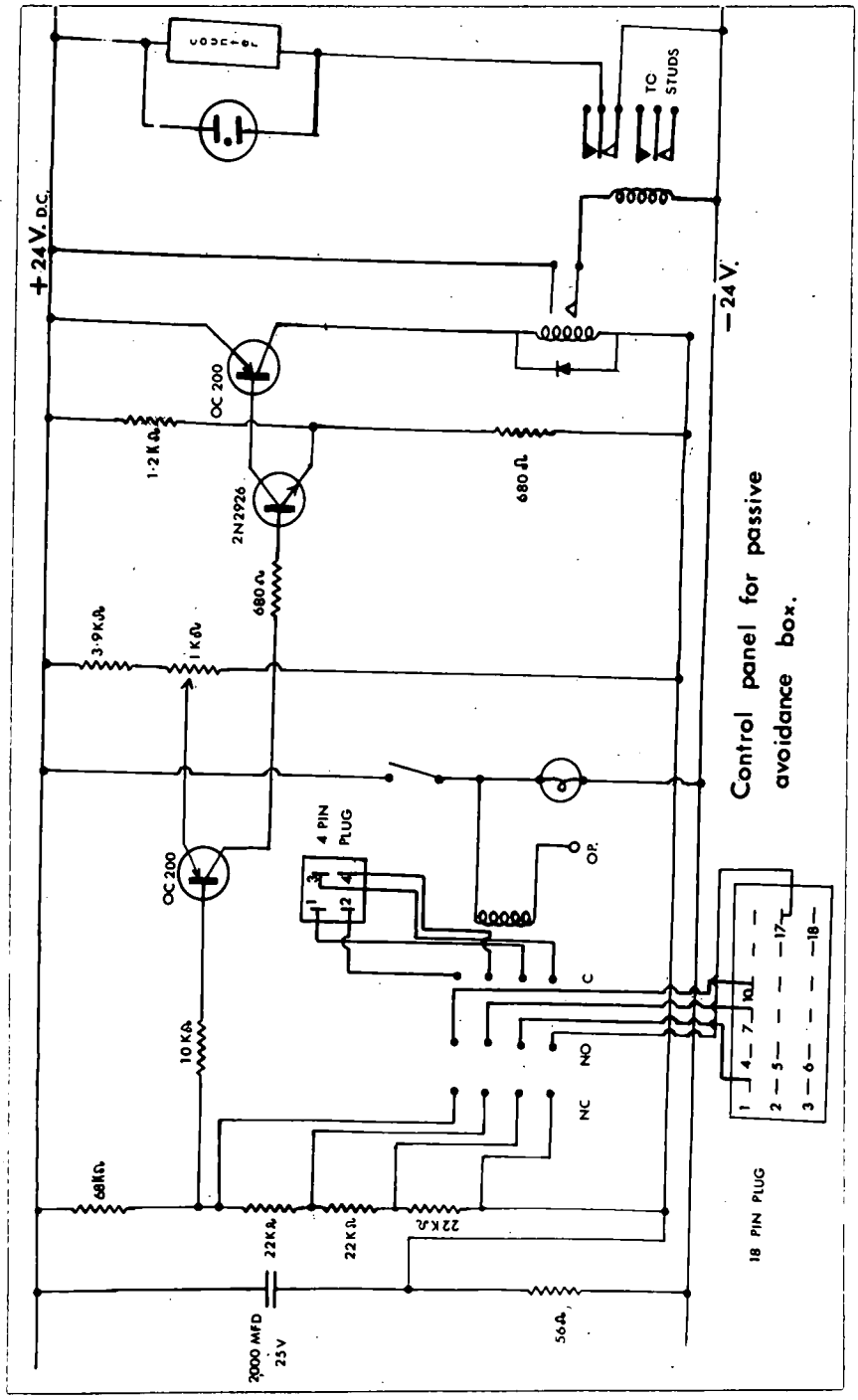

FIG. 20 Circuit diagram of control panel for the passive avoidance field.

