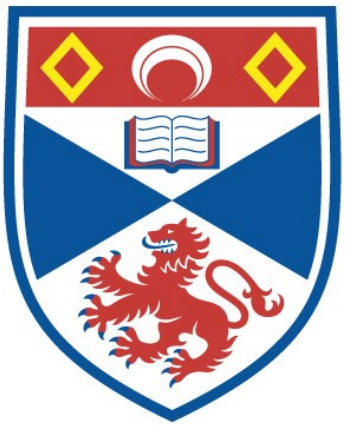
PROPERTIES AND DISTRIBUTION OF AN ACTIVE CEREBELLAR FACTOR

James Fabian Mitchell

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1957

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by

JAMES F. MITCHELL, B.Sc.

A Thesis submitted to the
University of St. Andrews
in canditature for the
DEGREE of DOCTOR of PHILOSOPHY

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DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a Higher Degree.

The research was carried out in the Department of Physiology in St. Salvator's College, St. Andrews under the direction of Dr J. Crossland.

(James F. Mitchell)

CERTIFICATE

I hereby certify that JAMES FABIAN MITCHELL has spent nine terms engaged in research work under my direction, that he has fulfilled the conditions of Ordinance 16 (St. Andrews), and is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

(James Crossland)

CAREER

I first matriculated in the University of St. Andrews in October, 1950 and followed a course of study leading to graduation in Science (Physiology and Natural History) in June, 1953. I was awarded 2nd Class Honours in Physiology in June, 1954.

I was accepted as a research student in August, 1954 and received a Maintenance Allowance from the Department of Scientific and Industrial Research from October, 1954 until February, 1957.

(James F. Mitchell)

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the advice and encouragement I have received from Professor A.E. Ritchie throughout the course of this work.

I am grateful to Dr Crossland who has supervised the course of my research and who originally suggested the subject of this investigation. For the many stimulating discussions I have had with him and for the valuable criticisms and encouragement which he has so often provided, I am indebted.

I am also grateful to Mr W.G.S. Stephens of the Department of Physiology, University of St. Andrews for permission to reproduce a lecture illustration previously presented by him to a meeting of the Scottish EEG Society; to Mr. P.R. Carnegie for carrying out much of the purely biochemical work described in Chapter 4 and to Mr Eric Carstairs for the photographic work involved in preparing the illustrations. The many generous gifts of drugs and chemicals that have been received are acknowledged in the text of this thesis.

St. Salvator's College,
St. Andrews.

February, 1957

CONTENTS

INTRODUCTION

Since the general acceptance of the chemical hypothesis of synaptic transmission, it has become evident that acetylcholine (ACh) is probably not the only mediator of transmission in the nervous system. Attempts to characterize a non-cholinergic transmitter substance followed the suggestion by Dale (1935) that the substance responsible for peripheral antidromic vasodilatation might also mediate transmission at some central non-cholinergic synapses, but these investigations have not so far met with success. In the present investigation, a new approach has been made to this problem which has led to the demonstration of a substance which, it is believed, might be of some importance in the non-cholinergic transmission of nerve impulses.

The cerebellum, despite a high cell density (Heller & Elliott, 1954), is poor in ACh (MacIntosh, 1941; Feldberg & Mann, 1946; Crossland & Merrick, 1954) and has a low choline acetylase activity (Feldberg & Mann, 1946; Feldberg & Vogt, 1948) which suggests that, unless synaptic transmission in the cerebellum is mediated by much smaller quantities of ACh than elsewhere in the nervous system, there may be few cholinergic neurones present

together with a correspondingly high density of non-cholinergic structures in this part of the brain. If this is so, and if transmission in the cerebellum is of a chemical nature, then it might be expected that this area of the brain would provide a relatively rich source of at least one transmitter substance other than ACh. It might reasonably be expected that one property of this substance would be, when suitably administered, to stimulate nervous activity in the cerebellum.

The experiments described in this thesis demonstrate the presence of a substance in extracts of cerebellum, which, when injected intra-arterially into the decerebrate preparation, increases the spontaneous electrical activity of the cerebellum. A study of the chemical, physical, physiological and pharmacological properties of this active substance suggests that it is not identical with any of the previously identified pharmacologically-active constituents of the brain. Attempts have been made to purify and identify the active factor and its action on various regions of the nervous system has been studied. The distribution of the active factor in nervous tissues has been found usually to bear an inverse relationship to the distribution of ACh, this relationship being particularly well demonstrated in the visual pathways.

No experiment in this thesis has been specifically

designed to show the direct participation of the active cerebellar substance in transmission across non-cholinergic synapses, but of the properties so far found for it, none is inconsistent with this possibility.

A preliminary account of some of the work contained in this thesis was communicated to the Physiological Society (Crossland & Mitchell, 1955) and was followed by a full report in the Journal of Physiology (Crossland & Mitchell, 1956). Some other results obtained during this investigation were communicated to the XX International Congress of Physiology in Brussels (Carnegie, Crossland & Mitchell, 1956).

CHAPTER I

THE DECEREBRATE TEST PREPARATION

A study of the effects of various substances on the electrical activity of the cerebellum can be made using one of several techniques. Many of these techniques have been used in this investigation, but the only one which has proved satisfactory and which has therefore been used extensively, was the decerebrate rabbit preparation in which test substances were introduced into the cerebellum through the internal carotid artery. The reasons underlying this choice and the experimental procedures used in its employment are outlined below.

1.1 Experimental Procedure

1.11 Surgical Procedure

Medium-sized rabbits of either sex were anaesthetized with ether and the depth of anaesthesia maintained at a level where the corneal reflexes remained sluggish. The trachea was cannulated and the right external carotid artery tied. If a blood pressure record was not required, the central end of the right common carotid artery was occluded and a glass cannula, attached to 12 cm of polythene tubing filled with 0.9% (w/v) sodium chloride solution, was inserted

pointing away from the heart. The tip of this cannula was arranged to lie in the internal carotid artery. If a blood pressure record was required, a second cannula was inserted into the right common carotid artery.

An extensive longitudinal incision was made in the skin overlying the skull and all muscular and connective tissue on the skull above and just behind the occipital cortical areas was removed. Two trephine holes (diameter, 0.8 cm) were made on either side of the midline of the skull. The bridge joining these holes was chipped away in order that the underlying, and sometimes adhering, dura mater should not be punctured. The head of the rabbit was secured in a conventional jaw clamp and the animal tied to the operating table. A Miller decerebration clamp was adjusted to secure partial occlusion of the vertebral arteries in the neck.

If respiration was now strong, the dura was cut and a blunt decerebration blade was inserted firmly into the brain. In order that the blood supply from the carotid arteries to the cerebellum should remain intact, a decerebration technique was used in which the blade moved forward through some parts of the brain but avoided the Circle of Willis. This is more fully described later in section 1.22. When the blade was removed, bleeding usually occurred and this could be stopped by the application of a warm

saline pad or haemostatic gauze (oxidized cellulose).

Ether anaesthesia was then discontinued and the rabbit left for 20-30 minutes. At the end of this time, the decerebration clamp was loosened and the clip on the left carotid artery was removed. The exposed brain was then covered by a warm saline pad and a rectal thermometer inserted. The carotid cannula and tube were connected to the injection apparatus and, if required, the second cannula was connected to a mercury manometer and the blood pressure recorded after heparin (600 I.U./kg) had been given intravenously through a marginal ear vein. A pin electrode was pushed into the skull overlying the anterior lobe of the cerebellum and the earth and indifferent electrodes were inserted into the muscles of the neck.

It was first reported by Dow (1938) that the spontaneous electrical activity of the cerebellum was dependent on the maintenance of the systemic blood pressure. In the present investigation, it has been found that directly the blood pressure falls below 55-60 mm Hg, the cerebellar electrical activity ceases abruptly. When medium-sized rabbits were used, provided that the rectal temperature was kept between 36 & 38°C and that bleeding had not been excessive, the blood pressure could usually be maintained for several hours.

Even when the precautions described had been taken, it was still found that some rabbits did not provide a successful assay preparation; an unpredictable and irreversible fall in blood pressure sometimes occurred and, less frequently, an apparently healthy preparation would fail to respond to the injection of an active solution. Possible reasons for this insensitivity of a healthy preparation are suggested in a later section.

1.12 The Injection of Test Solutions

The insertion and location of the arterial cannula has already been described. From this cannula a polythene tube was connected to one arm of a small three-way tap, the total internal volume of this cannula and tube being 0.2 ml. From the second arm of this tap a short length of tubing led to a reservoir containing 0.9% saline solution. The third arm received a 1 ml 'Tuberculin' syringe to which a signal switch was attached connected to a marker on the recording apparatus. A warm-water jacket surrounding the reservoir arm of the tap ensured that solutions injected into the animal were heated to 36-37°C.

Solutions and extracts for testing were stored in a frozen state and were thawed and placed in a water bath at 37°C shortly before use. When all traces of ether had been blown off

and a satisfactory electrical record from the cerebellum obtained, an injection of an active extract to test the sensitivity of the preparation was made. To make the injection, 0.2 ml of the active solution was injected through the upright arm of the tap so that it occupied the dead space in the polythene tube and cannula. The tap was then turned and 0.25 ml of saline solution was drawn into the syringe. The tap was turned again so that when the syringe plunger was depressed, the injected saline solution (0.25 ml) would force the test solution (0.2 ml) into the rabbit and leave the cannula and tube filled with saline.

If the preparation responded to a preliminary injection of an active solution, a similar volume of an inactive control solution was injected in the same way and, if this gave no response, the experiment was allowed to proceed. If the preparation had proved insensitive to the first injection, further doses of active solution were given every ten minutes. If a response had not been elicited at the end of 30 minutes, the preparation was discarded.

The sensitive preparation was used to study the action of various substances on the electrical activity of the cerebellum in the following manner. A rigid procedure was adhered to in

the administration of substances to the test preparation in order to reduce, as far as possible, any subjective or mechanical errors that might arise.

The solutions for testing were arranged in a predetermined order unknown to the operator; this order allowed, wherever possible, an active solution to be bracketed between inactive solutions. Modifications in the sequence and in the strengths of the injected solutions could not usually be made during the course of an experiment but, if it became evident to the operator that large numbers of active solutions were remaining unbracketed, or that a long series of injections had given no response, he could apply to the person knowing the order of injection for a suitable modification. The order of injection was not disclosed to the operator until the experiment was completed and the electrical records assessed.

For some of the experiments to be described, it was necessary to make some kind of quantitative estimation of the activity present in a given solution rather than simply to detect its presence. For this purpose a 'graded dose' procedure was used in which the minimum amount of stock cerebellar extract necessary to give a response was determined.

The activity present in a test solution was estimated quantitatively by gradually increasing the dose of this solution until a response was first obtained. The dose producing this response could be taken to contain the same concentration of active substance as the stock solution. The limitations of this procedure are discussed in a later section of this chapter.

1.13 Recording of the electrical activity of the cerebellum

In the present investigation, brain extracts and drugs have always been assayed by their actions on cerebellar nervous activity as reflected in the spontaneous electrical activity of the cerebellum. The action of substances on nervous structures within the cerebellum has not, however, been of primary interest and, for this reason, the electrical activity has usually been conveniently recorded with a pen-writing oscillograph. This method does not allow the fast activity of the cerebellum to be recorded faithfully, but it does show changes in the overall amplitude of the records (Appendix I). It is these changes that have been taken to reflect alterations in cerebellar activity.

The electrical activity of the cerebellum, besides being recorded on the pen-oscillograph was monitored on an oscilloscope

and loudspeaker. Some oscilloscope records from the stimulated and unstimulated cerebellum have been photographed and these are compared with similar pen records in an appendix to this thesis.

Monopolar recordings of cerebellar electrical activity were made from a pin electrode inserted into the anterior lobe of the cerebellum through the overlying skull. Electrical activity was recorded simultaneously on an oscilloscope, through a loudspeaker and on an Ediswan pen-oscillograph using conventional amplifying arrangements. Cerebellar records were made with an amplifier time-constant of 0.03 sec with the frequency filter set at 500 c/s. The sensitivity of the amplifying system remained constant throughout each experiment. Paper records were made continuously for about 5 sec before each test injection and for 15-80 sec afterwards.

1.14 Assessment of electrical records

At the conclusion of each experiment using the decerebrate test rabbit, the paper records taken for each test injection were placed in random order, each record having only a numeral on it for later identification. These records were then given to two separate observers who were asked to assess the amount of excitation, if any, shown in each record. The period of excitation

was defined as the length of electrocerebellogram (ECbG) record following an injection which showed an increase in amplitude combined with an increase or no change in frequency when compared with a control record taken before injection. The observers were asked to append an '0' to those records showing no increase in activity. A slight increase was indicated by the rating '1' and greater increases by '2' or '3' - the latter being given only for very large increases in electrical activity. The observers also measured and noted the time interval between the start of the injection and the beginning of any increase of activity and the duration of this activity.

The two observers, who were not conversant with the nature of the experiments, independently assessed all the records from the first 36 experiments and a good agreement was found between their detailed conclusions. Table 1 shows the results of their assessments of the first half of a typical experiment.

In some of the later experiments, notably those concerned with the solubility and other straightforward properties of an active substance found in the cerebellar extracts, only one observer assessed the records.

Rabbit BH Weight 2.0 kg; Sex, male; Cupar litter

11.15 a.m. anaesthetised, ether by mask
11.20 a.m. tracheal & carotid cannulae inserted; right external carotid artery tied.
11.25 a.m. carotid & vertebral arteries partially occluded; ether withdrawn
11.28 a.m. decerebrated
11.40 a.m. artery clamps released
11.41 a.m. skull cleaned and electrodes inserted
11.48 a.m. preliminary injection with ECbC recording

All extracts were alkali-boiled before injection

Order of Injection	Observer 1 (A.S.M.)			Observer 2 (E.J.C.)		
	Excit^{n}	Delay (sec)	Dur^{n} (sec)	Excit^{n}	Delay (sec)	Dur^{n} (sec)
1. Cb. (TCA) 20 mg	1	20	10	1	15	17
2. Cb. (saline) 20 mg	0	-	-	0	-	-
3. Cb. (boiled saline 20 mg	2	15	16	2	10	20
4. Cb. (saline) 20 mg	0	-	-	0	-	-
5. Cb. (boiled saline) 20 mg	2	15	20	2	10	12
6. Control, Cerebrum (TCA) 20 mg	0	-	-	0	-	-
7. Cer. (boiled saline) 20 mg	0	-	-	0	-	-
8. Cb. (boiled saline) 20 mg	1	20	14	2	15	12
9. Cer. (boiled saline) 20 mg	0	-	-	0	-	-
10. Cb. (TCA) 20 mg	1	20	8	1	15	15
11. Control (saline) 0.1 ml	0	-	-	0	-	-

The experiment was concluded at 2.10 p.m. following 20 injections (last injection, 1.45 p.m.). Rabbit killed by air embolism 2.15 p.m.

Table 1. Protocol of an experiment to test the extraction of the cerebellar excitatory factor from fresh cerebellar tissue by saline

1.2 Discussion of the technique

1.21 Selection of the test animal

The uncomplicated electrocerebellogram can only be recorded successfully from the decerebrate animal and, for this reason, the natural choice of laboratory test animal would have been the cat or dog. In both of these decerebration is a straightforward procedure and the operated animals usually live for many hours without artificial respiration and without deterioration in their general condition.

At the beginning of this investigation only rabbits were available in large numbers and so these were used as the experimental animals. It was soon realised that this choice had been fortunate because it was intended to introduce brain extracts and drugs into the cerebellum through the internal carotid artery and it has been demonstrated (Schmidt, 1950; Chungcharoen, Daly, Neil & Schweitzer, 1952) that the only common laboratory animal in which the internal carotid forms the main supply for the Circle of Willis is the rabbit. In most other animals, the main blood supply comes from the external carotid artery. In the cat, for example, the internal carotid artery is vestigial (Davis & Story, 1943) and the external carotid, besides supplying

the rest of the head with blood, also leads into the Circle of Willis. In the rabbit, on the other hand, not only does the internal carotid lead directly and without branching to the Circle of Willis, but it also provides the only source of blood, apart from the basilar artery, to the brain. It therefore provides an ideal route for introducing substances into the cerebral circulation. These points are illustrated in Fig. 1 which compares the anatomical distribution of blood vessels to the brain in the cat, rabbit and dog.

Although no series of experiments was specifically designed to establish this point, it was found preferable to select medium-sized rabbits weighing between 1.5 and 2 kg for the present experiments, since they appeared to withstand decerebration better than very small or large animals. By using these rabbits, and by following the experimental details to be described, it was usually possible to obtain a preparation which, after decerebration, required no artificial respiration, maintained a blood pressure of at least 65 mm Hg and retained its cerebellar electrical activity and sensitivity to injected substances for about two-and-a-half hours.

A preparation which did not fulfil these conditions could not be considered satisfactory for the purpose of assaying the

cerebellar excitatory factor.

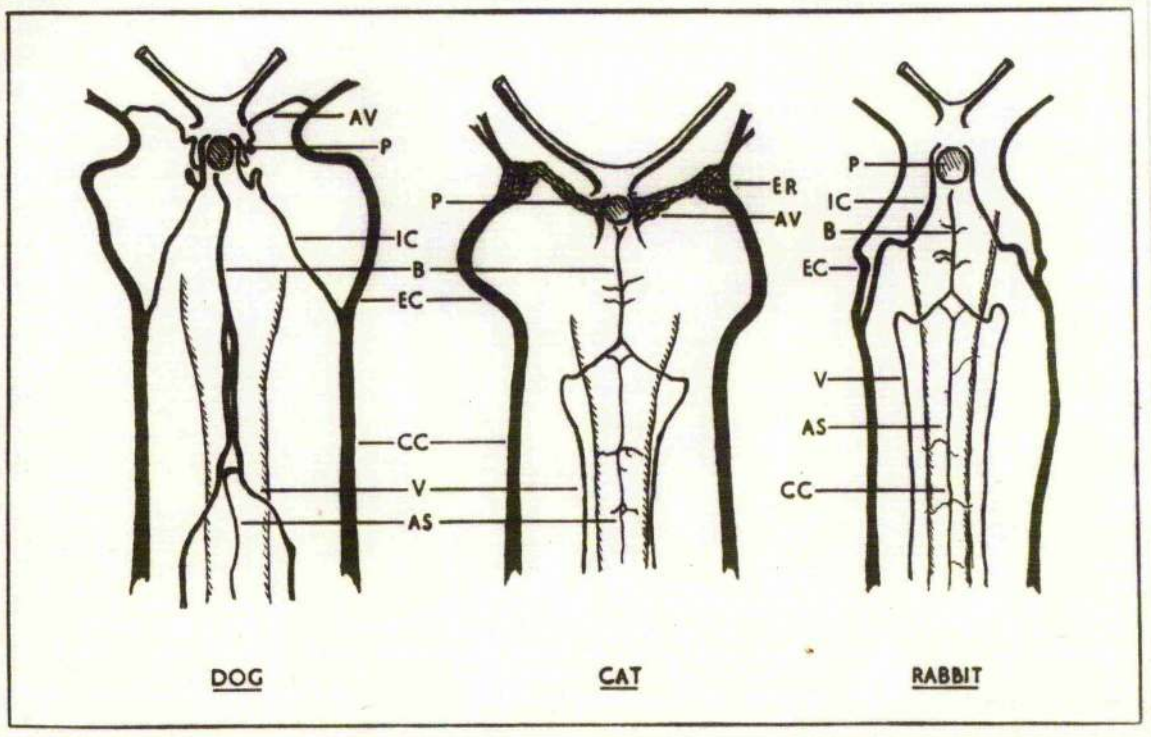


Figure 1. Illustrating the main arterial communications between the carotid and vertebral circulations in the dog, cat and rabbit. (Adapted from Chungcharoen et al., 1952)

Key: CC, common carotid artery; IC, internal carotid artery; EC, external carotid artery; ER, external rete; AV, anastomotic vessel; AS, anterior spinal artery; V, vertebral artery; B, basilar artery; P, pituitary

1.22 The administration of substances to the cerebellum

Although the internal carotid artery of the rabbit leads directly to the Circle of Willis in the base of the brain, substances injected by this route without further precaution would become distributed unevenly throughout most of the brain, the largest quantity probably entering that side of the brain on which the injection was made.

Apart from the topical application of substances to the surface of the cerebellum - a method with little to recommend it - the most direct vascular route to the cerebellum would be by way of the basilar artery, with the blood vessels anterior to the cerebral arteries partially or completely occluded. Methods for cannulating the basilar artery have been described by McDonald & Potter (1951), and by Feldberg, Gray & Perry (1952), the former authors stating that the operation lasts for several hours and might involve considerable loss of blood. On three occasions during this investigation, attempts were made to improve the assay technique by cannulating the basilar artery prior to decerebration, and on each occasion, the operation was completed within an hour. In none of these experiments, however, did the rabbits survive decerebration by more than about 15 minutes. This was probably due to the loss

of blood and length of the surgical procedure. Because the assay demanded a preparation in good condition, it was decided to abandon this laborious and hazardous technique.

If all the blood vessels leaving the Circle of Willis could be occluded except the cerebellar and internal carotid arteries, then substances injected into the carotid should reach the cerebellum without suffering great loss (Fig. 3). In practice these conditions could be realised to a large extent since the vertebral arteries could be temporarily occluded during each injection by means of the decerebration clamp, while the vessels leading from the anterior part of the Circle of Willis could be permanently occluded by destroying some parts of the brain during the course of decerebration.

In order to study the route taken and the parts of the brain reached by an injected solution under various conditions, use was made of Evan's blue dye (T.1824). This was injected in a similar manner to the normal test substances and then the experimental animal was killed. The dye distribution in the brain was inspected following wide craniotomy and then the cerebral hemispheres, upper brain stem, medulla and pons and cerebellum were removed separately and the dye present in each area was

estimated colorimetrically. Dye was extracted from the brain tissue by the method described by Crooke & Morris (1943) and was estimated colorimetrically (E.E.L. colorimeter, yellow filter 626) using externally dyed brain tissue as a control.

Using this procedure as a guide, it was possible to investigate the relative efficiency of several techniques which had been considered for occluding the blood vessels leaving the anterior part of the Circle of Willis.

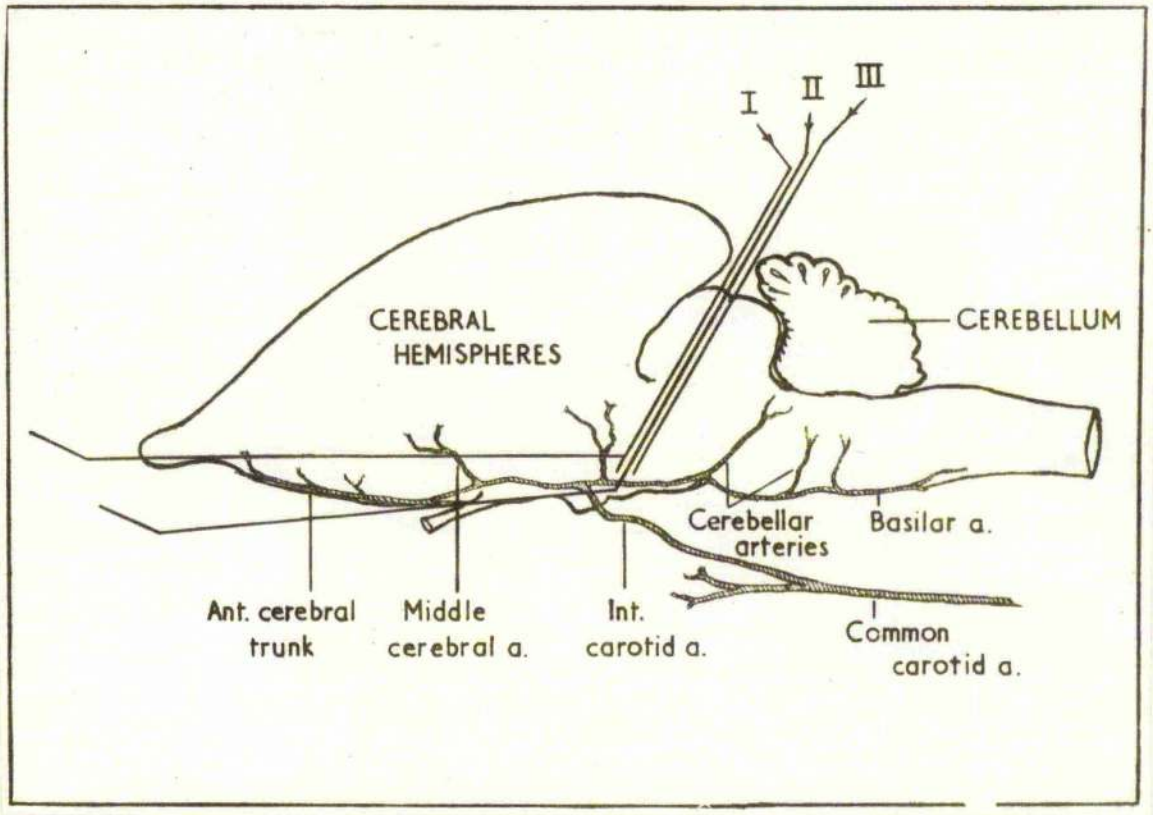


Figure 2. Illustrating the path of the decerebration blade through the brain in the three decerebration techniques described

Fig. 2 illustrates the three types of decerebration investigated. Method II involved a wide craniotomy and plunging the decerebration blade into the brain at mid-collicular level, passing it forward along the base of the skull and removing the upper brain stem and cerebral hemispheres. The space left in the skull was filled with warm bone-wax which was allowed to solidify. This procedure cuts through the communicating arteries and removes the anterior part of the Circle of Willis. It was hoped that the cut arteries would constrict and so stop injected fluid from passing forward along the base of the skull. Dye experiments showed, however, that injected material leaked forward and invariably large amounts were found anterior to the line of primary decerebration. Only small amounts, usually less than 20% of the total dye, were found in the cerebellum.

The second type of decerebration (Method III) was made through a small opening in the skull at mid-collicular level; the blade was pressed firmly down to the base of the skull and removed. It was hoped that this method might provide a fairly even distribution of injected material between the cerebrum and cerebellum because, although the complete cerebral circulation would be intact, the temporary occlusion of the vertebral arteries

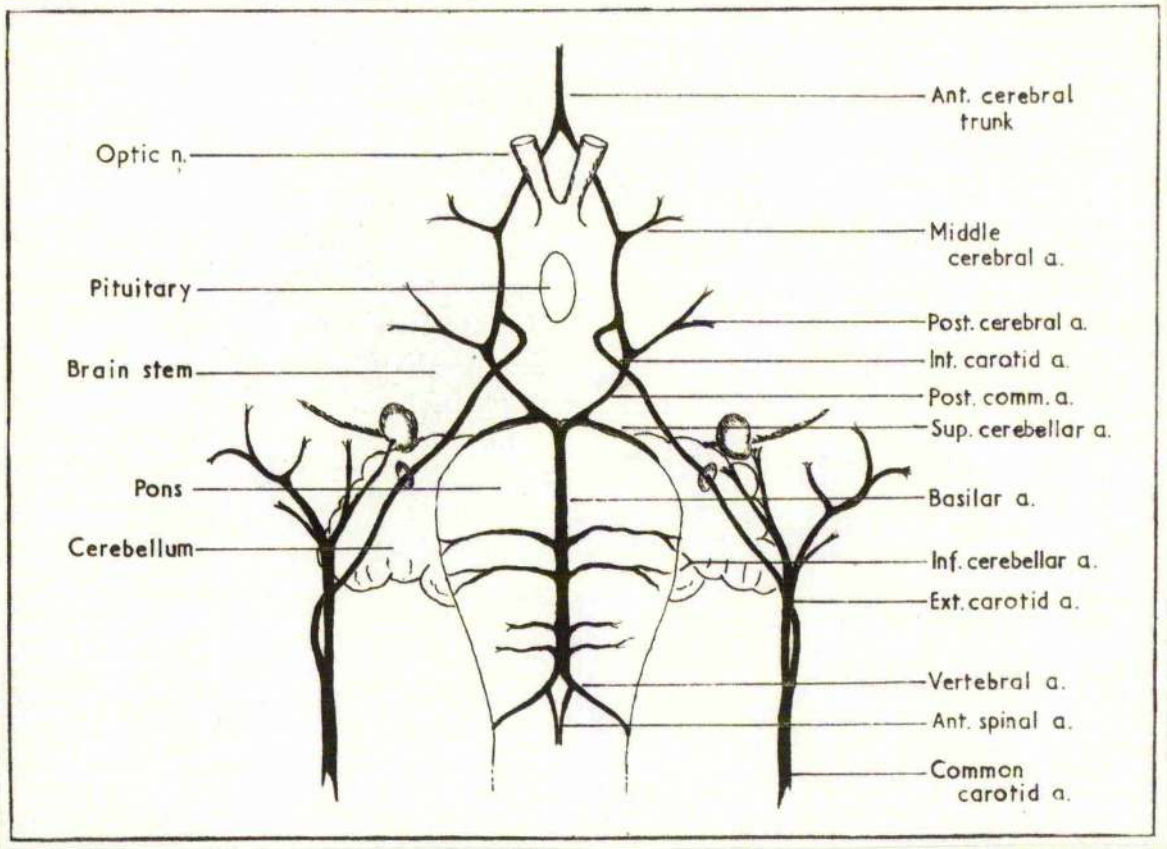


Figure 3. Arteries of the rabbit's brain seen from the ventral aspect

during injection would favour the backward passage of material. It was found that, in fact, nearly all the injected material passed forward, a detailed examination showing that most of it had passed down the large posterior cerebral arteries.

Finally, a type of decerebration (Fig. 2, Method I) was employed which did prove successful and which made it possible to ensure that of any material injected into the internal carotid

artery, about half would reach the cerebellum. The decerebration blade was inserted into the brain at mid-collicular level and was pressed down until it was estimated that the leading edge was a short distance from the base of the skull. When the blade was in position, the leading edge was moved forward, care being taken not to deflect it downwards. The blade was then pressed as far forward as possible, moved from side to side in a horizontal plane and removed.

The result of this manipulation was to leave the entire Circle of Willis intact, but to sever the anterior, middle and posterior cerebral arteries. The cerebral hemispheres were left in situ to aid occlusion of the cut arteries.

At the end of each of five successful experiments using this decerebration procedure, injections of Evans blue were made while the vertebral arteries were occluded. Table 2 shows the dye extracted from three areas of the brain. In each experiment, about half the dye entered the cerebellum.

When it is considered that the fresh weight of rabbit cerebellum is only about 15% (Latimer & Sawin, 1955) of the combined weights of the cerebral hemispheres and upper brain stem, it is evident that the actual concentration of injected material

Experiment	Medulla and Pons	Cerebrum, Brain stem, etc.	Cerebellum
	%	%	%
1	14	40	47
2	21	38	42
3	10	39	51
4	11	34	54
5	15	35	50

Table 2. Relative amounts of Evans blue extracted from different parts of the brain of the rabbit

will be about six times as great in the cerebellum as anywhere else in the brain. This conclusion has been confirmed by visual inspection of the dye distribution at the end of six other successful experiments.

At the end of six experiments, in which known active solutions failed to excite cerebellar activity, an injection of dye was made into the carotid artery. Following the removal of the brain, it was found that dye had leaked into the base of the skull and that, as a consequence, the cerebellum was not deeply stained.

Although the method described is not ideal for the introduction of substances into the blood vessels of the cerebellum, it has given satisfactory results for the purposes

of the assay. It provides a method of arterial injection into the cerebellum which is not difficult to perform and which minimizes the loss of injected material.

In conclusion it may be said that many other pharmacological and physiological preparations, both conventional and unconventional, have been investigated with a view to providing a more convenient and accurate assay, but none has proved more suitable than the decerebrate rabbit preparation.

CHAPTER 2

THE ACTION OF BRAIN EXTRACTS AND ACETYLCHOLINE ON THE ELECTRICAL ACTIVITY OF THE CEREBELLUM

2.1 Extraction methods

Early in this investigation it was found that extracts of cerebellar tissue, when injected into the cerebellum, excited cerebellar electrical activity and that this action could not be attributed to the ACh contained in the extracts. Experiments were performed in which the action of various brain extracts and ACh were studied in detail and unequivocal evidence was found for the existence of a substance, or substances, called for convenience a cerebellar excitatory factor (C.E.F.) in extracts of cerebellum. In these experiments, all the brain extracts were made in the same way using trichloroacetic acid (TCA) as the extraction medium.

Rabbits were anaesthetised with 'Dial' (Ciba) administered intraperitoneally (0.7 ml/kg corresponding to 70 mg/kg of diallylbarbituric acid and 280 mg/kg of urethane) and the rabbits were then widely craniotomized. The cerebral hemispheres were quickly removed and weighed, and then ground with 10% (w/v) TCA

(2 ml acid/g tissue) in a glass mortar. The cerebellum, and the remaining nervous tissues anterior to the cerebellum (the 'upper brain stem') were removed separately and similarly treated. During this time, the animal was kept in as good a condition as possible, artificial respiration being applied when necessary.

The three extracts were allowed to stand for 1½-2 hours in TCA at room temperature and were then centrifuged for three minutes and the volumes of supernatant fluid measured. Excess TCA was removed from the extracts with ether. Ether left in solution at the end of this procedure was removed in a stream of air. The extracts were neutralized and stored in a frozen state until required for testing, when they were thawed and suitably diluted.

It was often necessary to destroy the ACh contained in the extracts and this was done by making the solution weakly alkaline (pH 11) with N-sodium hydroxide, boiling briefly and neutralizing. The ACh content of the brain extracts was assayed on the neostigminized frog-rectus preparation, taking the usual precautions against the effect of sensitizing substances (Feldberg, 1944). Acetylcholine doses mentioned in the text refer

to the chloride.

In the early experiments, control solutions were prepared by mixing 0.8 ml of 0.9% saline solution with 2 ml TCA and then subjecting this mixture to the same process of extraction and dilution as the brain extracts themselves. It later became apparent that a more adequate control was furnished by ACh-free parts of the brain other than the cerebellum and by extracts of cerebellum whose activity had been destroyed by acid boiling. In order to simplify the nomenclature, doses of tissue extracts have been expressed as fresh brain equivalents (f.b.e.) throughout this thesis. Each fresh brain equivalent corresponds to one milligram of fresh tissue. Before testing, all the extracts were diluted to 200 f.b.e./ml while other solutions were diluted so that they could be injected in volumes not greater than 0.2 ml.

2.2 Results

This series of results was obtained from experiments on 29 decerebrate rabbits. The results of a typical experiment are shown in Figs. 4-6. The injection of 0.1 ml of the control solutions had no action on the cerebellar activity. Following the

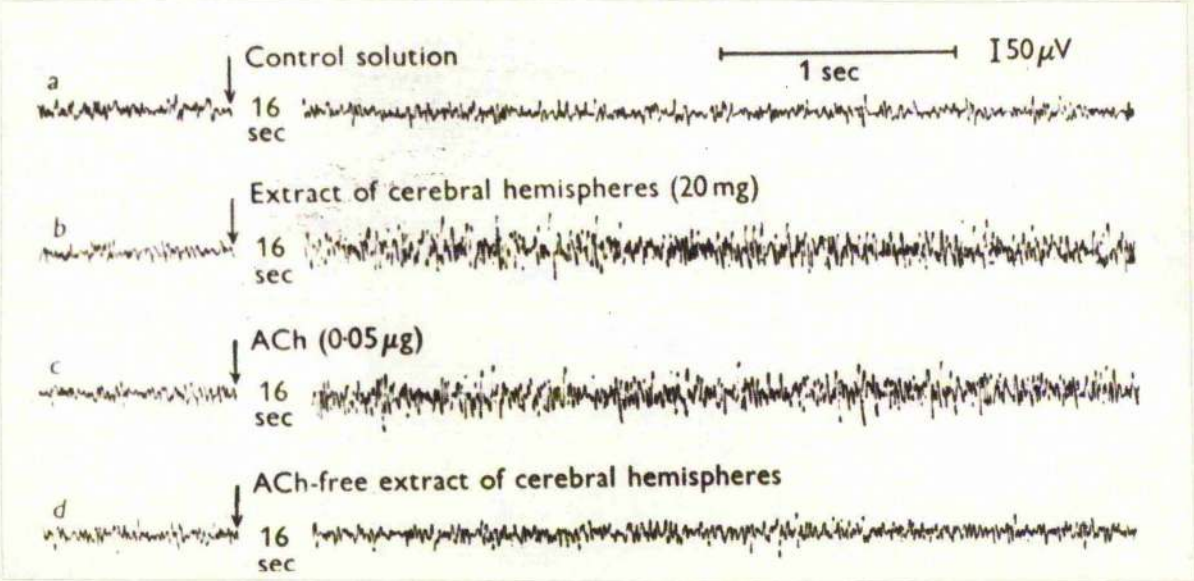


Figure 4 (Rabbit J; 21.3.55) Pen-oscillograph records of the electrical activity of the cerebellum before and after intra-carotid injections

a) 0.1 ml control solution
b) TCA extract of rabbit cerebral hemispheres (20 f.b.e.)
c) The ACh equivalent (0.05μg) of the extract
d) TCA extract of cerebral hemispheres after the removal of ACh (20 f.b.e.)

injection of 20 f.b.e. of cerebral hemispheres, the electrical activity of the cerebellum was increased (Fig. 4b). That this effect was due to the presence of ACh in the extract is shown by the third record (Fig. 4c) in which the ACh equivalent of 20 f.b.e. of cerebral hemispheres (0.05μg) is shown to have a similar effect. Further evidence that the excitation shown in Fig. 4b was due to ACh was found when alkali-treated extracts of cerebral hemispheres elicited no response (Fig. 4d).

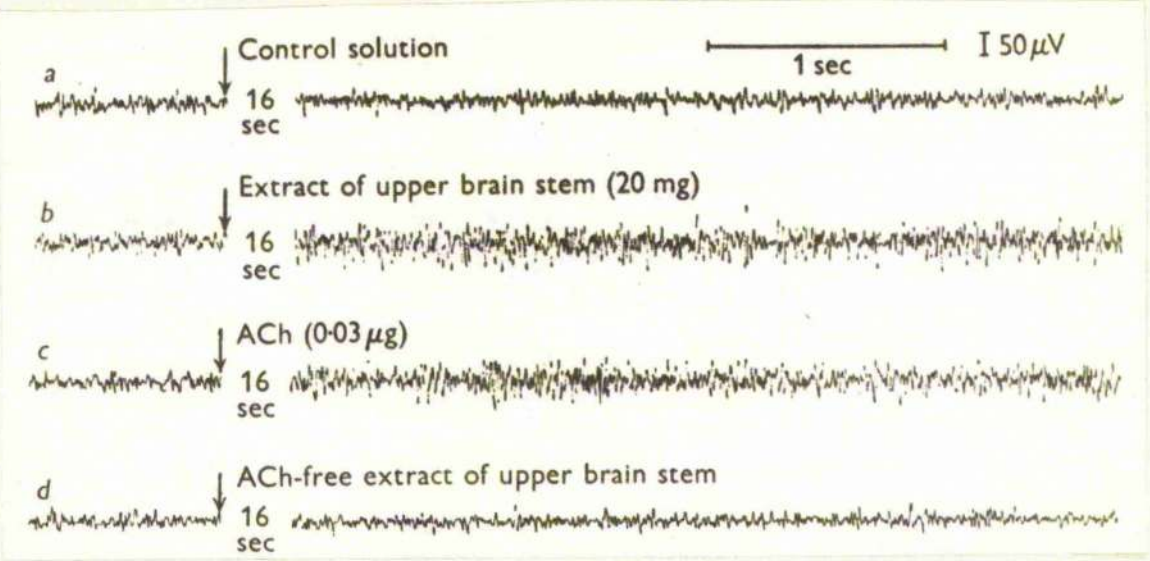


Figure 5 (Rabbit J; 21.3.55) Electrical activity of the cerebellum

a) Control
b) Upper brain stem extract (20 f.b.e.)
c) ACh equivalent (0.03μg) of upper brain stem (20 f.b.e.)
d) ACh-free extract of upper brain stem (20 f.b.e.)

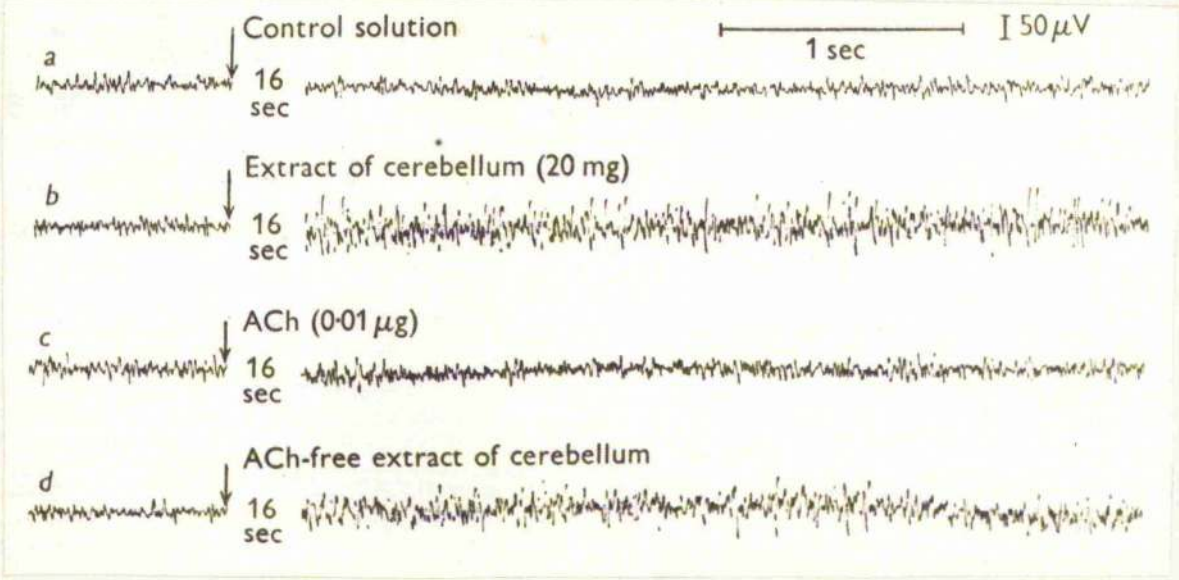


Figure 6 (Rabbit J; 21.3.55) Electrical activity of the cerebellum

a) Control
b) Cerebellar extract (20 f.b.e.)
c) ACh equivalent (0.01 μg) of cerebellar extract (20 f.b.e.)
d) ACh-free extract of cerebellum (20 f.b.e.)

Similar results were obtained with extracts of upper brain stem (Fig. 5) and its activity may again be attributed solely to its contained ACh (0.03 µg). The injection of cerebellar extracts, however, produced different results. The action of the untreated extract (Fig. 6b) produced a response similar to that of the other untreated extracts but, after destruction of the contained ACh, the response remained unaltered (Fig. 6d). The ACh equivalent of the cerebellar extract (0.01µg) was well below the threshold required for stimulation and no response occurred (Fig. 6c). The response to the injection of cerebellar extracts cannot be due to ACh and the existence of some other excitatory factor in these extracts must be postulated.

The results of 308 injections administered in the course of experiments on twenty-nine rabbits using brain extracts prepared separately from 21 animals are summarized in Fig. 7. Each histogram shows the percentage of each type of response provoked by the injection of control solutions, brain extracts and their ACh equivalent of the extracts. The actual number of each type of injection is given in brackets on the appropriate histogram.