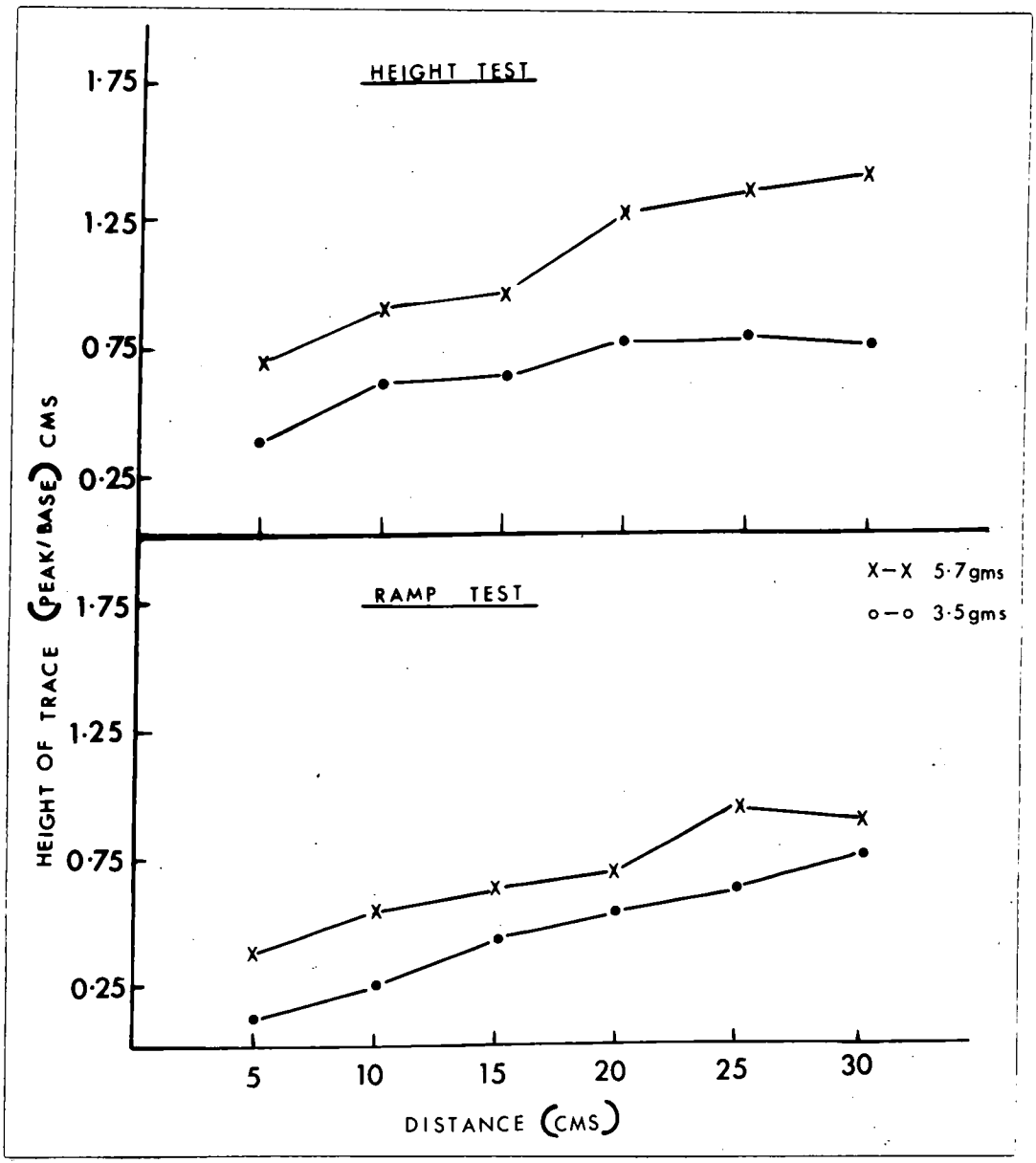

FIG. 21 Tests made on the Startle Chamber using two steel balls. Top graph shows the height of the trace when the two balls were released from a solenoid held at different heights above the centre of the Chamber. Lower graph shows the height of the trace when the two balls were released, from the solenoid, down an inclined groove to strike the mid-point of the front wall of the Chamber.