The histogram illustrating the results of 59 injections of control solution shows that 92% of the responses were assessed

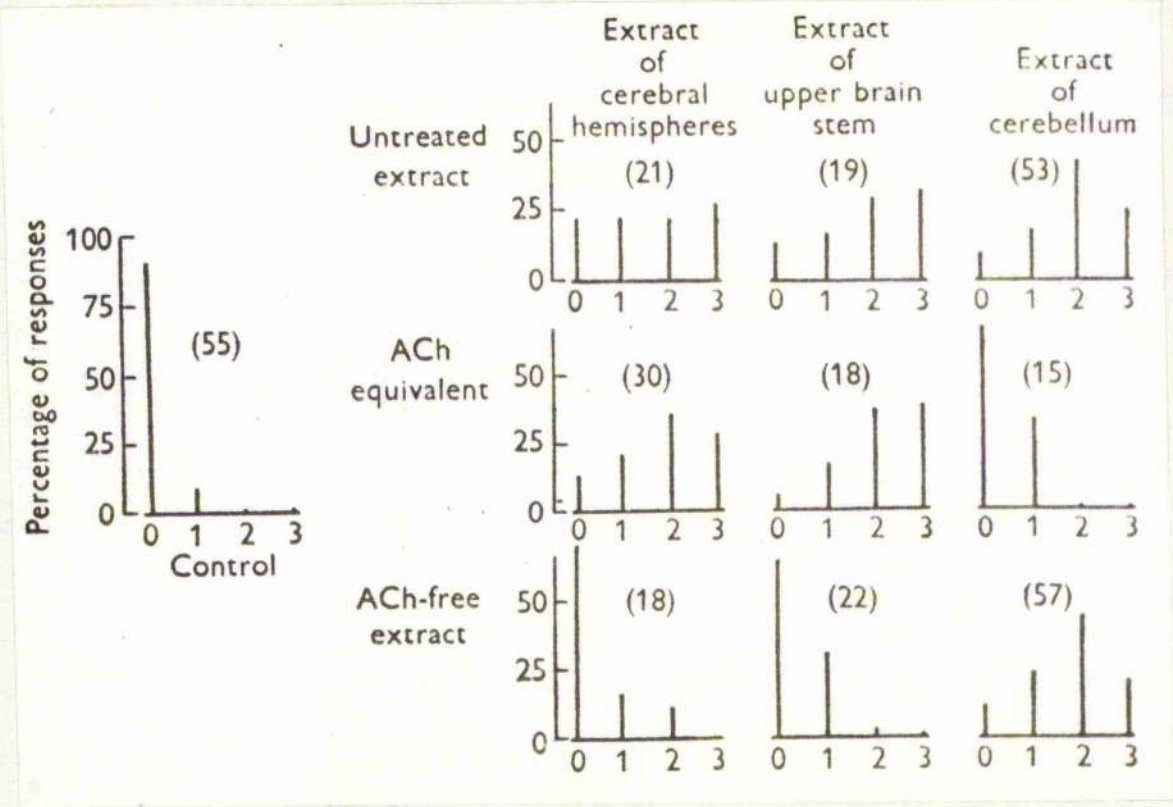


Figure 7 Summary of the results of 29 experiments

0 = no response
1 = slight response
2 = strong response
3 = very strong response

as having no effect on cerebellar activity. The remaining 7% were reported as producing slight excitation. A similar distribution of responses was shown by alkali-treated extracts of cerebral hemispheres and upper brain stem and by the subthreshold doses of ACh found in the cerebellum. The untreated extracts of cerebral hemispheres and brain stem, their

equivalent doses of ACh and the cerebellar extracts before and after alkali treatment all gave a pattern of responses similar among themselves but quite different from those of the control solution. Many of the responses in this group were described as being 'strong' or 'very strong'.

In some experiments of this series, the ACh equivalents of extracts of cerebrum and brain stem were injected in the presence of these extracts from which the ACh had been removed. This procedure had no effect on the cerebellar responses obtained.

Table 3 shows the time-courses involved in the excitatory responses of the electrical activity of the cerebellum to the injection of various substances. It is noticeable here that responses brought about by ACh and by cerebellar extracts follow similar time-courses. Another and more striking similarity results from the fact that the minimal effective dose of an ACh-free cerebellar extract, usually 20 f.b.e. of fresh tissue, is the same as that of an area of brain whose activity is attributable to ACh.

From these experiments, it would seem likely that a substance is present in cerebellar extracts which acts directly on the cerebellum to cause an increase in nervous activity.

Nature of Injection	Latency of Response	Duration of Response
	Mean ± S.E. of mean (sec)	Mean ± S.E. of mean (sec)
Extract of cerebral hemis.	10.8 ± 1.9 (15)	12.5 ± 2.4 (15)
Extract of upper brain stem	12.2 ± 2.5 (16)	13.1 ± 2.1 (15)
Extract of cerebellum	13.8 ± 2.4 (38)	11.9 ± 1.0 (38)
Extract of cerebellum (alkali-treated)	12.0 ± 2.1 (39)	13.9 ± 1.4 (39)
Acetylcholine	13.3 ± 1.2 (39)	10.4 ± 1.0 (31)
ACh + ACh-free brain extracts	13.0 ± 2.2 (12)	10.9 ± 1.8 (12)

Table 3 Time-course of excitatory responses of the electrical activity of the cerebellum

This substance is not acetylcholine and is not apparently present in the cerebrum or upper brain stem in sufficient amounts to cause excitation at the dose levels used.

CHAPTER 3

THE ACTION OF BRAIN CONSTITUENTS AND DRUGS ON THE CEREBELLUM

Although the effects of strychnine on the electrical activity of the cerebellum have been studied in some detail by previous workers, the effects of other drugs have received only slight attention. The results of work on the action of strychnine show considerable disagreement. A 1% solution topically applied has been reported to depress cerebellar activity (Dow, 1938) and to increase spike activity (Brookhart, Moruzzi & Snider, 1950). Johnson, Browne, Markham & Walker (1950) found that a 2½% solution of strychnine, when applied topically, excited cerebellar activity but only after a lapse of 1½-2 hours.

The action of ACh has only previously been studied by Dow (1938) when it was found that the topical application of a 1:100,000 solution had no effect. Anticholinesterases, however, do appear to affect the cerebellum. Miller (1937) placed a 'tablet of eserine' on the exposed cerebellum and observed that 'almost at once the right foreleg was thrust forward, the left

likewise, but more slowly. The hind legs relaxed promptly then stiffened, later relaxed and stiffened alternatively.' These signs are similar to those observed in some of the decerebrate rabbit experiments described here following the injection of a solution containing C.E.F. Dow (1938) placed three crystals of physostigmine in contact with the anterior lobe of the cerebellum and found a reduction in amplitude, and sometimes in frequency, of cerebellar activity. He also showed that a similar effect occurred with cocaine (2%), which confirmed Bremer's observation (1922) that when cocaine was topically applied, cerebellar activity was reduced and decerebrate rigidity enhanced.

The experiments of greatest interest are those concerning ACh and anticholinesterases but, unfortunately, previous workers have only briefly investigated the action of these drugs on the cerebellum and no clear conclusions can be based on the information they provide.

The results described in this chapter provide a further study of the action of various substances of pharmacological interest on the electrical activity of the cerebellum. All doses given in this chapter are quoted in terms of the relevant bases.

3.1 Action of brain constituents

Histamine Of the pharmacologically active constituents of the brain, apart from ACh whose action has been described, only histamine has been found to have any excitatory effect on cerebellar activity following intra-arterial injection.

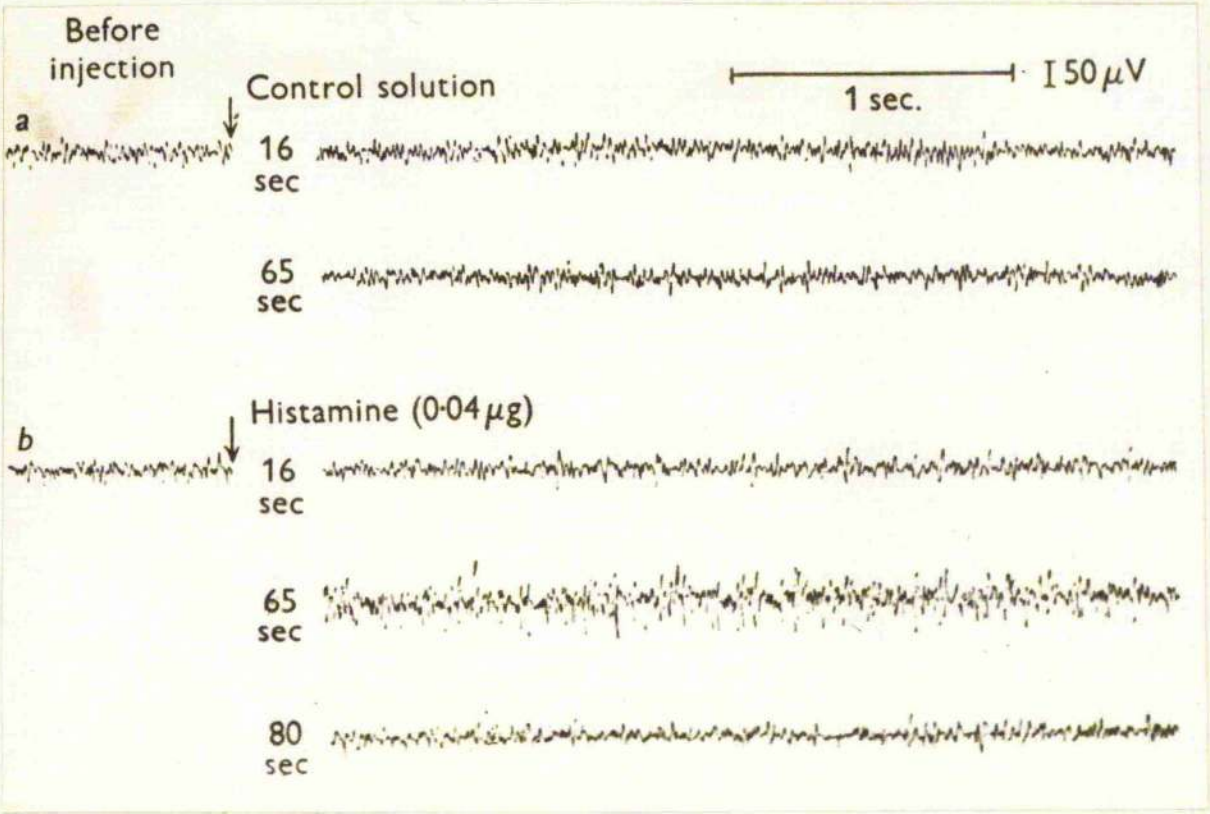


Figure 8 Effect of histamine (0.04 μg histamine base) on the electrical activity of the cerebellum of a rabbit (b). Cerebellar excitation occurred only after a delay of 65 sec.

Figure 8b shows the excitatory effect of 0.04 μg of histamine on cerebellar activity. This response differed from that produced by ACh and brain extracts in that the interval between injection and the appearance of excitation was 61.5 ± 4.1 seconds (26 injections) and the duration of the response was 18.1 ± 2.2 seconds (26 injections). The latency of the response for other substances (Table 3) lay between 10.8 and 13.6 seconds, while the duration was 10.4-13.9 seconds.

When histamine was injected in the presence of inactivated cerebellar extracts, no large changes occurred in the time-course of the response (response latency 71.7 ± 5.2 sec, 11 injections; response duration 21.6 ± 2.2 sec, 11 injections). Injections of 0.04 - 0.13 μg of a solution of histamine inactivated by alkali treatment in the presence of brain tissue extracts gave no cerebellar response. The action of histamine was abolished when the anti-histamine chlorcyclizine ('Diparalene') was given intravenously (1.5 mg/kg) to the test rabbit five minutes before the injection. This treatment did not affect the excitatory action of 20 f.b.e. of a cerebellar extract.

Histamine was injected in doses of 0.04-0.08 μg on the assumption that the histamine concentration in the cerebellum was

about 2.0 μg/g tissue (Kwiatkowski, 1943). More recently, Harris, Jacobsohn & Kahlson (1952) have found only minute traces of histamine in the cerebellum of the dog (<0.2 μg/g) and cat (<0.5 μg/g), although their results for the histamine content of other regions of the nervous system agree with those reported by Kwiatkowski. Recent experiments suggest that the concentration of histamine in the cerebellum may be about 0.2 μg/g tissue in the cat and rabbit (Garven, unpublished). The possible significance of the action of histamine on the cerebellum will be discussed in a later section.

'Sympathin' Vogt (1954) has shown that nor-adrenaline and adrenaline are widely distributed throughout the nervous system, but that the concentration in the cerebellum is relatively low (0.07 μg/g). The action of adrenaline on cerebellar activity was tested by injecting adrenaline (0.01-0.05μg) in 0.9% saline solution and in the presence of TCA extracts of cerebellar tissue which had been acid and alkali boiled and which therefore contained no nor-adrenaline or adrenaline (Gaddum & Schild, 1935; D'Silva, 1937; Weil-Malherbe & Bone, 1954). Under none of the conditions described did adrenaline excite cerebellar activity.

ATP No figures for the concentration of ATP in the

cerebellum are available, but the concentration of ATP in the whole brain of cats and rats has been found to be 1.06-1.54 mg ATP/g fresh tissue (Mcllwain, 1955). Kratzing & Narayanaswami (1953) found the ATP content of the guinea pig cerebrum to be 1.7-1.75 mg/g tissue. Assuming that the ATP concentrations are of the same order in the brains of cats, rabbits and guinea pigs, it is evident that, after the relative sizes of the cerebrum and cerebellum have been allowed for, the ATP concentration in these two areas will be similar. The sodium salt of ATP (Light & Co. Ltd.) was administered to the test preparation in the usual manner in doses of 20-100 µg, both with and without the addition of inactivated brain extracts. These produced no change in cerebellar activity.

The possibility that cerebellar electrical activity might only be excited by the breakdown products of ATP was tested by injecting solutions of ATP which had been incubated for an hour at 30°C with fresh cerebral tissue from which the contained ACh had been removed before testing. Similar amounts of ATP were boiled in a weakly acidic solution, thus effecting a non-enzymic breakdown as described by Barsoum & Gaddum (1935). These solutions were all tested on the decerebrate preparation

(20-100 µg ATP), but none produced any change in the cerebellar activity.

5-Hydroxytryptamine (Serotonin) The possibility that 5-hydroxytryptamine might affect cerebellar activity was tested in the following experiments. Doses of 5-hydroxytryptamine (serotonin creatinine sulphate) ranging from 0.1 - 0.4 µg were injected with or without inactivated brain extracts into the cerebellum. None of these injections caused any change in the normal electrical activity of the cerebellum.

Propionylcholine Propionylcholine iodide (kindly prepared by D.R. Marshall, Chemistry Department, St. Andrews University) was injected in doses of 0.05-0.2 µg in saline solution with inactivated cerebellar extracts. No effect on the ECbG was recorded.

Choline Choline chloride, when administered in small amounts (10-50 µg) gave no cerebellar response. The injection of more than 1.5 mg, however, did enhance cerebellar activity. The production of a response under these circumstances might be expected since large doses of choline often mimic the effect of small quantities of ACh when applied to sensitive biological preparations.

Thiamine Small amounts of thiamine (0.8-1.5 µg/g tissue)

are found in the brain (Mallwain, 1955) and it has been suggested that this substance has an important role in the nervous system (von Muralt, 1943). Injections of a range of doses (0.03-0.06 µg) of thiamine failed to produce any effect on cerebellar activity.

Substance P Small amounts of substance P (0.43-2.8 units/g tissue) are found in the cerebellum (Amin, Crawford & Gaddum, 1954). The injection of substance P (preparation P_5, containing 13.8 Euler units/mg, prepared by Dr. Crawford) alone and in the presence of inactivated brain extracts in doses ranging from 0.35 µg (0.005 units) to 7.0 µg (0.1 unit) had no effect on the electrical activity of the cerebellum.

γ-amino butyric acid Florey (1954) has described the extraction, from mammalian nervous tissue, of an inhibitory principle (Factor I) which appears to be similar to γ-amino butyric acid (Bazemore, Elliott & Florey, 1956). This substance is present in whole brain in a concentration of about 230 µg/g fresh tissue. (Tallan, Moore & Stein, 1954).

γ-amino butyric acid was tested in the usual way using doses of 4-10 µg dissolved in saline or inactivated brain extracts. These injections did not effect the electrical activity

of the cerebellum. When 4-10 μg of γ-amino butyric acid was injected in the presence of 15 and 20 f.b.e. of active cerebellar extract, no inhibition of the excitatory response was observed.

Glutathione and ethanolamine Glutathione and ethanolamine are found in the whole brain in concentrations of 270 μg and 210 μg/g fresh tissue respectively (Tallan, Moore & Stein, 1954). In addition, glutathione has been detected in the cerebellum, but not in the cerebrum (Flock, Block, Grindlay, Mann & Bollman, 1953) and ethanolamine has Rf characteristics similar to those of the cerebellar factor (Chapter 4). Injection of these substances (4-10 μg) alone and in the presence of inactivated brain extracts produced no cerebellar response. The injection of 10 mg of ethanolamine had no effect.

3.2 Actions of other drugs

Strychnine Doses of strychnine (0.03-0.08 mg) dissolved in saline solution were tested for an effect on cerebellar activity. Doses above 0.05 mg produced an excitation of cerebellar activity within 15 seconds of the injection. The response at 0.05 mg was similar in appearance to the response

to 20 f.b.e. of cerebellar extract, but had a longer duration - often as much as 30 minutes.

Doses of strychnine in higher concentrations caused a progressively greater response, the increase in 'spike' activity being particularly noticeable. The effect with these high concentrations lasted about three-quarters of an hour and was often accompanied by muscular twitching.

Eserine The injection of 100 µg of eserine gave strong excitation of cerebellar activity beginning 5 seconds after the injection and continuing for about 4 minutes. At the height of the response, large spike potentials of the type found after the injection of strychnine appeared. 50 µg of eserine gave a smaller response lasting about 2½ minutes, while 25 µg produced a short response identical to that occurring after the injection of 0.03 µg of ACh or 20 f.b.e. of cerebellar extract.

CHAPTER 4

PHYSICAL AND CHEMICAL PROPERTIES OF THE CEREBELLAR EXCITATORY FACTOR

To investigate the physical and chemical properties of the cerebellar excitatory factor, use has been made of the decerebrate rabbit preparation for both qualitative and quantitative determinations. The experimental procedures used in the assay of C.E.F. have been described in Chapter 1. The number of times that each experiment was repeated is shown in the text. This refers to the number of times the complete procedure was repeated and not to the number of test injections. Except in the counter current and chromatographic eluate tests, each unknown solution was injected at least three times and was bracketed with suitable controls.

4.1 Extraction methods

The preparation of TCA extracts of fresh brain tissue has been described in Chapter 2. After the first series of experiments using TCA extracts had been completed, it became

desirable to extract large quantities of tissue in a form suitable for storage over long periods without the loss of activity. It was also interesting to study the extraction of C.E.F. using a variety of procedures. The experiments in this section were designed to investigate these two points.

Acetone dried powders Acetone dried powders of brain tissue were frequently used in later experiments because of their convenience for storing; they also formed a good starting material for the preparation of active solutions containing C.E.F. since the process of acetone drying will extract many other pharmacologically active substances.

After successful pilot experiments had been performed to ensure that C.E.F. was not lost in the preparation of these powders, brain extracts were prepared from sheep killed locally with a humane killer. The complete cerebellums and a sample of the cerebral hemispheres of 100 Half Bred and Black-face sheep were removed within ½-1 hour of death. A total of 1100 g of cerebellum and 2,000 g cerebrum was obtained and this was immersed in sufficient chilled acetone to ensure a minimum final concentration of 80% acetone. Four to five hours later the tissue was homogenized with excess fresh acetone. The homogenate was then

filtered, washed with acetone and ether and dried with constant grinding at room temperature (23°C). The dry powders were stored in an evacuated desiccator over phosphorus pentoxide.

No loss of activity was found on testing samples of the powder at the end of nine months. C.E.F. could be extracted from the acetone dried powders by brief boiling with 0.9% sodium chloride solution, acid ethanol or 10% TCA solution.

Extraction by saline Fresh brain tissue was ground with 1 ml 0.9% saline solution/200 mg tissue and boiled for three minutes. The mixture was cooled, centrifuged and the supernatant fluid decanted. This extract was alkali-boiled (pH 11) to remove acetylcholine and was tested on the decerebrate preparation. These extracts yielded similar quantities of the cerebellar factor as did crude TCA extracts (3 experiments).

If the acetone dried powder was extracted in a similar way but without boiling the mixture, no C.E.F. was found to be present in the extract (3 experiments).

Extraction by acid ethanol Acid ethanol was prepared by mixing 1 ml of concentrated hydrochloric acid with 1 litre of ethyl alcohol, redistilled from charcoal. 2 ml of alcohol were used for each gram of fresh brain and the mixture was ground and

allowed to stand for 90 minutes. The extracts were centrifuged and the supernatant solution evaporated to dryness *in vacuo* at 35-40°C. The residue was dissolved in 0.9% saline and diluted to 200 f.b.e./ml. The solution was boiled with alkali and tested.

The result of testing this solution showed that C.E.F. was extracted by acid ethanol, but not apparently as efficiently as by TCA or boiling saline. The minimum excitatory dose of alcohol extract needed to produce a response was 30 f.b.e. while TCA and boiled saline extracts gave a response with 20 f.b.e. (1 experiment).

The necessity of using acid solvents or an extraction procedure involving boiling indicates that C.E.F. might be loosely bound to some cell constituent, perhaps a protein or lipoprotein.

4.2 Solubility

Solubility in absolute alcohol and 96% ethanol

Acetone dried powder of sheep cerebellum (100 mg) was boiled under a reflux condenser with 10 ml absolute alcohol for two minutes. The resultant mixture was centrifuged and the supernatant solution was decanted off and evaporated to dryness *in vacuo* at 40°C.

The residue was taken up in 2 ml 0.9% saline solution and tested. The experiment was repeated using 96% ethyl alcohol.

The injection of these alcohol-extracted solutions (30 f.b.e.) produced typical cerebellar excitation. C.E.F. is therefore soluble in absolute alcohol and 96% ethyl alcohol (absolute alcohol, 3 experiments; 96% ethyl alcohol, 1 experiment).

Chloroform, ether and benzene 2 ml of a boiled saline extract of 400 mg of acetone dried powder of sheep cerebellum were shaken separately with the appropriate solvent and the solvent phase removed. This procedure was repeated twice. The solvent phases were mixed and evaporated to dryness at 40°C *in vacuo* and the residue was taken up in 0.2 ml 0.9% saline and tested. The aqueous phase was also kept. Any trace of solvent was removed in a stream of air and the solution was tested for activity.

It was found that the aqueous phase was active following extraction with ether (3 experiments) and chloroform (1 experiment) and the solvent phase was inactive. C.E.F. is therefore not freely soluble in these solvents. Activity was detected in both phases of the benzene extraction, but the concentration of C.E.F. was greater in the aqueous phase.

The starting material, a boiled saline extract of

acetone dried powder of sheep cerebellum, gave a cerebellar response with 20 f.b.e. The aqueous phase of the benzene extraction gave a response with 30 f.b.e., but the solvent phase required 120 f.b.e. to produce an effect. The solubility of C.E.F. in benzene under these conditions is therefore about 15% (1 experiment).

Butyl alcohol 2 ml of a boiled saline extract of 400 mg acetone dried powder of sheep cerebellum were saturated with sodium chloride in order to reduce the miscibility of the aqueous and butyl alcohol phases. This solution was extracted twice with 1.5 volumes n-butyl alcohol. The combined butanol layers were evaporated to dryness in vacuo at 40°C and the residue taken up in 2 ml saline and tested.

Activity was found only in the butanol phase, even when large doses of the aqueous layer (120 f.b.e.) were injected. C.E.F. is therefore freely soluble in butanol under these conditions (1 experiment).

4.3 Stability

In the first series of experiments, brain extracts known to contain C.E.F. were boiled briefly (5 sec) at a pH ranging from

2-12. Those solutions were then cooled, neutralized and volumes of 0.9% saline added to provide a suitable dilution for testing (200 f.b.e./ml). Further portions of active brain extracts (400 f.b.e.) were left to stand for periods of time up to 24 hours at room temperature (20°C) at a pH ranging from 1.5 - 7 before being neutralized, diluted and tested.

It was found among the boiled solutions that, when 20 f.b.e. were injected, activity had only been lost where the solution had been boiled at a pH of less than 5, indicating that C.E.F. is unstable to boiling in acid solution. Of the solutions left standing at room temperature under acid conditions, no activity was lost (1 experiment).

During the extraction of fresh brain tissue with 10% TCA, it is necessary to leave the extract at about pH 1.5 for 1½ hours. This treatment caused no apparent loss of activity, since the final solution containing C.E.F. was approximately equipotent with extracts prepared by methods involving less drastic treatment.

The effect of subjecting active solutions to more prolonged heating under various conditions of pH was next investigated. To three 1 ml portions of a boiled saline extract

of acetone dried powder of sheep cerebellum was added 0.25 ml 5N hydrochloric acid to give a final acid concentration equivalent to that of N-HCl (pH 1). This was repeated on further portions of active solution using 5N sodium hydroxide, thus giving a pH of 14 in each tube. Control solutions were provided by three more 1 ml portions of active solution which were subjected to similar treatment as the test solutions, except that 0.9% saline solution was added to them in place of HCl or NaOH.

The solutions were then placed in a boiling water bath. At the end of two minutes, an acid-treated, an alkali-treated and a control solution were taken out. The next set of solutions was removed after 5 minutes and the final set at the end of 10 minutes. The solutions were cooled, neutralised, suitably diluted and then tested on the decerebrate rabbit.

When the active extract had been boiled in acid solution, the activity was lost. In the solutions boiled under alkaline conditions, no detectable loss occurred after boiling for 2 or 5 minutes. In the alkali-treated extract which had been boiled for ten minutes, no activity was found despite the fact that four times the previously active dose was injected. Some loss of

activity occurred in the control solution which had been boiled for ten minutes, 40 f.b.c. being required to produce cerebellar excitation instead of the 30 f.b.c. of the original extract required in this assay. No loss of activity was detected in solutions kept in acid conditions at room temperature for 24 hours. (2 experiments).

pH	Room Temp. (20°C)	Length of time at 100°C (min)			
	24 hours	0.08	2	5	10
1	+	-	-	-	-
1.5	+	-			
2,3,4	+	-			
5	+	-			
6	+	+			
7	+	+	+	+	±
8,9,10,11		+			
12		+			
14		+	+	+	-

Table 4 Stability of the cerebellar excitatory factor to acid and alkali treatment

Summary of results from three experiments.

+ indicates the activity of C.E.F. is not destroyed
- indicates the activity of C.E.F. is destroyed
± indicates the activity of C.E.F. is partially destroyed

C.E.F. is therefore stable to boiling in solutions above

pH 5 if the boiling lasts for less than five minutes. These results are summarized in Table 4 and are discussed in a later chapter.

4.4 Other properties

Chromatographic analysis The application of chromatographic techniques was of somewhat limited value because of the difficulty of performing quantitative assays for C.E.F. on a very large number of column fractions. Some further knowledge of the chemical nature of C.E.F. was gained by using these methods.

C.E.F. was found not to be taken up on the hydroxyl form of the anionic resin I.R.A.-400 which indicates that it is not of an acidic nature (1 experiment). On the hydrogen form of a Dowex 50 column, a strongly cationic resin, C.E.F. was found to be retained. Elution of this column with 4% HCl released the retained C.E.F. This experiment confirmed the basic nature of the substance (1 experiment).

An attempt was made to determine whether C.E.F. was strongly or weakly basic by adsorbing it from an acid solution (pH 4.5) on to a column of Dowex 50 (hydrogen form) and eluting

it with a weakly acid citrate buffer (pH 5.5) followed by N-NaOH. A weakly basic substance is removed with this buffer whereas a strongly basic substance (e.g. histamine) requires NaOH for elution. Although this experiment was repeated twice, no C.E.F. was eluted from the column by the buffer or by the NaOH, possibly because it was evenly distributed throughout all the eluted fractions and thus became diluted to a degree beyond the sensitivity of the assay preparation.

Although ion exchange chromatography is useful for separating mixtures of small molecular weight substances, it was not used for this purpose in this investigation for two reasons. First, efficient use of the technique requires the collection of very large numbers of fractions for testing on decerebrate rabbits and secondly, the method would have required the injection into the living rabbit of unphysiologically high concentrations of the buffer salts used in eluting the column.

The active extracts of acetone dried powder of sheep cerebellum were passed through a column of activated alumina and the resultant effluent tested. Activity was found in concentrations similar to that of the original extract indicating that C.E.F. is not retained on activated alumina (1 experiment).

Paper Chromatography Rf values for C.E.F. were obtained using the following solvent mixtures; butanol:acetic acid:water (4:1:5), phenol:water, collidine:water. Untreated Whatman No. 1 paper was used for the chromatograms. All chromatograms were run at 18-20°C in glass battery tanks. Ascending runs were made for the butanol and phenol chromatograms, while the collidine chromatogram was made using a descending run.

The solvent layers were prepared in the following ways. The butanol-acetic acid-water mixture was shaken for 24 hours and the layers separated. The butanol layer was used. A mixture of phenol and water was heated to 60°C to ensure that they became totally miscible before shaking. After cooling the phenol layer was used. For the collidine-water solvent, redistilled collidine and water were shaken for 24 hours and allowed to separate. The collidine layer was used.

An acid ethanol extract of acetone dried powder of sheep cerebellum was made up to a concentration of 1200 f.b.e./ml acid ethanol. 1 ml of this solution was then applied in bands 2 cm in length to the chromatogram paper and developed overnight in the appropriate solvent. The solvents were removed from the paper by washing in ether and drying.

Since the nature of C.E.F. was unknown, its position on a chromatogram could not be detected by spraying techniques. Therefore, after removal of the solvents, the paper was cut into horizontal strips 3-4 cm wide. These strips were placed between two microscope slides and in contact with a strip of filter paper which dipped into a trough of distilled water. The strips were eluted by allowing the water to pass down them by gravitational attraction for about 12 hours. At the end of this time, 2-3 ml had passed down each strip. The whole system was enclosed in a damp atmosphere within a large vessel to minimize evaporation during the procedure.

Any trace of solvent left in the eluates was removed by extraction with several small quantities of ether, the residual ether from this procedure being removed in a stream of air passed through the solutions. The eluates were tested for activity in the usual way.

When activity was detected in an eluate, the distance from the edge of the corresponding strip and the starting point was measured and this figure was divided by the distance of the solvent front from the starting point. From these figures, an Rf value could be calculated for C.E.F. The active eluates were

boiled at pH 2, cooled, neutralized and tested for activity. In all cases, the activity of the eluates was destroyed by this treatment indicating that, in all probability, their original activity was due to the presence of C.E.F.

Three approximate Rf values were obtained and, although the dangers of speculative identification from these figures alone cannot be over-emphasized, it is interesting to note that, out of many hundreds of substances for which three Rf values have been obtained under conditions similar to those used in this investigation, only one substance has values similar to those shown in Table 5 by C.E.F. This exception is ethanolamine, whose Rf values for these solvents are butanol, 0.33; phenol, 0.65 and collidine, 0.44.

Solvent mixture	Range of Rf values	
	C.E.F.	Ethanolamine
Butanol:acetic acid: water (4:1:5)	0.24 - 0.36 (1 experiment)	0.33
Phenol:water	0.7 - 0.8 (2 experiments)	0.65
Collidine:water	0.4 - 0.5 (1 experiment)	0.44

Table 5 Comparison of Rf values of C.E.F. and ethanolamine

Dialysability 10 ml of a 200 f.b.e./ml solution of saline-boiled extract of sheep cerebellum (acetone dried powder) were placed inside a dialysis tube and dialysed against 5 ml of distilled water for 24 hours. The dialysate was concentrated *in vacuo* at 40°C to 5 ml and tested for activity. It was found that C.E.F. was dialysable, about 70% passing through the membrane in 24 hours (3 experiments).

The fact that C.E.F. is dialysable eliminates the possibility that it is a large molecular weight substance or that it is a protein, polysaccharide or one of the large polypeptides. It does not, however, eliminate the smaller polypeptides, such as substance P, which could pass through the membrane.

Barium precipitation Barium precipitates ATP, its related compounds and Holton's vasodilator substance of the spinal roots (Holton, 1954). 2 ml of a 200 f.b.e./ml solution of a saline-boiled extract of sheep cerebellum (acetone dried powder) were divided into three equal parts and placed in separate test tubes. 0.05 ml of 0.9 M-barium acetate was added to the first two tubes and, after mixing, 0.02 ml M-sodium sulphate was added. These samples were stored at 2°C for 48 hours when

0.07 ml M-sodium sulphate was added to the third tube and a fourth tube, the control, was made up containing 2 ml 0.9% saline, 0.05 ml 0.9 M-barium acetate and 0.02 ml M-sodium sulphate. These solutions were stored under similar conditions. At the end of this time, the solutions were centrifuged and the supernatant portions tested for activity.

No apparent loss of C.E.F. was detected following the precipitation with barium (the barium control solution gave no response) suggesting that C.E.F. is not identical with Holton's vasodilator substance, nor is it ATP, ADP, UTP or other nucleotides which are precipitated by barium.

4.5 Purification methods

Before the action of C.E.F. on various pharmacological and physiological preparations could be examined, it was considered essential to attain some measure of purification of the active factor. Purification of the crude extracts was also necessary before certain chemical tests could be applied and would be necessary before a final identification of the excitatory substance could be made. The aim of the purification procedures was to produce a solution of C.E.F. which would be free from all

other pharmacological substances and yet would leave C.E.F. in a sufficiently high concentration for carrying out the experiments contemplated.

The purification of crude brain extracts presented several difficulties, the chief of which was the certain elimination of the many pharmacologically active substances of low molecular weight in the brain. Acetylcholine and other substances which have been suggested as being nervous transmitters and which are pharmacologically active, such as histamine, substance P, 5-hydroxytryptamine, nor-adrenaline and ATP, would have to be eliminated.

The purification procedures to be described were conducted on a pilot scale at first using only small quantities of active material. This led to the development of a final purification scheme which is described and which is suitable for the large scale production of a purified solution in which the final yield of C.E.F. is high. Quantitative estimations of C.E.F. were made at the end of each purification stage using the procedure already described (Chapter 1).

In all the purification procedures used, the starting material selected was the acetone-dried powder of fresh brain tissue.

This extraction removes several pharmacologically-active substances found in brain tissue (ACh, histamine and 5-hydroxytryptamine). It also removes cholesterol and some free amino acids.

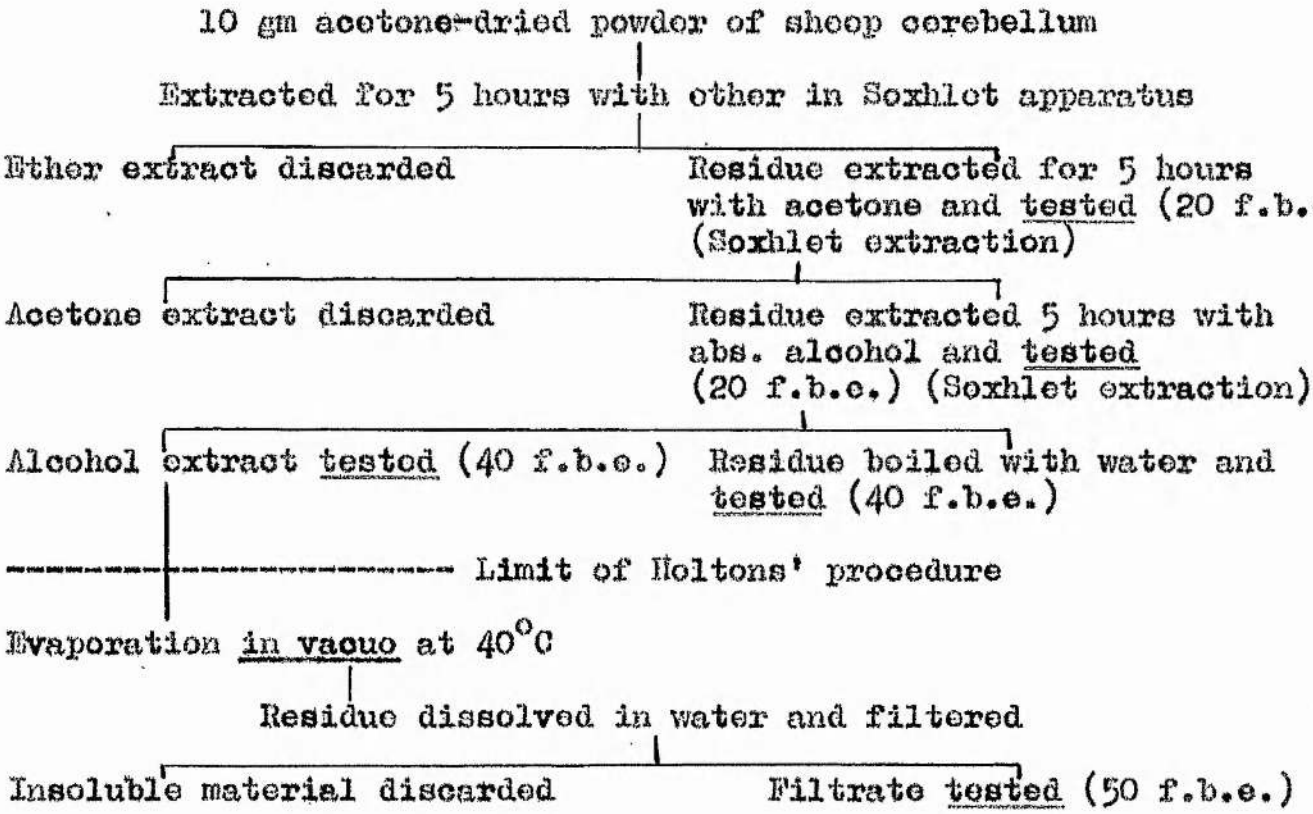
Purification procedure I

Many of the properties of C.E.F. are similar to those of Holton's vasodilator substance found in the spinal roots. The only major dissimilarity results from barium precipitation in which Holton's substance is precipitated while C.E.F. is not.

A purification of crude brain extracts, based on Holtons' method (1945) was used in order to compare further the properties of C.E.F. with Holton's substance and, since the physical and chemical properties are so similar, to attempt the preparation of a concentrated solution of C.E.F. The purification scheme is shown below. A similar procedure was carried out on a sample of acetone-dried powder of sheep cerebrum to act as control during chromatographic and assay investigations.

This purification procedure was somewhat laborious and uneconomical (60% loss of activity), but its interest lay in the comparison it provided between the properties of C.E.F. and

Purification Scheme I



* Dose required to excite cerebellar activity shown in brackets.

Holton's vasodilator substance. Both of these passed through the first two stages (ether and acetone extraction) without loss. In the third stage (absolute alcohol extraction), however, Holton's substance suffered no loss while C.E.F. was detected in approximately equal amounts in both the alcohol extract and the extracted residue. In previous experiments, C.E.F. was found to

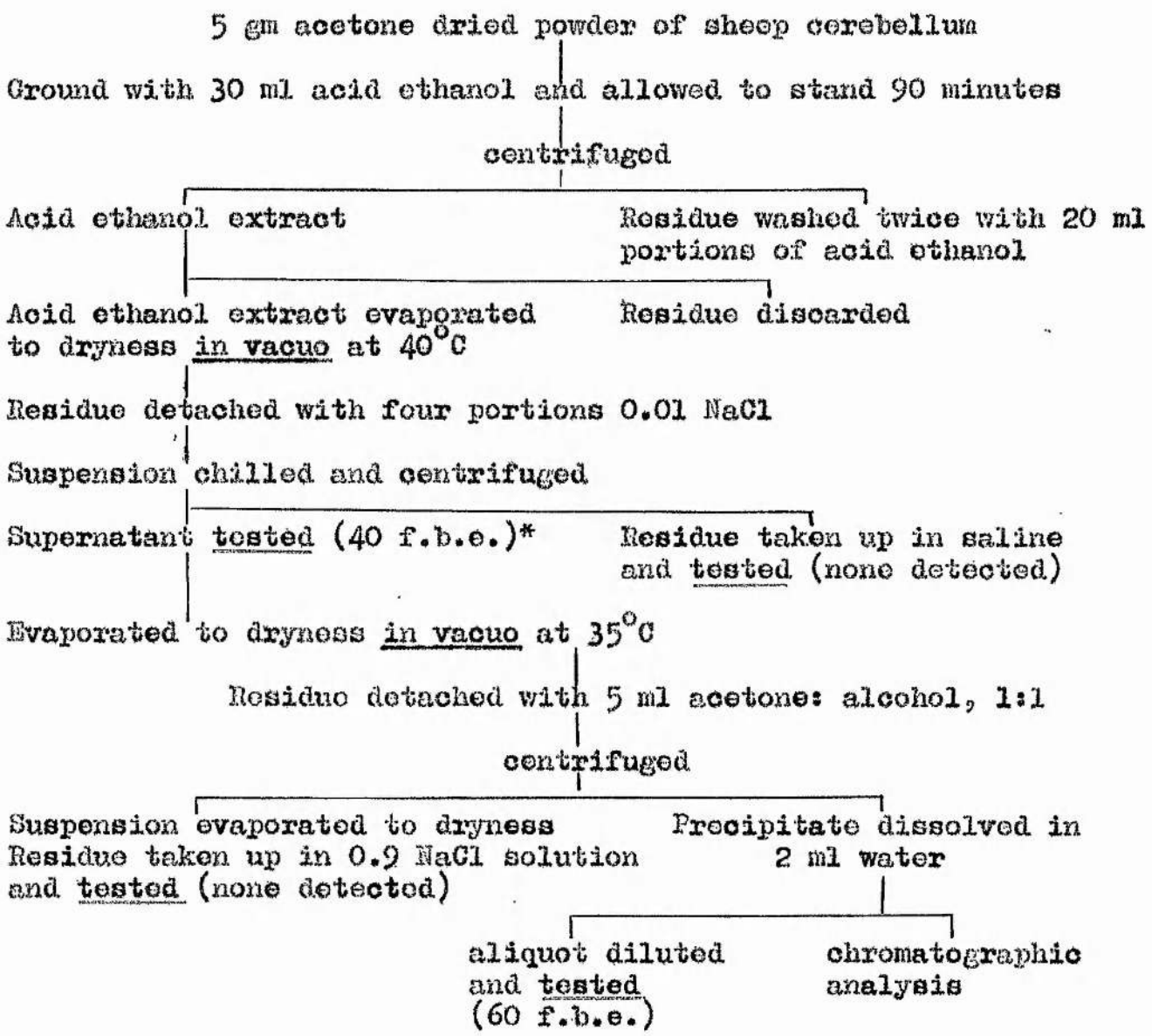
be 33% soluble in absolute alcohol (Chapter 4.2). Following this loss, the purification was continued through the remaining stages of the procedure in order to investigate the efficiency of other extraction methods and to provide a relatively pure but rather small sample of C.E.F. to test chromatographically.

Purification procedure II

In an attempt to obtain purified C.E.F. more conveniently purification procedure II was evolved. This procedure resulted in considerable loss of C.E.F (65%). This might be attributable, as in the previous method, to the large numbers of transfers involved and the drastic treatment accorded to the active solution at various stages.

This procedure was finally abandoned because of its unsuitability for dealing with large amounts of tissue and because of the substantial loss of activity encountered.

Purification scheme II



*Dose required to excite cerebellar activity shown in brackets.

A 5 g sample of acetone dried powder of sheep cerebrum was treated in the same way.

Purification procedure III

The two previous procedures achieved the elimination of compounds of high molecular weight, proteins, polysaccharides and lipids. Some pharmacologically-active substances of small molecular weight were also removed. The methods used to attain this were laborious and unsuited to the extraction of large amounts of material. Virtually the same results might be obtained by starting with an acetone dried powder extract and subjecting it to dialysis. This would achieve the same degree of purification as the previous two methods with a smaller loss of activity, and it has the great virtue of being a simple method applicable to large amounts of material.

The previous attempts at purification had shown that chemical extraction methods alone were not proving satisfactory and it was therefore decided to use a simplified form of Craig's (1950) counter current distribution procedure which, it was thought, might provide an efficient method of separating dialysable substances of low molecular weight.

A twelve tube counter current distribution apparatus was used. 10 ml of the solvent forming the lower layer of the solvent system were placed in each tube and an equal volume of the upper

layer solvent was placed in the first tube; the lower layer in this tube contained C.E.F. in solution. The tubes were revolved by hand for 90 seconds and, when the layers had separated, the top layer in the first tube was transferred to the second tube. 10 ml of fresh solvent were added to the first tube and the cycle repeated until a total of 12 transfers had been made by the original upper layer.

The final position of a substance after counter current extraction is dependent upon its partition coefficient between the solvents used. The fraction of the substance present in the rth tube after n transfers is given by the formula:

$$T_{n-r} = \frac{n!}{r!\,(n-r)!}\left(\frac{1}{k+1}\right)^{n} K^{r}$$

where K is the partition coefficient.

The assay preparation was not able to provide sufficiently accurate quantitative estimations to determine a partition coefficient for C.E.F. between the two solvents, but, as will be shown, some degree of purification was obtained by this method; the original amount of C.E.F. being confined to about three counter current tubes at the end of the extraction.

This technique was incorporated in purification procedure III.