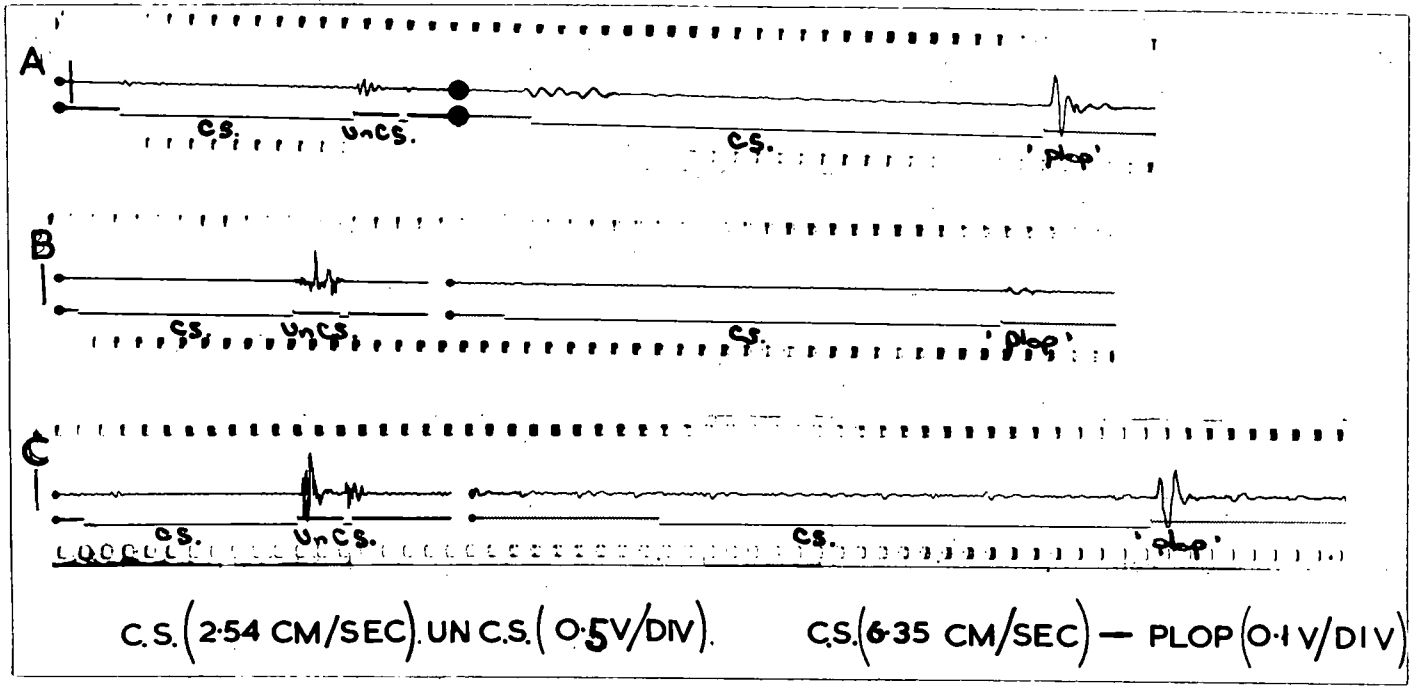

FIG. 22 Typical traces recorded from mice during startle chamber trials. The top trace is a record of the animal's movement; the bottom trace shows the onset and offset of the CS or UnCS. Values shown below the traces are paper speeds and oscilloscope settings for the trial being recorded.

TRACE A: shows a mouse making a movement shortly after the shock UnCS onset. The CS (light) onset produces an adjustment movement and is followed after the CS offset and explosive plop by a typical startle record.

TRACE B: similar to trace A, with a more vigorous movement made to the shock and a more limited startle reaction.

TRACE C: shows the startle reaction occurring in a mouse who produced continuous body movement throughout the trials. The difference between body movement and the startle response is clearly shown.

TABLE 12

ANALYSIS OF VARIANCE FOR SPLIT-PLOT DESIGN USED TO ANALYSE DATA FROM THE STARTLE RESPONSE EXPERIMENT REPORTED IN CHAPTER V

SOURCE	df.
Treatment	1
Sex	1
Shock	1
Treatment x sex	1
Treatment x shock	1
Sex x shock	1
Treatment x sex x shock	1
Between subjects within groups	40
Between subjects	47
Between days	3
Days x sex	3
Days x treatment	3
Days x shock	3
Days x sex x shock	3
Days x treatment x shock	3
Days x treatment x sex	3
Days x treatment x sex x shock	3
Error term	120
TOTAL	191

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