Purification scheme III

30 g acetone dried powder of sheep cerebellum

↓

Boiled 2-3 min. with 6 times its weight of water

↓

Mixture transferred to 1" dialysis tubing and dialysed against 100 x its weight of distilled water for 60 hours at room temperature (20°C) with constant stirring

↓

Residue in dialysis tubing discarded

Dialysate concentrated to 10 ml in vacuo at 40°C

↓

Extracted three times with ether

↓

Ether extract discarded

Aqueous solution evaporated to dryness at 40°C in vacuo

↓

Residue detached with 10 ml butanol:acetic acid:water (4:1:5) or butanol:NaCl and water

↓

centrifuged

↓

Insoluble material discarded

Supernatant subjected to counter current extraction (I and II) and tested on decerebrate rabbit preparation and chromatographically

The first solvent system for counter current extraction (counter current I) consisted of butanol:acetic acid:water (4:1:5), the residue from the previous extraction stage being dissolved in 10 ml of the lower layer of the solvent mixture. An equal volume of the upper layer of the mixture was added. After the

counter current procedure was completed, each of twelve solutions was evaporated to dryness at 30-35°C *in vacuo* and the residue taken up in 5 ml ethyl alcohol. 0.5 ml of the alcoholic solutions was evaporated to dryness *in vacuo* (30-35°C) and the residue dissolved in 0.9% saline and tested for activity and analysed chromatographically.

The second solvent system (counter current II) consisted of butyl alcohol:water saturated with NaCl. The residue from the extraction was dissolved in 10 ml of the lower layer of the solvent, the upper layer (10 ml) forming the mobile phase. The counter current extraction was carried out as before, but instead of assaying the combined top and bottom layers for activity as is normally done, the top layer only was taken to dryness and the residue dissolved in 3 ml water and tested for activity. This separation of the layers was necessary because the high salt concentration of the lower layer would have rendered chromatographic and physiological assessment impracticable. The lower layer was diluted and tested on the decerebrate rabbit preparation.

The counter current extraction procedures were only partially successful. It was found on testing counter current I on the decerebrate rabbit that activity was distributed over at

least three tubes (detected in 1, 2 and 3) with the second tube containing most of the activity. The actual amounts required to excite cerebellar activity were: tube 1, 550 f.b.e.; tube 2, 400 f.b.e.; tube 3, 500 f.b.e. The activity in these tubes was destroyed on acid boiling. This represented a total C.E.F. loss during the extraction and purification process of about 85%.

The second extraction (counter current II) resulted in the distribution of C.E.F. over tubes 8, 9 and 10 in the following manner: tube 8, 190 f.b.e.; tube 9, 100 f.b.e.; tube 10, 180 f.b.e. The activity in these tubes was destroyed by acid boiling. This represents a 60% loss of C.E.F. during the extraction.

The solutions obtained from counter current extractions I and II were subjected to paper chromatographic analysis for two reasons. First, it was hoped it might be possible to demonstrate the cerebellar factor as a chromatogram spot and secondly, that the analysis would provide good evidence as to the purity of the solutions.

In all the attempts at chromatographic analysis when samples of a purified cerebellar extract were being tested, an equal quantity of similarly treated cerebral extract was run on

a second chromatogram and treated with the same sprays. By comparing cerebellum and cerebrum strips, it was hoped that some difference might be detected. Spots appearing on the cerebellum strip but not on the cerebrum strip would be tested for activity on the ECbG. If the cerebellum spot proved to be the cerebellar factor, the type of spray reagent to which it was sensitive would be known, thus greatly facilitating future analysis and identification.

The difficulties likely to arise when attempting to demonstrate the cerebellar factor on a chromatogram by spraying will be appreciated when it is realised that, for instance, for amino acids at least 2-10 µg must be present before a positive ninhydrin reaction becomes visually identifiable. If the concentration of C.E.F. in the cerebellum is of the same order as ACh in the cerebral cortex, then the entire C.E.F. content of 1-5 g of fresh cerebellum would be required to give one ninhydrin spot.

Spots from each concentrated counter current solution (1,000 f.b.e. each) were placed on the chromatograms and were developed in butanol:acetic acid:water (4:1:5) and phenol:water (Chapter 4.3) for 15 hours. After removal of the solvents and

drying, they were sprayed with suitable reagents to show amino groups, imidazole groups, adrenaline, indoles and carbohydrate derivatives.

Analysis of tube 2 (counter current I), which had been found to excite cerebellar electrical activity, showed that it contained about ten ninhydrin positive compounds in very large amounts. A similar result was obtained with the corresponding cerebrum strip and no difference between the two strips was observed.

When the most active solution from counter current II (tube 9) was examined chromatographically, it was found to contain only three ninhydrin positive spots, all with a low Rf value. Most of the ninhydrin positive spots appeared in tubes 1 and 2 (cf. counter current I). Despite the use of sprays sensitive to the presence of imidazole groups, adrenaline, indoles and carbohydrate derivatives, no further spots were found from tube 9. The corresponding cerebrum strip showed no differences when compared with the cerebellar strip.

It must be concluded that, although C.E.F. was known to be present in tube 2 (counter current I) and tube 9 (counter current II) because of the action of these solutions on the

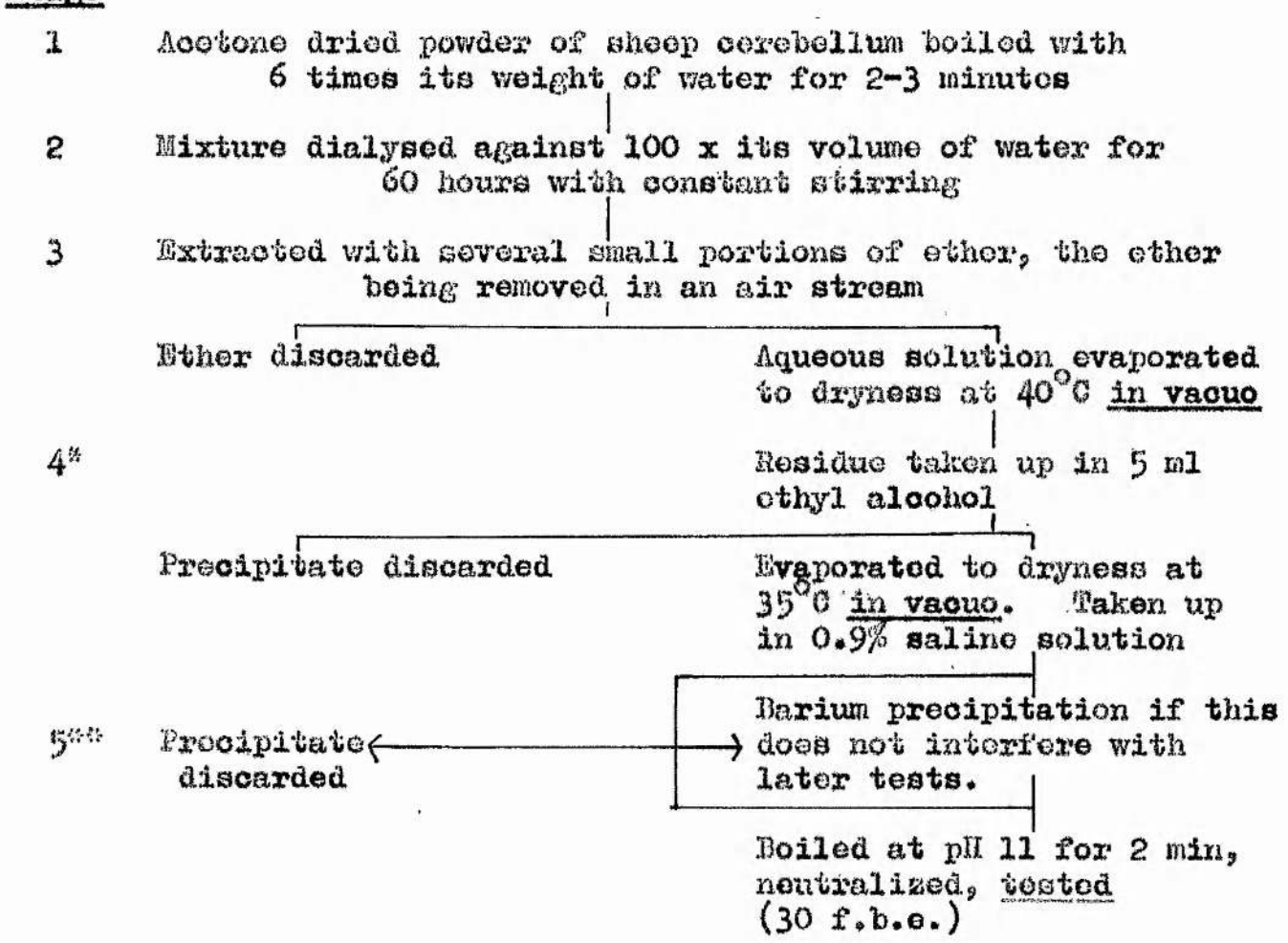
cerebellum, C.E.F. could not be demonstrated as a chromatogram spot, either because it was not present in sufficient amounts or, more probably, because it was not sensitive to the sprays used.

Purification procedure IV

None of the purification procedures so far described has proved entirely satisfactory. Their main faults are their uneconomical yields of C.E.F., their laborious nature, their unsuitability for use with large quantities of material and the doubtful extent of purification achieved. They have, however, provided a basis for the purification procedure (procedure IV) now in use. This purification does not provide the cerebellar substance in a sufficiently pure state for chemical analysis, but it does provide a solution which is free from all known pharmacologically-active substances, except C.E.F., and from cell constituents such as lipids and proteins. It is therefore a satisfactory purification for testing the activity of C.E.F. on physiological and pharmacological preparations. The procedure also has the advantage of being applicable to large quantities of material and gives high yields of C.E.F. (the loss of C.E.F. is approximately 33%).

From a knowledge of the characteristics of various brain

Purification scheme IV



*Stage 4 (ethyl alcohol) was usually omitted

**Stage 5 was omitted when it was considered that barium ions might have a detrimental effect on the preparation on which the solution was to be tested.

constituents, it is possible to say at what stage in this extraction process they have been eliminated. Substances have

only been included in this list if their elimination is certain. If any doubt was found to exist (for example, in the destruction of histamine by alkali treatment) that substance has not been included.

List of substances eliminated in Procedure IV

Stage 1 (Acetone dried powder)
ACh, histamine, 5-hydroxytryptamine, bradykinin.

Stage 2 (Dialysis)
Large molecular compounds including proteins and most lipids.

Stage 3 (Ether extraction)
Further lipids, substance I, Ungar & Parrot's substance.

Stage 4 (Ethyl alcohol)
Holton's vasodilator substance, Hellauer & Umrath's vasodilator substance.

Stage 5 (Barium precipitation)
ATP, UTP, etc., nor-adrenaline, adrenaline, further histamine, 5-hydroxytryptamine and Holton's vasodilator substance.

Stage 6 (Alkali boiling)
Thiamine, further ACh, adrenaline, 5-hydroxytryptamine, adenosine and derivatives, Hellauer & Umrath's vasodilator substance.

Samples of sheep cerebral hemispheres and of inactivated (acid-boiled) cerebellum which had been subjected to this purification procedure were tested on several conventional pharmacological assay preparations, but, even in fairly high

concentrations, it was found that they exerted no obvious effects (Chapter 5.2). Brain tissue extracted and purified by this method has been used in the investigation of the physiological and pharmacological properties of the cerebellar factor reported in the next chapter.

CHAPTER 5

PHYSIOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF THE CEREBELLAR EXCITATORY FACTOR

5.1 Physiological properties

5.11 The enzymic destruction and synthesis of the cerebellar factor

A study of the enzymes associated with possible transmitter substances has received almost as much attention as the active substances themselves and some interesting results have been obtained. It was thought appropriate, therefore, to investigate the enzymic destruction and synthesis of the cerebellar excitatory factor.

Enzymic destruction In a preliminary series of experiments, the brains of anaesthetised, craniotomised rabbits were frozen *in situ* with liquid oxygen (Crossland & Merrick, 1954). One half of the cerebellum was then removed, weighed and quickly ground in cold saline and boiled. The other half was removed, weighed and allowed to thaw before being ground in saline and boiled. On testing these solutions, activity was found only in

the extract which had not been allowed to thaw.

This result is similar to that obtained for acetylcholine in the brain (Crossland, 1951; Crossland, Pappius & Elliott, 1955) and suggests that, in the intact cell, C.E.F. is protected from an inactivating enzyme in rather the same way that ACh is protected from the action of cholinesterase. Further evidence that the destruction was of an enzymic nature was provided by the observation that, when the frozen cerebellum was ground, without thawing, in 10% TCA solution, no destruction of C.E.F. occurred - presumably due to the destruction of the inactivating enzyme.

In the second series of experiments, TCA extracts of rabbit cerebellum were prepared, the TCA removed and the extracts neutralized. Two rabbits were anaesthetised with pentobarbitone sodium (Nembutal, 30 mg/kg I.V.) and the cerebellums removed, weighed and ground with 0.9% saline. To 1 ml of the TCA extract (200 f.b.e.) was added 0.5 ml of the fresh cerebellar homogenate (100 mg) and the mixture was incubated for one hour at 37°C. At the end of this time, the mixture was boiled to destroy the enzymes. It was found on testing these solutions that all their activity had been lost. Further experiments were therefore

designed to study details of this destruction. Mixtures for incubation were again made from TCA extracts of cerebellum (TCA removed) with fresh cerebellar homogenate providing the inactivating enzyme. Fresh cerebellar homogenates which had been boiled were used as controls. It was realized that this treatment, which destroyed any C.E.F. inactivating enzyme, would also release C.E.F. from the brain tissue into the control solution. Any loss of C.E.F. from these boiled solutions during incubation would not be due to enzymic activity. By boiling the test solutions at the end of incubation, C.E.F. would be released from the homogenate unless it had been enzymatically destroyed during the incubation. The test and control solutions were made up as shown in Table 6 before incubation at 37°C.

Table 6 shows that the activity in mixtures of TCA extracts and unboiled homogenates (tubes 1,2,7 and 8) was destroyed on incubation, but that no loss of activity occurred in active mixtures where the enzyme was absent or had previously been destroyed by boiling. This suggests the presence in fresh cerebellar homogenate of an enzyme capable of destroying the cerebellar factor.

The possibility that strychnine or eserine would affect

Tube No.	TCA ext. of Cb.	Fresh Cb. Homog.	Fresh Cb. Homog. (boiled)	Incub. Time	Result
	200 fbe/ml	200 mg/ml	200 mg/ml	Hours	
	ml	ml	ml		
1	1	0.5	-	1	-
2	1	0.5	-	2	-
3	1	-	0.5	1	+
4	1	-	0.5	2	+
5	1	-	-	1	+
6	1	-	-	2	+
7	1	0.5	-	1	-
8	1	0.5	-	2	-

+ indicates no destruction of C.E.F.
- indicates the destruction of C.E.F.

Table 6

the destruction of the cerebellar factor was considered in the following experiments. A boiled saline extract of acetone dried powder of sheep cerebellum was made up to a concentration of 600 f.b.e./ml. The inactivating enzyme was derived from rabbit cerebellar homogenate, 0.5 g fresh cerebellum in 2.5 ml of 0.9% saline solution. For this extraction, the rabbit was killed by a blow on the head, since the use of anaesthetics was considered inadvisable. The following mixtures were then prepared and incubated, with shaking, at 37°C for two hours. After this time

they were centrifuged, decanted, boiled and tested for activity.

Tube No.	Boiled saline extract of cerebellum	Fresh Cb. Homog.	In the presence of	Result
	(600 f.b.e./ml)	(200 mg/ml)		
1	2 ml	0.6 ml	0.3 ml saline	-
2	2 ml	0.6 ml	0.3 ml strych. (1 in 10,000)	+
3	2 ml	0.6 ml	0.3 ml eserine (1 in 50,000)	-
4	2 ml	0.6 ml (boiled)	0.3 ml saline	+
5	2 ml (acid boiled)	0.6 ml saline	0.3 ml strych. (1 in 10,000)	-
6	2 ml (acid boiled)	0.6 ml saline	0.3 ml eserine (1 in 50,000)	-

+ indicates no destruction of C.E.F.
- indicates the destruction of C.E.F.

Table 7

It will be seen that the destruction of C.E.F was unimpaired by the addition of eserine (1:50,000) (tube 3) but, in the presence of strychnine (1:10,000), the destruction was inhibited (tube 2). Control solutions in which the active factor in the original solutions had been destroyed by acid boiling (tubes 5 & 6) and which had no homogenate added were inactive although

they contained similar amounts of eserine and strychnine as were placed in tubes 1-3. This indicates that the responses obtained were not due to the presence of strychnine or eserine. These results suggest that strychnine inhibits the enzymic destruction of C.E.F., whereas eserine has no apparent effect. This observation is fully discussed in Chapter 7 since it is of some interest in view of the action of strychnine on the enzymes associated with other suggested transmitter substances.

Polypeptides, such as substance P, are destroyed by proteolytic enzymes including trypsin. 7 mg of crystalline trypsin (Canada Packers Ltd.) were dissolved in 5 ml distilled water. The activity of the trypsin was shown by its ability to hydrolyse crystalline ovalbumin, the hydrolysis products of which were demonstrated by spraying paper chromatograms with ninhydrin. 2 ml of the trypsin solution were added to a 2 ml (1,200 f.b.e./ml) solution of C.E.F. and incubated at pH 7 for one hour at 37°C. A further 2 ml of the trypsin solution were boiled for four minutes and added to an extract containing C.E.F. to serve as a control. After one hour, these solutions were boiled, cooled and tested for activity. Both of them proved to be active indicating that C.E.F. is not susceptible to hydrolysis by trypsin.

It has been shown that histamine exerts an excitatory action on the electrical activity of the cerebellum. To eliminate the possibility that C.E.F. is identical with histamine, a solution containing C.E.F. was incubated with histaminase. A solution of histaminase was prepared from pig kidney (by Dr Jean Garven, Physiology Department, St. Andrews University) and was shown, in a dilution of 1 in 10, to destroy 0.1 µg of histamine in 40 minutes. 2 ml of this histaminase solution were incubated at pH 7 and at 37°C with 2 ml (1,200 f.b.e.) of a boiled saline extract of sheep cerebellum (acetone dried powder). A solution of histaminase boiled for five minutes was used as a control under similar conditions. After incubation, the solutions were boiled, cooled and tested. No detectable loss of activity occurred in either solution which suggests that C.E.F. is not inactivated by histaminase.

The significance of the results obtained in this study of the enzymic destruction of C.E.F. will be discussed in Chapter 7.

Enzymic synthesis The ability of cerebellar tissue to synthesize the cerebellar factor was tested on two occasions. Since the conditions favouring the synthesis of C.E.F. were unknown, it

was not possible to use acetone-dried powders of cerebellar tissue in this investigation. Fresh tissue was used and a method similar to that described by Quastel, Tonnenbaum & Wheatley (1936) for the synthesis of ACh from fresh brain was employed but with the addition of small amounts of strychnine (1:10,000) to preserve the active substance. After incubating these solutions for up to three hours, they were tested for activity in the usual way, but no evidence was found to suggest that synthesis of the cerebellar factor had taken place. These experiments do not exclude the possibility that synthesis can occur under more favourable conditions.

5.12 The release of the cerebellar excitatory factor from the cerebrum and the cerebellum

One of the characteristics one might expect of a chemical transmitter substance would be its release from a 'bound' form during excitation of the nervous structures with which it is associated. In this investigation, the release of the cerebellar factor from both the cerebellum and from the cerebrum was studied under various conditions of excitation and anaesthesia. Medium-sized rabbits were used and exudates were collected simultaneously from the surface of the cerebellum and cerebrum for periods of up

to three hours.

Three types of rabbit preparation were used for the collection of exudates; the anaesthetized, the decerebrate and the anaesthetized and decerebrate animal. From each of these preparations samples of brain exudates were collected from the animal at rest, from the leptazol-stimulated animal and from the electrically-stimulated animal. The first type of preparation was anaesthetized with 'Dial' (70 mg/kg I.P.) and a wide incision was made in the skin overlying the skull and the dorsal muscles of the neck. The skin and muscles were reflected and trephine holes (area 0.5 sq. cm) made in the skull over the cerebrum and anterior lobe of the cerebellum. The underlying dura was then removed to expose the brain. When all bleeding had ceased, irrigation of the surfaces of the cerebellum and cerebrum was begun using Ringer-Locke solution (0.15 g $NaHCO_3$, 0.24 g $CaCl_2$, 0.42 g KCl, 9.2 g NaCl, 1 g dextrose/litre). It was not considered necessary to buffer this solution, since pH changes occurring while the fluid was in contact with the brain would only become serious if the fluid and brain were in contact for long periods of time (Jasper & Elliott, 1949). This did not occur in these experiments, but the fluid was made slightly alkaline

(pH 7.4) as recommended by Jasper & Elliott, so that any acid production by the brain would not cause the fluid to become acidic.

The fluid, which contained strychnine (1 in 10,000) in order to preserve any liberated C.E.F., was passed from a flask through a drop counter and a constant pressure head. The flow was arranged to be 0.1-0.02 ml/min by means of an adjustable clip. This rate was kept constant throughout an experiment.

After leaving the pressure head, the fluid passed through a water jacket at 37°C and thence on to the exposed cerebrum. An identical apparatus was used to irrigate the cerebellum. Narrow bore glass tubes led off from the cerebellum and cerebrum to which suction was constantly applied from a vacuum pump. The irrigation fluid was sucked up these tubes and trapped in small test tubes. When the irrigating fluid was perfusing steadily, its collection was begun and was continued for either 30 or 60 minutes, depending on the state of the animal. In order to provide strong stimulation to the cerebellum and cerebrum, blunt, bipolar silver electrodes were placed on the exposed surfaces of these areas. The distance between the electrodes was 4 mm and the strength of the stimuli (1 msec. duration via an induction coil) was kept constant at 3 volts. Finally, leptazol (60 mg/kg I.P.)

was injected and a third sample obtained.

The second type of preparation was decerebrated in the usual way, respiration being supported artificially if necessary. As in the previous experiments, control, leptazol-convulsed and electrically-stimulated brain exudates were collected over periods of 30 or 60 minutes. The third type of preparation was anaesthetized with 'Dial' (70 mg/kg I.P.) and then decerebrated and the experiment continued as before.

The volume of each sample obtained was noted and the solutions were briefly boiled, neutralized and frozen until required for assay. The samples were assayed for C.E.F. in the usual way on the decerebrate rabbit. The results are presented in Table 8.

Whenever practicable, pen-oscillograph records were made of the electrical activity from the cerebrum and cerebellum during the collection of exudates, but the number of records that could be obtained from the different preparations was limited, for the uncomplicated activity of the cerebrum cannot be recorded from a decerebrate animal and cerebellar activity cannot be recorded from a non-decerebrate or deeply anaesthetized animal. Records of activity during electrical stimulation could not be

Condition of animal preparation			
	Anaesthetized	Unanaesthetized decerebrate	Anaesthetized decerebrate
Cerebrum			
Normal	0 0 0 0	0 0 0	
Leptazol stimulated	0 0 0	0 0 0	0 0 0
Electrical stimulation	0 0	0 0	
Cerebellum			
Normal	0 0 0	0 0 0 0	
Leptazol stimulated	0 0 0	85 75	0 0
Electrical stimulation	95 120 80	225 145 90	

Figures for the release of C.E.F. are expressed as fresh brain equivalents released/cm^2 cortex/hour

Table 8 Release of the cerebellar factor from the cerebrum and cerebellum

made because of interference from artifacts. Records were therefore taken following directly on the cessation of electrical stimulation.

The main conclusions that may be drawn from the results shown in Table 8 are that C.E.F. is liberated from the surface of the cerebellum under certain conditions of stimulation but not, however, from the surface of the cerebrum even during extreme excitation.

The anaesthetized and decerebrate preparation was studied to find whether the damage to nervous structures caused by decerebration could have been responsible for the release of C.E.F. It was found that when the anaesthetized and decerebrate animal was given leptazol no C.E.F. was released from the cerebellum. This suggests that the act of decerebration did not itself cause the release of C.E.F. This conclusion was confirmed in the experiments on the unstimulated, decerebrate animal when no C.E.F. was produced.

It will be seen that the amount of C.E.F. released from the cerebellum bears a direct relationship to the level of excitation of the brain; the greater the excitation, the greater the release of C.E.F.

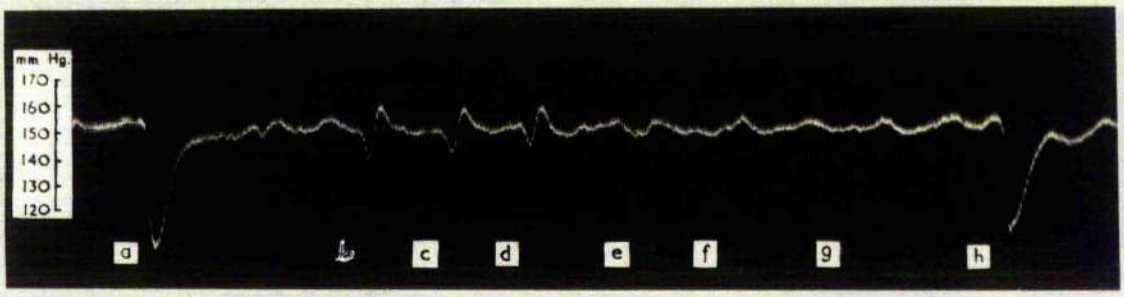
It was evident from a study of the electrical records that those made following electrical stimulation of the brain were of an amplitude never found under physiological conditions. The results of this strong stimulation must therefore be regarded as being physiologically abnormal. The results of this series of experiments will be discussed in Chapter 7.

5.13 Action of the cerebellar factor on blood pressure

In order to detect any action of brain tissue extracts which might be specifically ascribed to the cerebellar factor, extracts were tested on the blood pressures of cats and rabbits. It would be extremely difficult, when testing an extract containing the cerebellar factor together with other pharmacologically active substances, to determine which effects were due to C.E.F. and which to some other compound. This difficulty was overcome in two ways. First, when crude extracts were tested, they were controlled by solutions made by preparing an ACh-free extract of cerebral hemispheres or by acid-boiling cerebellar extracts. Neither of these solutions then contained C.E.F. and a comparison of their effects and the effects of extracts which did contain C.E.F. could be made on the cat and rabbit blood pressures. Secondly, a purified extract was produced (Chapter 4,

procedure IV) which contained no known pharmacologically-active constituent of brain except C.E.F. These purified solutions were tested on cat and rabbit blood pressures, using acid-treated, purified extracts of cerebellum as a control.

Figure 9 shows the blood pressure record from a cat anaesthetized with pentobarbitone sodium (nembutal, 30 mg/kg).



Cat anaesthetized with nembutal (30 mg/kg I.P.) and heparinized (600 I.U./kg I.V.). Injections shown on lower signal trace. Continuous record. Extracts (e)-(g) purified by procedure IV. ACh removed from all brain extracts.

a) ACh 0.25 µg
b) Crude cerebrum (30 f.b.e.)
c) Crude cerebellum (30 f.b.e.)
d) Crude cerebrum (30 f.b.e.)
e) Acid-boiled cerebellum (30 f.b.e.)
f) Cerebellum (30 f.b.e.)
g) Cerebellum (60 f.b.e.)
h) ACh 0.25 µg

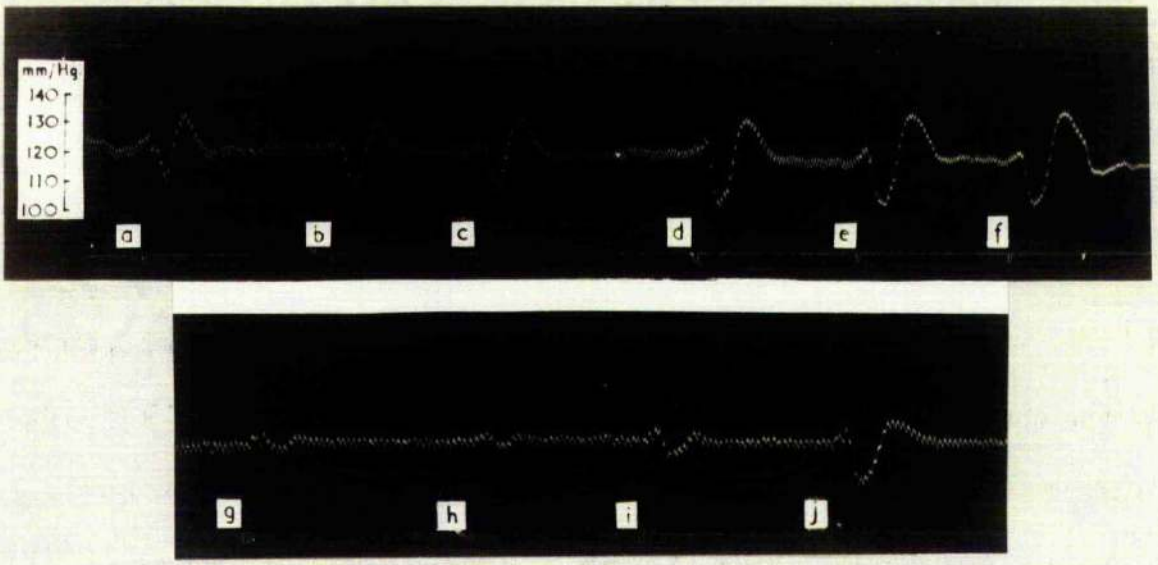
Figure 9 The effect of ACh and brain extracts on the blood pressure of the cat

Substances for testing were introduced into a cannula in the femoral vein in small volumes (0.3 ml) and were washed in with 0.4 ml 0.9% saline solution.

Injections (b), (c) and (d) show the effect of injecting 30 f.b.e. of crude TCA extract (TCA and ACh removed) of cerebrum and cerebellum. It will be seen that, although a small biphasic response did occur, it was the same whether extracts of cerebellum or cerebrum were injected. Injections (e), (f) and (g) show the response to injections of similar amounts of purified cerebellar extract and its acid-boiled control. No alteration in blood pressure occurred, despite the fact that one of the doses injected was 60 f.b.e. of cerebellar extract. Brain extracts were tested on the blood pressures of two other cats with similar results.

In another series of experiments, crude and purified extracts were prepared as before and tested on the blood pressure of an atropinised rabbit (2 mg/kg). Figure 10 shows the results of some of these injections. Injections (a)-(f) show the effect of injecting 20 and 40 f.b.e. of a crude TCA extract (TCA and ACh removed) of fresh cerebellum and cerebrum. As in the cat blood pressure experiments, a biphasic response occurred

and was always similar for equal doses of cerebellum and cerebrum. Injections (g) and (h) demonstrate the ineffectiveness of the purified extracts and their controls (40 f.b.e.) to produce a response, while injection (i) represents the administration of



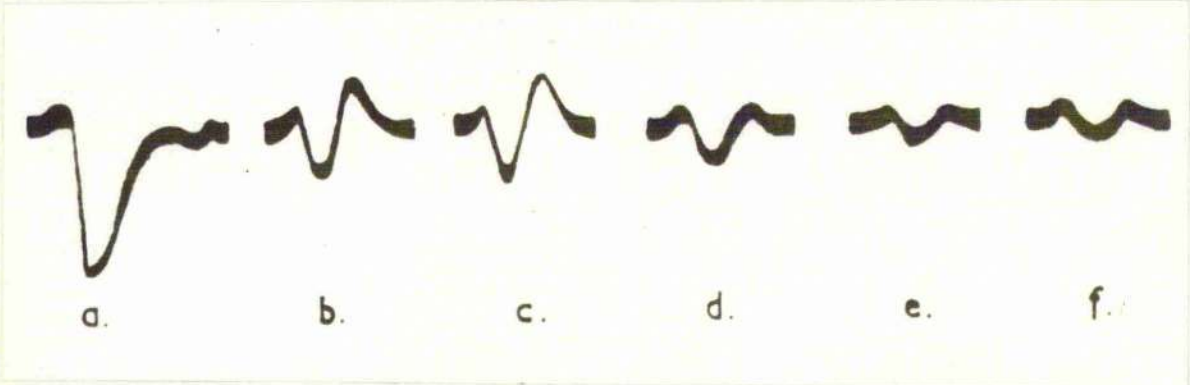
Rabbit anaesthetized with nembutal (30 mg/kg I.V.) and heparinised (600 I.U./kg I.V.). Atropine 2 mg/kg (I.V.). Injections shown on lower trace. All extracts have their contained ACh removed. Extracts (g)-(i) purified by procedure IV.

a) Cerebellum, TCA extract, 20 f.b.e.
b) Cerebrum, TCA extract, 20 f.b.e.
c) Cerebellum, TCA extract, 20 f.b.e.
d) Cerebellum, TCA extract, 40 f.b.e.
e) Cerebrum, TCA extract, 40 f.b.e.
f) Cerebellum, TCA extract, 40 f.b.e.
g) Cerebellum, 40 f.b.e.
h) Cerebrum, 40 f.b.e.
i) Cerebellum, 80 f.b.e.
j) Cerebellum, TCA extract, 20 f.b.e.

Figure 10 The effect of ACh and brain extracts on the blood pressure of an atropinized rabbit

80 f.b.e. of cerebellum (purified extract), still without producing a response. These results were confirmed on two further rabbit blood pressure preparations.

After subjecting crude brain extracts to the different extraction procedures already described, a comparison of their effects on the blood pressure was made. This was done to find whether extracts which had undergone purification had less effect on the blood pressure than the crude extracts. Figure 11



Rabbits anaesthetized with nembutal (30 mg/kg I.V.) and heparinized (600 I.U./kg). Extracts (b)-(f) have their contained ACh destroyed. Records (a)-(c) from one rabbit, records (d)-(f) from a second rabbit.

a) Untreated cerebrum, TCA extract, 40 f.b.e.
b) Cerebrum, TCA extract, 40 f.b.e.
c) Cerebellum, TCA extract, 40 f.b.e.
d) Saline extract of cerebrum (acetone dried powder) 40 f.b.e.
e) Cerebrum, 80 f.b.e. (purification procedure IV)
f) Cerebellum, 80 f.b.e. (purification procedure IV)

Figure 11 Blood pressure response to brain extracts in various stages of purification

summarizes the blood pressure responses following the injection of extracts in various stages of purification. Figure 11 (a) shows the effect of injecting a crude TCA extract of cerebrum without the removal of its contained ACh. Removal of the ACh by alkali treatment destroys most of the depressor response and reveals some pressor activity (Fig.11 (b)). Fig. 11 (c) shows a similar response after injecting 40 f.b.e. of cerebellar extract treated in the same way. On a second rabbit the response to an extract made from acetone dried powders of sheep cerebrum (Fig. 11 (d)) is small. These powders have always been used as the starting material for the preparation of purified extracts of C.E.F. for testing on pharmacological preparations. Finally, Fig. 11 (e) and (f) show the responses obtained from extracts of cerebrum (80 f.b.e.) and cerebellum (80 f.b.e.) purified by procedure IV (Chapter 4).

From these experiments, it may be concluded that C.E.F. has no marked action on the blood pressure of the cat or rabbit under the conditions described. The investigation has, however, confirmed that the purification procedures used have probably eliminated all the pharmacologically-active constituents of brain known to have a pressor or depressor action on blood pressure and

that C.E.F. is unlikely to have affected cerebellar electrical activity indirectly by acting on the systemic blood pressure.

5.14 The effect of the cerebellar factor on the blood vessels of the brain

The dependence of the spontaneous electrical activity of the cerebellum on the maintenance of a good blood pressure has been emphasized by Adrian (1935) and by Dow (1938). Slight variations in blood pressure cause considerable changes in the amplitude of the electrical fluctuations, while a fall of blood pressure below about 55 mm Hg usually causes their abolition. It is reasonable to suppose that variations in blood flow brought about by small changes in the calibre of the blood vessels of the brain might affect the electrical activity of the cerebellum in a similar way. In this connection, it is perhaps relevant to mention that it has been suggested (Waterson & Macdonald, 1939) that acetyl-β-methylcholine and carbaminoyl choline, when delivered intravenously, exert an action on nervous activity, not by acting directly on nervous tissue, but by dilating the cerebral blood vessels.

The possibility that C.E.F. caused an increase in cerebellar activity by altering the blood supply to the brain rather than by

acting on nervous tissue must be considered. That C.E.F. does not affect the systemic blood pressure of rabbits in the doses used in the assays was shown in Chapter 5.13, and it therefore remained to study the effect of the active factor on the cerebral and cerebellar vascular systems.

The method used for the investigation has already been described in detail (Mitchell, 1954) and was based on the principle used by Kingsolver, Dontas & Hoobler (1953) for the bioassay of vasodilator substances. An external column of blood is forced into the cerebral vascular system under a constant pressure after the injection of a test solution. The speed at which this column of blood enters the brain is noted and this gives a measure of the vascular resistance being encountered. This procedure can be repeated many times using saline controls to bracket the test solutions.

The technique is subject to a number of variable factors which make it difficult to obtain consistent results from consecutive control injections. The blood column, as it runs into the brain under constant pressure, tends to gather momentum since the resistance offered by the external column becomes reduced as the column shortens. This increase in momentum is not always

constant, for, with a raised vascular resistance, the momentum of the column will take longer to develop. The second and more serious variation in response is that the apparent vascular resistance increases steadily and the blood column moves more slowly during the course of an experiment. No reason has been found for this change; it could not be attributed to blood pressure or temperature changes, nor was it due to any variation in the experimental procedure.

This difficulty could be satisfactorily overcome by bracketing unknown solutions with their control as quickly as possible and then testing the next unknown solution and its control in the same way. This means that the results obtained for each individual injection cannot be satisfactorily compared with one another, but can only be compared with the results from their own controls.

For these experiments, rabbits were anaesthetized with ether, the trachea cannulated and the left external carotid artery tied. A 'T' piece with cannulae attached by polythene tubing to each horizontal arm was filled with saline and the cannulae inserted into the left common carotid artery, one facing towards and one away from the heart. The vertical arm of the 'T'

piece was attached to a three-way tap and thence to a length of polythene tubing 90 cm long, 1 mm internal diameter. The distal end of this tube could be attached to a mercury manometer and pressure bottle which provided a constant pressure of 140 mm Hg. The polythene tube was marked off in 10 cm lengths (internal volume 0.078 ml) and a morse key connected to a signal marker writing on a fast-moving smoked drum.

With the double cannula in place in the temporarily clamped left carotid, the right carotid was clipped and the rabbit decerebrated in the manner used to prepare the assay preparation (Chapter 1). When all bleeding had ceased, the rabbit was given heparin (700 I.U./kg I.V.) and both carotid arteries were unclipped. The blood in the left carotid passed through both cannulae and the horizontal arm of the 'T' piece, but the continuity of the flow was unimpaired.

It was hoped that the experimental conditions prevailing during the assay of C.E.F. would, as nearly as possible, be duplicated in this experiment and so injections were made in a way which mimicked the assay procedure injection method. All injections were given in 0.1 ml volumes from a 'tuberculin' syringe inserted in the upright arm of the three-way tap. At the

beginning of each experiment, the three-way tap was turned to allow blood from the carotid to pass up and fill the length of polythene tube. The distal end of this tube was then connected to the manometer and, after the tap had been turned, 140 mm Hg pressure was applied to the end of the blood column. It was found to be necessary to use this pressure, which is higher than is normally encountered in the decerebrate rabbit, in order to force the blood column into the brain at a suitable rate.

The test fluid was then injected into the dead space between the tap and the 'T' piece. The proximal side of the cannulated carotid was clipped, the drum started and the tap turned so that the column of blood was forced into the brain pushing the injected solution before it. An air bubble, which had been introduced at the distal end of the blood column, was followed visually as it passed down the polythene tube and, as it passed each 10 cm mark, the morse key was depressed. When the bubble had passed the length of the tube, the tap was turned, the pressure released and the carotid circulation restored by removing the clip. This procedure was repeated for each injection.

Every 30 minutes the systemic blood pressure of the

rabbit was checked by putting the cannulated carotid into 'open circuit' with the mercury manometer. If the blood pressure was found to have dropped more than 15 mm Hg since the start of the experiment, or was below 100 mm Hg, the preparation was discarded.

The only difference between this injection procedure and the one used during the assays is that this one forces the test fluid in with a column of blood (0.78 ml) for 15-20 seconds whereas the assay solutions were forced in with 0.25 ml of saline in 1-5 seconds. This difference was reduced in some experiments (injection series (g) and (h)) by forcing the injected solution in with saline (0.25 ml) and then, after a delay of 12 seconds, releasing the blood column as before. The delay would provide sufficient time to allow solutions to affect the cerebellar electrical activity before further disturbances occurred.

At the end of the experiment, the distances between the signal marks on the drum were measured and the rate of flow in each 10 cm length of the column calculated. The figures obtained give a measure of the vascular resistance encountered by the moving blood column and provide an indication of the state of the blood vessels (dilated or constricted) and the changes produced by injecting various substances. It is in this form

Substance Injected	Time taken for blood column to empty after each injection (sec)						Mean Difference ± S.E.(sec)
a) Cerebellum	12.0	13.2	13.0	13.0	13.5	14.5	
b) Cerebrum	12.5	13.8	14.7	11.1	13.8	14.5	
Difference	+0.5	+0.6	+1.7	-1.9	+0.3	0	0.2 ± 0.4
c) Cerebellum	15.5	14.0	15.5				
d) Cerebellum*	16.2	14.0	15.5				
Difference	+0.7	0	0				0.2 ± 0.2
e) ACh 0.1 µg	14.6	18.8	19.7	18.6			
f) Saline 0.1 ml	16.4	15.4	17.5	19.3			
Difference	+1.8	-3.4	-2.2	+0.7			0.8 ± 0.9
g) Cerebellum D	18.0	18.8	22.0	19.3	20.0	17.8	
h) Cerebrum D	20.5	19.0	18.8	19.0	15.2	19.6	
Difference	+2.5	+0.2	-3.2	-0.3	-4.8	+1.8	0.6 ± 0.9

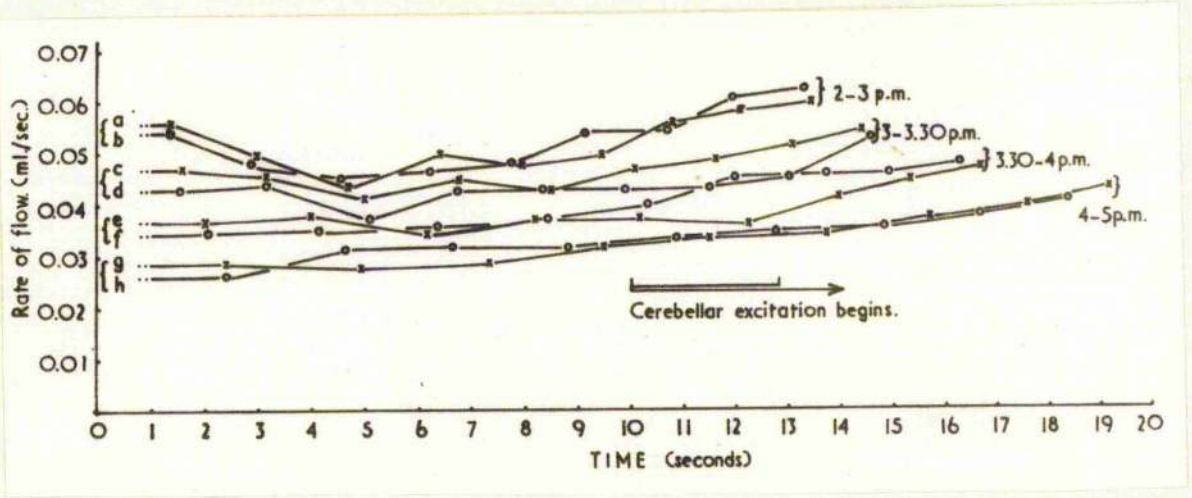
Decerebrate rabbit. All brain extracts purified by procedure IV and therefore ACh-free. All doses 50 f.b.e. in 0.1 ml, except where stated. * indicates alkali and acid-boiled extract. D indicates delayed injection (see text).

Injection series (a) and (b) made between 2 & 3 p.m.
Injection series (c) and (d) made between 3 & 3.30 p.m.
Injection series (e) and (f) made between 3.30 & 4 p.m.
Injection series (g) and (h) made between 4 & 5 p.m.

Table 9

that the results have been finally expressed.

Care was always taken to bracket active solutions (those that enhanced the cerebellar activity) with inactive solutions and, on some occasions, cerebellar activity was recorded and the latency of the appearance of excitation noted.



Results of a typical experiment on a decerebrate rabbit. Graphs show the average rate of flow of blood into the brain following injections of various substances. All brain extracts purified by procedure IV and therefore ACh-free.

a) Cerebellar extract (50 f.b.e.) 6 injections
b) Cerebral extract (50 f.b.e.) 6 injections
c) Cerebellar extract (50 f.b.e.) 3 injections
d) Acid-boiled cerebellar extract (50 f.b.e.) 3 injections
e) ACh 0.1 µg, 4 injections
f) Saline 0.1 ml, 4 injections
g) Cerebellar extract, delayed, (50 f.b.e.) 6 injections
h) Cerebral extract, delayed, (50 f.b.e.) 6 injections

For further details, see text.

Figure 12 The effect of ACh and brain extracts on the blood vessels of the brain

Table 9 summarizes the results of a typical experiment in which the cerebral vascular actions of acetylcholine, cerebellar extracts, acid-boiled cerebellar extracts and cerebral extracts were investigated. These figures are presented graphically in

Figure 12. Similar results were obtained from two other experiments.

From inspection of the rate of flow graphs and the figures in Table 9, it is evident that there is no significant difference between the vascular resistance encountered by the blood column after the injection of test solutions and their controls. The steady overall increase in vascular resistance occurring throughout the course of the experiment is also evident. No attempt can be made to compare the effects of injections from different pairs (e.g. series (a) with series (h)). This does not affect the conclusions that may be drawn from these experiments.

The solutions that usually excited cerebellar activity were found to do so under the conditions of this experiment also (18 injections) and were found to have the same time-courses as before. Figure 12 shows that 10-13 seconds after the injection no change in vascular resistance occurred and no difference between the response to the active solutions and their controls took place. This suggests that the excitation of cerebellar electrical activity by solutions containing the cerebellar factor or ACh is not due to any action on the cerebral vascular system.

This conclusion was confirmed on three separate occasions when the blood vessels on the surface of one half of the cerebellum were exposed while electrical records were taken in the usual way. The injection of active material appeared to have no effect on the calibre of these vessels.

5.15 Action of the cerebellar excitatory factor on neuromuscular transmission

The testing of C.E.F. on the neuromuscular junction was most conveniently studied on a perfused frog muscle preparation in situ. Some experiments were carried out to find whether the isolated nerve-muscle preparation (sciatic-gastrocnemius) with drugs applied topically as described by Schäfer (1912) would be suitable, but it was soon found that even strong doses of curare (1 mg) did not affect transmission for at least 30 minutes after their application.

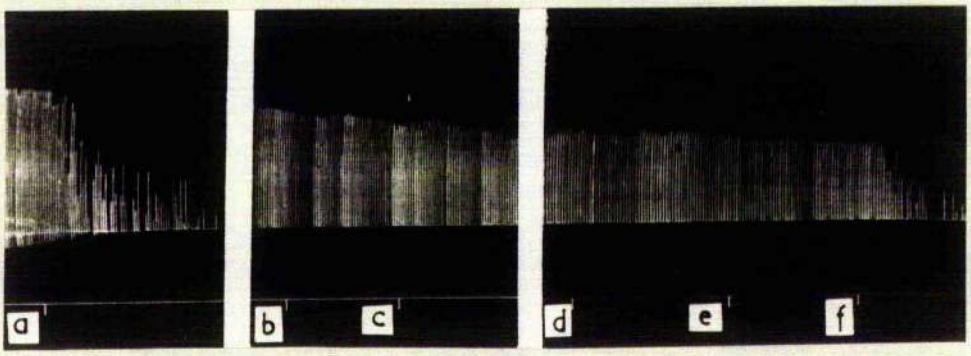
The brain and spinal cord of a large frog were destroyed and an incision made in the skin on the dorsal aspect of the right leg. The skin was deflected and any large cutaneous blood vessels arising from the sciatic artery were ligatured. The Achilles tendon was severed and the gastrocnemius raised to expose the peroneal and tibial arteries. These vessels were tied distal to the origin of the gastrocnemius.

The sciatic nerve was cut high up in the thigh and a length dissected out for stimulation. A fine glass capillary cannula was inserted and secured in the sciatic artery and perfusion with buffered amphibian Ringer-Locke solution was begun. It was found necessary to force the perfusion fluid through the vascular system under slight positive pressure (10 mm Hg). The rate of flow under these conditions remained constant at 0.15 ml/min.

Drugs and brain extracts were introduced into the perfusion fluid through a fine hypodermic needle inserted into rubber tubing just below a drop counter; the rate of injection of the solutions was made just sufficient to maintain a normal rate of perfusion through the muscle. Solutions were injected in 0.1 ml volumes in amphibian Ringer-Locke solution. The sciatic nerve was stimulated every 5 seconds through shielded electrodes to give a muscle twitch which was recorded isotonically on a kymograph.

The first injection to be made was always d-tubocurarine (10 μg in 0.1 ml). The effect of these injections is shown in Figure 13 (a) and (f). The interval between the injections and the beginning of transmission blockage was usually less than

10 seconds indicating that the technique allowed the injected substances ready access to the muscle end-plate.



Frog sciatic nerve stimulated once every 5 seconds and contractions of gastrocnemius muscle recorded. Brain extracts purified by procedure IV (ACh-free) from fresh sheep tissue. 20 minute intervals between (a) and (b), (c) and (d).

a) d-tubocurarine (10 μg)
b) Extract of cerebellum (100 f.b.e.)
c) Extract of cerebellum, acid-boiled, (100 f.b.e.)
d) Extract of cerebrum (100 f.b.e.)
e) Extract of cerebellum, acid-boiled (100 f.b.e.)
f) d-tubocurarine (10 μg)

Figure 13 Effect of curare and brain extracts on neuromuscular transmission

Figure 13 (b)-(d) shows that high doses of brain extracts containing the cerebellar factor did not affect neuromuscular transmission.

5.16 The action of C.E.F. on nervous transmission in the sympathetic ganglion

Solutions containing C.E.F. were applied by close-arterial injection to the superior cervical ganglion of the cat anaesthetised with ether while contractions of the ipsilateral nictitating membrane were recorded. The method described by Kibjakow (1933) for the perfusion of the sympathetic ganglion was used to allow retrograde close-arterial injection of test solutions to be made to the ganglion while still ensuring continuity of the local blood supply.

The right external carotid was cannulated high in the neck with the cannula directed towards the heart. The internal carotid and all minor blood vessels which did not lead directly to or from the sympathetic ganglion were ligatured. The right cervical sympathetic chain was dissected out and severed low in the neck. A fine silk thread was then passed through the central edge of the right nictitating membrane and tied. The cat was turned on to its abdomen and the spinal cord severed in the cervical region (C2) and the brain destroyed. Ether was withdrawn and artificial respiration (95% oxygen, 5% carbon dioxide) begun. In one experiment, the cat was not made spinal, but was anaesthetised with chloralose (80 mg/kg)

administered while warm into the jugular vein. The preparation was then set up in the usual way and injections were made in volumes of 0.1 ml into the carotid cannula and their delivery signalled on the kymograph. Some of the injections were given while the sympathetic chain was stimulated.

Although ACh and adrenaline both affected transmission in the sympathetic ganglion, purified brain extracts, with and without C.E.F., had no effect.

5.17 Actions of the cerebellar factor on the spinal cord

The investigation of problems of transmission within the spinal cord is complicated because, with anything but the most refined experimental techniques, the application of drugs or the recording of their effects will certainly embrace the responses of large numbers of heterogeneous neurones and reflex paths. In the present investigation, standard methods have been used to investigate the monosynaptic and polysynaptic reflexes which, although they do not illustrate uncomplicated reflex responses, do allow the action of C.E.F. to be compared with the actions of drugs used by many previous workers under similar conditions. In addition, a less conventional technique has been used to investigate the action of C.E.F. on the

monosynaptic reflex arc in a pure form. This series of experiments has, however, produced controversial results.

The monosynaptic reflex The first series of experiments was designed to test the effect of C.E.F. on a monosynaptic extensor reflex (the knee-jerk) of the rabbit. Rabbits (1.5-2 kg) were anaesthetized with ether, a tracheal cannula inserted and both common carotid arteries temporarily occluded. The rabbit was decerebrated in the usual way and the ether withdrawn. The spinal cord was severed in the lower thoracic region (T8-T12).

The intra-arterial injection of substances into the spinal cord was made by a technique first described by Holmstedt & Skoglund (1953). The animal was laparotomized and a suitably bent and blunted hypodermic needle was inserted into the right external iliac artery and guided forward into the aorta until its tip lay just below the branches of the renal arteries. The needle was then firmly tied at its point of insertion into the iliac artery. All the major arteries leaving the aorta posterior to the renal arteries, except the lumbar arteries, were tied. A thread was passed round the left external and internal iliac arteries and was passed outside the animal.

Later this thread served to occlude these arteries during injections. The technique enabled solutions to be injected into the blood stream above the lumbar arteries and so pass directly to the spinal cord through these vessels. The temporary occlusion of the arteries to the left leg during injections prevented drugs from passing into the muscles concerned with the knee-jerk.

Injections were made with a 'Tuberculin' syringe into a polythene tube leading from the needle cannula. When the effects of an injection had worn off, the cannula and tube were freed of injected solution by allowing blood from the aorta to pass up them. The blood was then forced back with a fresh solution to be tested.

The femur of the left leg was fixed in a vertical position and the sciatic nerve was cut. A cord was tied to the left ankle and, after passing under a pulley, was attached to an isometric lever which magnified the records about six times. Taps were applied every five seconds to the tendon of the quadriceps muscle by means of a Schweitzer & Wright automatic knee-jerk hammer. The intensity of these stimuli was adjusted to give a maximal reflex response. This was found to be

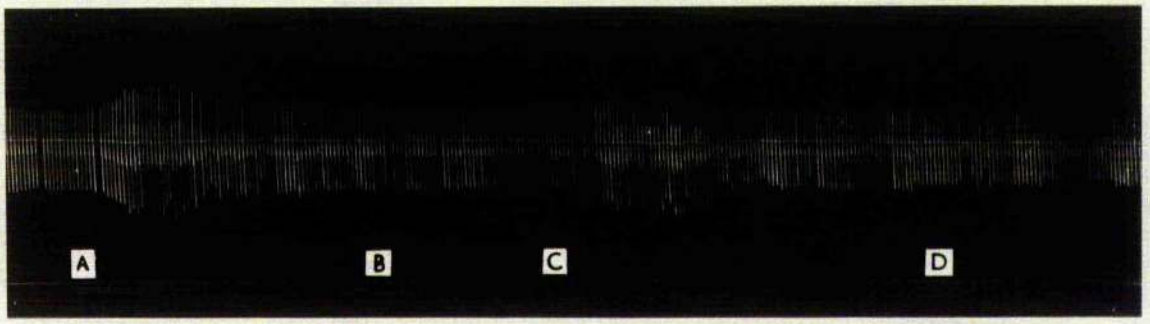
necessary in order to produce regular responses for a long period.

This method for studying the effect of C.E.F. on monosynaptic reflexes has several disadvantages, the greatest of which is that the knee-jerk may not represent a pure monosynaptic reflex (Eccles, Fatt, Landgren & Winsbury, 1954). The pure reflex arc was studied in the next experiment, but it was still considered of interest to determine the action of C.E.F. on the classically monosynaptic knee-jerk.

Another drawback of this method is that some of the injected fluid will enter the back muscles causing fasciculations or other direct effects changing the afferent inflow from these structures. Attempts to deafferentate these muscles were not made because of the technical difficulties, and it was decided that the use of suitable numbers of carefully selected control injections could obviate errors from this source.

There are several advantages of using an intra-arterial injection technique. An intravenous route involves secondary effects upon peripheral organs and requires the injection of large amounts of material; intrathecal or intracisternal application give an uncontrollable penetration into the cord and, like topical application or microinjection methods, risks uneven

absorption and structural damage. The technique has the disadvantages that injected substances will have to pass through a selective barrier between the blood vessels and the nerve cells and might well become inactivated in the blood stream.



Downward deflection represents the knee-jerk (mechanical rebound of lever caused upward deflection). Knee-jerk every 5 seconds. Continuous record. All brain tissue extracts were subjected to purification procedure IV and therefore had their contained ACh destroyed.

A) Cerebellum (40 f.b.e.)
B) Cerebellum, acid-boiled, (40 f.b.e.)
C) Cerebellum (40 f.b.e.)
D) Cerebrum (40 f.b.e.)

Figure 14 The effects on the knee-jerk reflex of applying brain extracts intra-arterially to the spinal cord of a decerebrate rabbit

Figure 14 illustrates the effect on the knee-jerk reflex following intra-arterial injections of brain extracts into the spinal cord of a decerebrate rabbit. It will be seen that

extracts of cerebellar tissue potentiate the reflex (Fig. 14 (A) and (C)) and that acid-boiling destroys this activity (Fig. 14 (B)). A similar extract of cerebral tissue left the reflex unaffected. It would appear that, under these experimental conditions, C.E.F. potentiates the knee-jerk. From this evidence, it is not possible to say that C.E.F. excites the monosynaptic reflex arc but, nevertheless, its actions on the knee-jerk (whatever reflexes this may involve) are interesting to compare with those of other substances.

A more direct approach to the study of the cerebellar factor on the monosynaptic reflex has been made by investigating its action on the negative synaptic potentials of the ventral roots resulting from monosynaptic activity in the anaesthetized spinal cord first described by Eccles (1946). The existence of prolonged negative potential changes and their connection with synaptic activity had been recognized by several workers (Umrath, 1933; Eccles & Pritchard, 1937; Barron & Matthews, 1938). That similar phenomena occur at the synapses of sympathetic ganglia (Eccles, 1943, 1944) and at the neuromuscular junction of skeletal muscle has also been demonstrated (Eccles & Kuffler, 1941; Kuffler, 1942).

These synaptic potentials are best studied in isolation after the superimposed spike discharges have been abolished by blocking transmission. In the spinal cord Eccles (1946) achieved this as well as the blocking of interneuronal activity by anaesthetizing the isolated frog spinal cord. By using pentobarbitone sodium (1 in 100,000), he was able to record potentials from the ventral roots arising only from activity in monosynaptic reflex arcs. This preparation appeared to be ideal for testing the actions of C.E.F. on monosynaptic transmission, for there can be little doubt that monosynaptic activity alone is being recorded and, as a result of direct experimental evidence, it is possible to demonstrate that the recorded potentials are unlikely to result from a cholinergic transmitter mechanism.

For these experiments, large frogs were decerebrated and the entire length of spinal cord was widely exposed from the dorsal surface. The spinal root pairs 1-4 were cut close to their origin at the cord, while roots 5-9 were cut at the point where they entered their respective intervertebral foramen. The 9th spinal roots were always chosen for stimulating and recording purposes. The cord was lifted out of

the vertebral canal and transferred to an airtight chamber containing Ringer's solution at 20°C through which a continuous stream of moist oxygen was passed. The cord was then divided longitudinally to ensure efficient oxygenation and more ready access of drugs to the deeper tissues. The 9th dorsal root was placed over a pair of silver stimulating electrodes which were connected through an induction coil (earth-free secondary coil) to an R.A.F. stimulator (Type 5 S.S.E. Ltd.). Throughout the experiments, the frequency of stimulation was maintained at 200 c/s and the pulse length at 1 msec. The strength of the stimulus was adjusted at the beginning of each experiment to be supramaximal with respect to the synaptic potential response. Recording of the monosynaptic potential was from the ipsilateral 9th ventral root by means of silver/silver chloride electrodes. The earth electrode was bent into a hook shape and placed on the origin of the spinal root and in contact with the closely adjacent body of the cord. The grid electrode was placed in contact with the cut end of the ventral root. The slow synaptic potentials were recorded with a pen-oscillograph and were monitored on an oscilloscope. Amplification was by means of conventional Ediswan apparatus.

The experiment was started by replacing the Ringer's solution surrounding the cord by Ringer's solution containing pentobarbitone sodium (1 in 10,000). After 30 minutes, this solution was sucked off and control records were made of the synaptic potentials by briefly stimulating the dorsal roots. A permanent paper record was made of the response and the electrical resistance between the recording electrodes was noted. During this recording period, the airtight chamber was not opened and the spinal cord was surrounded by an atmosphere of moist oxygen. When the recording was completed, Ringer's solution containing a substance for testing, in suitable concentration, was run into the chamber and allowed to surround the cord for 30 minutes. At the end of this period, the solution was sucked off and electrical recordings again made, and the electrode resistance checked.

This procedure was repeated every 30 minutes using various test and control solutions until the synaptic response to stimulation of the dorsal root showed signs of deterioration when the preparation was discarded. Provided that the pure oxygen content of the chamber was maintained with only the minimum of interruptions for manipulation, the reflex activity of

the cord could be maintained for several hours.

It will be seen from Figure 15 that the synaptic potentials have been recorded in a summated form. A single stimulus produced a response which exhibits an approximately exponential decay falling to I/e in 40 msec. This was not considered a suitable form for recording purposes and, consequently, the more prolonged and summated response was used.

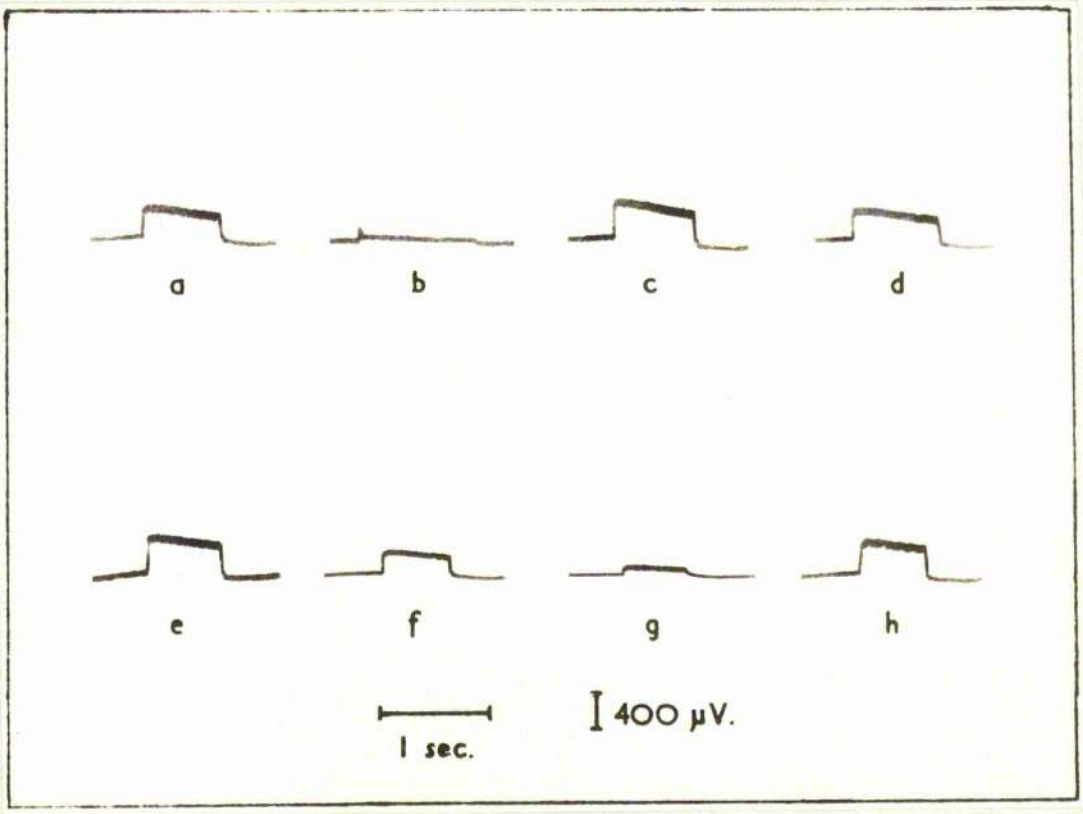
At stimulus frequencies above 125/sec, the summated potentials rose to a smooth plateau which declined only slowly with continued stimulation. When stimulation was stopped, there was a rapid decay of the potentials. In this summated form, the potentials were very easily recorded, the maximum height of the smooth plateau being taken to reflect the level of excitability of the synapses concerned (Eccles, 1946). The effect of drugs and brain extracts on the height of this plateau was investigated.

Several tests were undertaken to ensure that the potential changes recorded from the dorsal roots were true synaptic potentials and not due to stimulus or other artifacts. When a ventral root record was obtained which was believed to represent a true synaptic potential, the strength of the dorsal

root stimulation was gradually increased. Provided the recorded potentials were of synaptic origin, they would increase in size to a maximum level and then, whatever further increases might be made in the stimulus strength, the height of the responses would remain unchanged. If the record had resulted from a stimulus artifact, it would be expected to rise in proportion to the increase of the stimulus strength. By taking fast paper records and by observing ventral root responses on the oscilloscope, the rising phase of the response could be seen to have a duration of about 5 msec and an exponential decay form. These characteristics would not be consistent with the recording of an electrical artifact.

At the end of each experiment, the cord was soaked in a strong 'Nembutal' solution (1:100) and recordings made from the ventral root during stimulation as before. Under these conditions, it was always found that the previously recorded potentials had been abolished leaving only a very small stimulus artifact.

The cord was removed from the electrodes and was replaced by a length of Ringer-soaked thread which was laid across the stimulating and recording electrodes. Records made

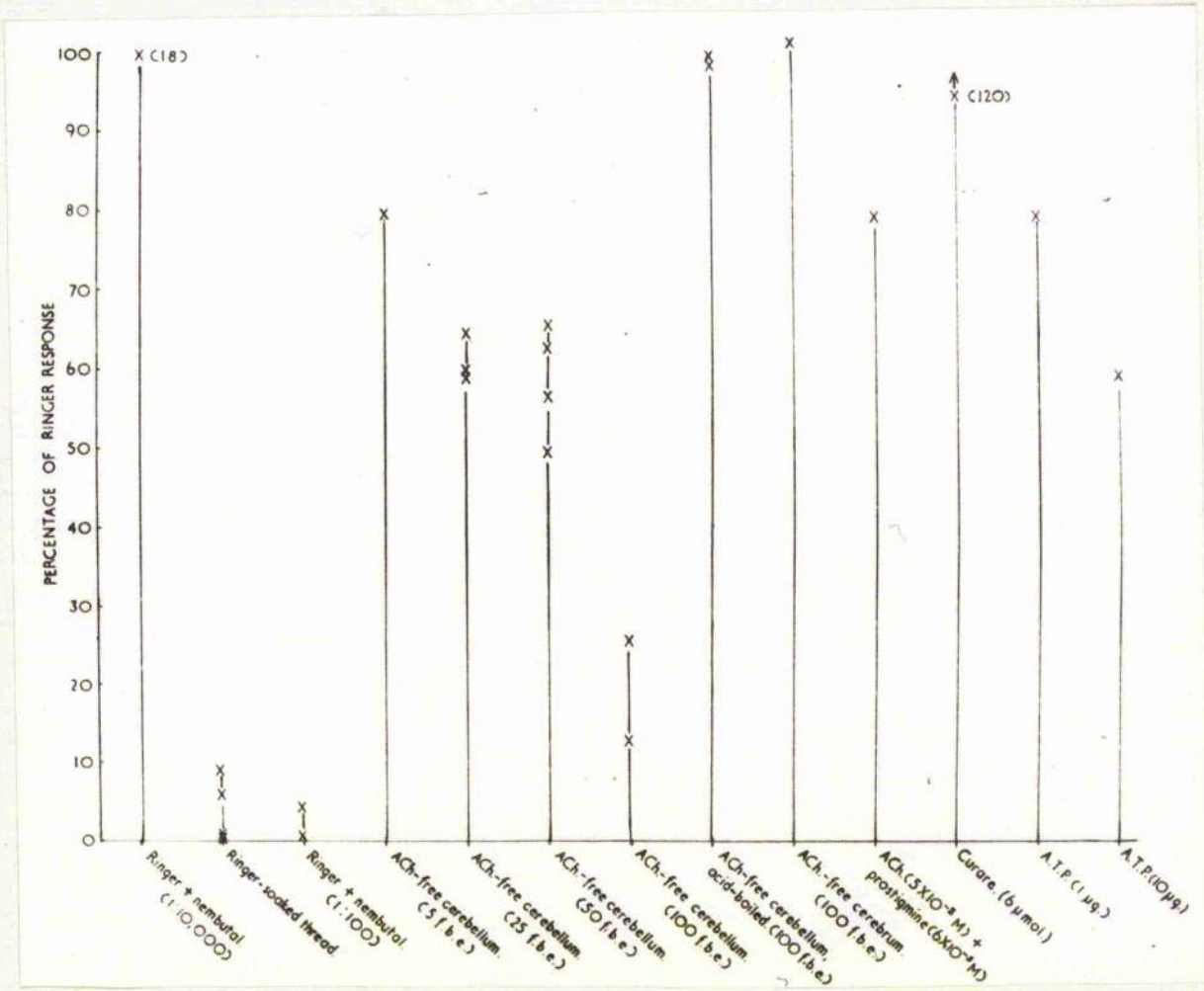


Pen-oscillograph records of synaptic potentials recorded from 9th ventral root and evoked by stimulation of the dorsal root at 200 stimuli/sec. Cord soaked for 30 min in each solution before recording. All brain extracts purified by procedure IV.

a) Ringer + Nembutal (1:10,000)
b) Ringer + Nembutal (1:100)
c) Ringer + d-tubocurarine (6µ mol)
d) Ringer + ACh (5×10^{-3}M) + prostigmine (6×10^{-5}M)
e) Ringer + cerebellar extract (25 f.b.e.)
f) Ringer + cerebellar extract (50 f.b.e.)
g) Ringer + cerebellar extract (100 f.b.e.)
h) Ringer + acid-boiled cerebellar extract (100 f.b.e.)

Figure 15 The action of various substances on the synaptic potentials evoked from the ventral roots of an anaesthetized, isolated frog spinal cord

in this way during stimulation showed only a slight stimulus artifact (Figure 16).



Modification of the synaptic potentials by various substances are expressed as a percentage of the response obtained when the spinal cord was bathed in a Ringer + Nembutal solution (1:100,000). The cord was soaked in Ringer + Nembutal and a record taken before and after each test; each unknown therefore had its own standard (100%) for comparison.

Figure 16 Individual results from seven experiments on the anaesthetized, isolated frog spinal cord

The most satisfactory evidence that the true synaptic potentials were, in fact, being recorded was provided by the effect that various substances had on the magnitude of these potentials; even grading of the response was sometimes possible by changing the concentrations of the drug bathing the cord.

Figures 15 and 16 illustrate typical modifications of the ventral root potentials by the application of various substances to the spinal cord. The application of a test solution to the cord was always preceded and followed by a control recording with Ringer + Nembutal bathing the cord. The response of the synaptic potentials in Fig. 16 are expressed as percentages of the Ringer + Nembutal response. The individual results of seven experiments are represented in Fig. 16.

The results obtained by Eccles for the actions of ACh (5×10^{-3}M) + prostigmine (6×10^{-5}M) and for curare (6μ mol) have been confirmed. It also appears that ACh-free extracts of cerebellar tissue depress the potentials, this effect being abolished by acid-treating the extracts. ACh-free cerebral extracts do not appear to have any action (100 f.b.e.) while ATP depresses the potential. The significance of these results will be discussed in a later chapter.

Polysynaptic Reflex A convenient preparation for the study of polysynaptic reflexes is the spinal frog in which the spinal cord is continuously perfused. The technique used is the same as that described by Bunzl, Burgen, Burns, Pedley & Terroux (1954), but without perfusing the entire vascular system.

The brain of a large frog was destroyed with a pithing needle and an incision made in the skin over the biceps femoris muscle. The distal two-thirds of this muscle was dissected free and a thread attached to the tendon. The spinal cord was exposed and divided in the region of the brachial enlargement. A small hole was then made into the vertebral canal between the urostyle and the base of the vertebral column and a blunt No. 14 hypodermic needle was inserted into the canal.

With the needle in position, perfusion was immediately started, the fluid entering the vertebral canal through the needle and leaving it from the exposed area of the canal in the brachial region where the fluid was sucked off and discarded. The perfusion fluid was Ringer's solution (6.5g NaCl, 0.14 g KCl, 0.12 g $CaCl_2$, 2.0 g dextrose, 3 ml 0.15 M-sodium phosphate buffer, pH 7.4/litre) which was continually oxygenated. This solution, but without the addition of dextrose, was used to

keep the skin of the frog moist. The perfusion head was 50 cm above the level of the frog and the fluid, after oxygenation, passed through a cotton wool filter, a drop counting chamber and thence to the vertebral canal. The rate of perfusion was adjusted to be about 2 ml/min in order to ensure efficient oxygenation of the spinal cord. This rate was kept constant throughout each experiment.

Solutions for testing were injected in small volumes from a 'Tuberculin' syringe into a rubber tube immediately below the drop counting chamber. It was possible to give these injections without altering the perfusion rate by injecting test solutions at a rate just sufficient to stop the formation of drops in the counter. With perfusion taking place, the frog was pinned down and the thread from the biceps femoris was passed under a pulley and attached to an isotonic myograph lever. Electrical stimuli were applied to the ipsilateral foot by means of two pin electrodes. Stimuli were given every 30 seconds from a Ritchie-Sheath stimulator (square pulses of 10 msec duration, 50 pulses/sec for 0.5 sec once every half-minute).

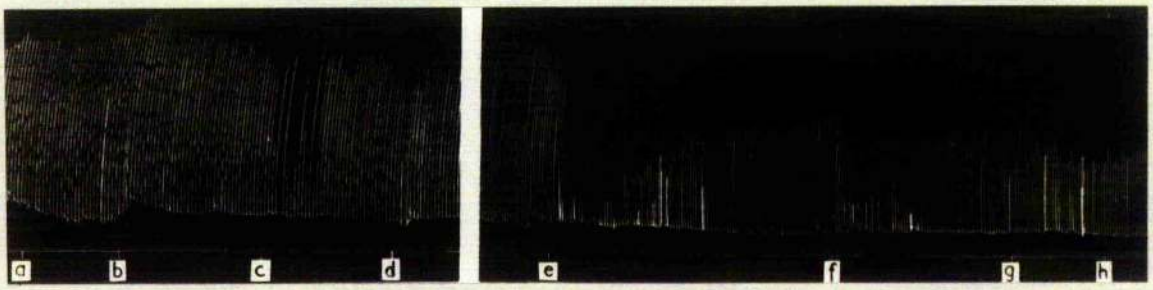
No test solutions were injected until the reflex

responses had remained regular for about half-an-hour. At the conclusion of each experiment, the reflex nature of the muscle contractions was tested by adding 'Nembutal' (1 in 100) to the perfusion fluid. This procedure always abolished the flexor response to electrical stimulation and so indicated that the response was mediated through a reflex arc and not by direct stimulation of an efferent motor nerve.

In early experiments, it was found that certain brain extracts containing C.E.F. were affecting the flexor reflex, but only when applied in amounts greater than 300 f.b.e. Strychnine is known to inhibit the enzymic destruction of the cerebellar factor (Chapter 5.11) and, for this reason, all brain extracts in the later experiments were injected in the presence of an amount of strychnine (1:10,000) which alone was insufficient to affect the reflex (Fig. 17(a)).

Injections into the perfusing fluid were continued until the reflex showed signs of deterioration or irregularity. This usually occurred 2-5 hours after beginning an experiment.

The result of injections of purified brain extracts containing C.E.F. on the flexor reflex is shown in Figure 17. Extracts of ACh-free cerebellum caused inhibition of the reflex



Record of the reflex contraction of the biceps femoris following stimulation every 30 seconds of the ipsilateral foot. Spinal cord continuously perfused at a rate of 2.1 ml/min. (a)-(d) and (e)-(h) continuous records. All brain extracts purified by procedure IV. Strychnine added was always 1 in 10,000.

a) Strychnine
b) Nicotine (1 in 25,000)
c) Cerebellum (50 f.b.e.) + strychnine
d) Acid-boiled cerebellum (50 f.b.e.) + strychnine
e) Cerebellum (100 f.b.e.) + strychnine
f) Cerebellum (100 f.b.e.) + strychnine
g) Cerebrum (100 f.b.e.) + strychnine
h) Acid-boiled cerebellum (100 f.b.e.) + strychnine

Figure 17 The action of drugs and brain extracts on the flexor reflex of the frog

while the same extracts had no effect after boiling in acid. Extracts of cerebrum with their contained ACh removed did not affect the reflex. The experiments suggest that C.E.F. exerts an inhibitory effect on the polysynaptic flexor reflex.

No quantitative comparison may be made between the action of C.E.F. and of other substances on these reflexes because

of the difficulty in accurately estimating the amount of an injected solution which reaches the spinal cord. This difficulty occurs because of such variable factors as dilution, loss into the general circulation and washing away in the perfusate of active material before it has diffused into the tissue of the cord. The method does, however, allow a qualitative comparison to be made. This, and the significance of the action of C.E.F. on these reflexes, are discussed in Chapter 7.

5.18 The action of the cerebellar factor on the cerebral cortex

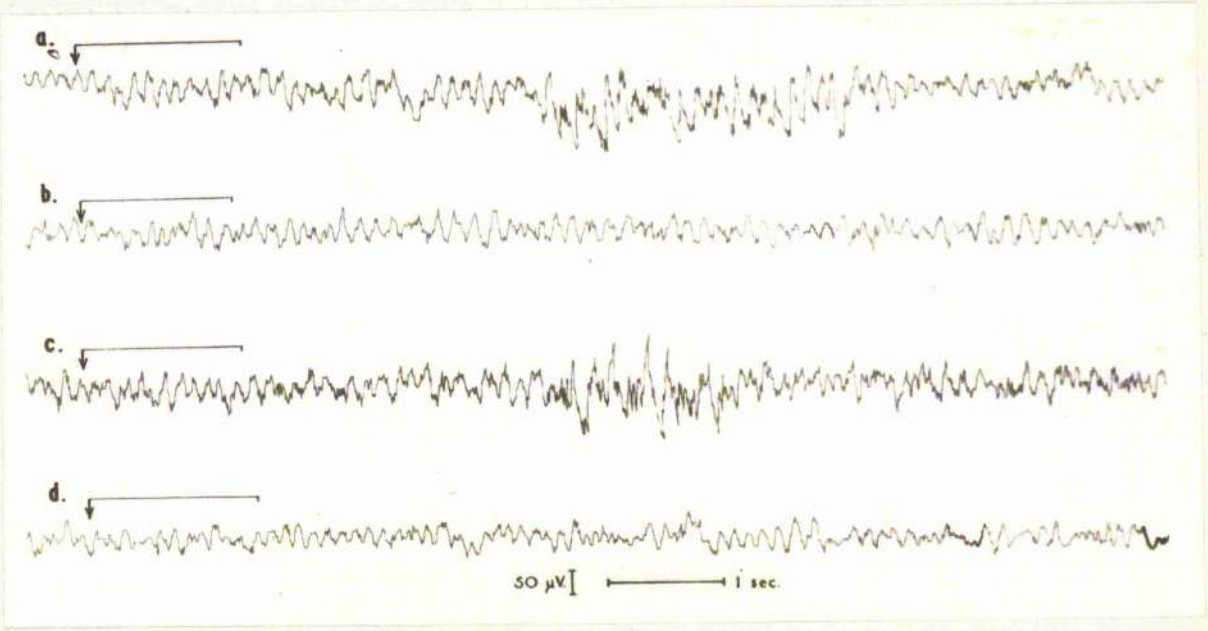
Experiments were performed on rabbits anaesthetized with 'Dial' (Ciba) given intraperitoneally (70 mg diallylbarbituric acid + 280 mg urethane/kg). A small trephine hole was made in the skull over the right cerebral hemisphere and the dura removed. When drugs were not being applied, the exposed brain was lightly covered with gauze soaked in warm saline (0.9%). Pin electrodes were inserted into the adjacent skull and the electroencephalogram (EEG) recorded with an Ediswan pen-writing oscillograph and monitored on an oscilloscope. Substances were applied to the cortex on small cotton-wool pads soaked in the

test solution which had previously been warmed to 37°C and suitably diluted with 0.9% saline.

It was found that, while the application of ACh (25 μg) caused marked excitation of the EEG, doses as high as 300 f.b.e. of cerebellar extract produced no effect.

In order to test further whether C.E.F. had a cerebral excitatory action, brain extracts were delivered via an intra-arterial route into the cerebrum. In these experiments, the external carotid was occluded while the test solution was injected into the internal carotid from a cannula placed in the common carotid. The rabbits were anaesthetized with 'Dial' (Ciba) and the EEG recorded as before. All the injections were made into the carotid cannula in 0.1 ml volumes at 37°C. Other details concerning the injection technique were similar to those described in Chapter 1 for the administration of substances to the cerebellum except, of course, that the animal was not decerebrated. The EEG was recorded on a pen-writing oscillograph and permanent records were taken for 10 seconds before and at least 40 seconds after the injection.

It was found that the injection of ACh (0.05 μg) produced an excitatory effect on the EEG (Fig. 18(a)). This



Rabbit anaesthetized with 'Dial' (80 mg/kg I.P.). All brain extracts prepared by extracting acetone dried powders of sheep brain with boiling saline. ACh destroyed in all extracts.

a) ACh (0.05 μg)
b) Acid-boiled cerebellar extract (50 f.b.e.)
c) Cerebellar extract (50 f.b.e.)
d) Cerebral extract (50 f.b.e.)

Figure 18 The effect of intra-arterial injections of brain extracts and ACh on the electroencephalogram of the anaesthetized rabbit

was a considerably lower dose than had been necessary previously when application was made topically or without the occlusion of the external carotid artery. If the external carotid was not occluded, it was found that this small dose of ACh gave no

response.

Boiled saline extracts of acetone dried powders of sheep cerebellum were always tested in 0.1 ml volumes. It was found that no EEG response occurred until 50 f.b.e. of active extract was injected. At this dose a response similar to that following the injection of ACh (0.05 μg) was elicited. Greater increases in the dose were not attempted because of the difficulties of producing extracts of such high concentrations (greater than 1,000 f.b.e./ml).

Control solutions of 50 f.b.e. of boiled saline extract of acetone dried powder of sheep cerebrum with the ACh removed and acid-boiled cerebellar extracts were used, but these solutions produced no response (Fig. 18 (b) and (d)).

It would appear that, under the favourable conditions occasioned by using a restricted arterial injection route, the cerebellar excitatory factor does exert an excitatory action on the spontaneous electrical activity of the cerebral hemispheres, although only on the application of rather large doses. The significance of this observation will be discussed in a later chapter.

5.2 Pharmacological properties of the cerebellar excitatory factor

Of the pharmacological preparations so far tested, none has proved responsive to the action of the cerebellar factor. A summary of the results obtained from six different preparations is reported below.

Rabbit duodenum (Garven, personal communication) This preparation was sensitive to the action of 100 µg ACh in a 10 ml bath. 20-30 f.b.e. of ether-washed dialysates of sheep cerebrum and cerebellum produced no response, but 140 f.b.e. of these extracts produced identical reductions in tone. This action was unaffected by boiling the extracts in acid or alkali.

Cyprina heart (Garven, personal communication) Acetylcholine reduced the strength of the heart beat. Crude extracts of sheep cerebellum (20-60 f.b.e./10 ml bath) decreased the frequency and amplitude of the beat, but this response remained unaltered after boiling the extract in acid and alkali.

Guinea-pig ileum (Garven, personal communication) The ileum was sensitive to histamine (20-40 mg/2ml bath). Boiled saline extracts of sheep cerebrum and cerebellum (100

f.b.e.) gave no response whether acid-boiled or not.

Guinea-pig ileum (Garven, personal communication) In the presence of atropine (10^{-6}), the ileum responded to histamine. Small, identical responses were obtained with 40 f.b.e. of sheep cerebrum or cerebellum. This response was not affected by acid or alkali boiling.

Frog rectus (Thompson, 1956) Similar doses of boiled saline extracts of sheep cerebrum and cerebellum (acetone dried powders) caused slight stimulation of the muscle.

Perfused frog heart (Mitchell, present investigation) The heart could be blocked with 0.5 µg ACh. TCA extracts of sheep cerebrum and cerebellum (10 f.b.e.) both caused an increase in heart rate leading to a short block after removal of their contained acetylcholine. The effect was unaffected by acid boiling the extracts.

Isolated rabbit ear (Mitchell, present investigation) The blood vessels of the ear were dilated by ACh (0.02 µg) and constricted by adrenaline (0.12 µg). TCA extracts of cerebrum and cerebellum (20 f.b.e.) taken from cats and rabbits each produced a slight dilation which was not altered after acid boiling the extracts.

These results suggest that, under the conditions of the experiments, the cerebellar factor had no specific action on smooth, striated or cardiac muscle. Most of the results have been confirmed indirectly using very different techniques which have been previously described. The results of the experiments will be integrated and discussed in Chapter 7.

At the end of this section containing, as it does, so few positive results, it is perhaps heartening to mention that one property of C.E.F. has become apparent and that is its singular lack of activity anywhere except on the spinal cord and brain.

CHAPTER 6

THE DISTRIBUTION OF THE CEREBELLAR EXCITATORY FACTOR

It might reasonably be expected that any chemical substance which plays a part in nervous transmission would be widely distributed, not only throughout the nervous system, but also throughout the animal kingdom. In the nervous system, a distribution pattern for a non-cholinergic transmitter might be expected in which high concentrations were present in nervous tracts poor in ACh and choline acetylase while low concentrations were present in parts rich in these substances. With this in mind, certain regions of the nervous system have been assayed for the presence of C.E.F.

Cerebellar extracts from six different animal species and samples of non-nervous tissue from the rabbit have also been assayed for the presence of the cerebellar factor. Since many pharmacologically-active substances are present in nervous tissue, the active cerebellar factor has been defined as a substance, which, on intra-arterial injection into the cerebellum, causes an increase in the electrical activity of this

structure. The active factor in the extracts is stable to brief boiling in alkali, but not to brief boiling in acid solution.

In order to ensure that the cerebellar factor and not some other substance was being assayed, all the extracts were alkali-boiled before testing. If they showed activity, they were then acid-boiled and tested again. If the activity was destroyed by the acid treatment, it was assumed that the substance responsible for the activity was the cerebellar excitatory factor.

6.1 Species distribution of the cerebellar factor

Boiled saline extracts of acetone dried powders of cerebellum were prepared from the rabbit, cat, dog, sheep, horse and ox. These extracts were tested in the usual way on the decerebrate rabbit. Control solutions were prepared either by boiling the active solutions at pH 3, neutralizing and bringing to a suitable dilution or by using extracts of cerebral hemispheres. It was found that all the untreated extracts of cerebellum enhanced cerebellar electrical activity and all the extracts appeared to be approximately equipotent,

about 20 f.b.e. being required to produce excitation. The control extracts had no effect.

6.2 Distribution of C.E.F. in tissues other than nervous tissue

Trichloroacetic acid (10% w/v) extracts were prepared from the following rabbit tissues: uterus (non-pregnant), spleen, skeletal muscle, small intestine and liver. These extracts were alkali boiled to destroy ACh, neutralized and tested on the decerebrate test preparation.

Although 60 f.b.e. were injected, none of the extracts had any effect on the electrical activity of the cerebellum in this dosage. Active control injections of 20 f.b.e. of a TCA extract of rabbit cerebellum did enhance the electrical activity of the cerebellum.

6.3 Distribution within the nervous system

Acetone dried powder extracts were prepared separately from the two optic nerves and the dorsal and ventral roots of a horse 2-3 hours after it had been killed with a humane killer. Boiled saline extracts of these powders were alkali-boiled,

neutralized and tested on the decerebrate preparation. It was found that all the extracts produced cerebellar excitation. Both the dorsal and ventral root extracts, however, required 40 f.b.e. to give a response while the optic nerves only required 15 f.b.e.

These experiments were repeated using extracts from rabbits (2 experiments) and cats (2 experiments) when the optic nerve and the spinal root extracts were found to be equipotent with cerebellar extracts, 20 f.b.e. of fresh tissue producing a response.

The distribution of the cerebellar factor in other parts of the nervous system was studied in tissue obtained from dogs. Three dogs (weights 14, 12 & 14 kg) were anaesthetized with chloroform and killed by bleeding. The brain and spinal cord were exposed and the nervous tissues required were all removed within one hour of death. These tissues were made into acetone dried powders in the usual way. The powders were stored over phosphorous pentoxide until they were required for testing when they were extracted with boiling saline, alkali-boiled, neutralized and suitably diluted. Control solutions consisted of acid-boiled nervous tissue extracts.

	C.E.F.			Choline acetylase
	Dog 1	Dog 2	Dog 3	
Dorsal roots	100	100		0
Optic nerves	100	100		16
Cerebellum (whole)	100	100		-
Cerebellum (white)	100			-
Cerebellum (cortex)	100		100	26
Dorsal columns	<10	<10		33
Pyramids	15			42
Sensory nerve		100		70
Optic tracts		80		100
Cerebellar peduncles			100	142
Visual cortex	<10	<10	<10	148
Motor cortex		15		185
Lateral geniculate body	100			325
Ventral roots	100	100		573
Sympathetic chain	<10			-

The figures for C.E.F. (present investigation) and for choline acetylase activity (Feldberg & Vogt, 1948) were obtained from dog tissue extracts with the exception of the figure for the choline acetylase content of dorsal roots which was obtained from oxen (Hebb, 1955). The concentration of the cerebellar factor is expressed as a percentage of its concentration in an extract of whole cerebellum. Choline acetylase concentrations are shown as μg acetylcholine synthesized/g powder/hour.

Table 10 Distribution of the cerebellar excitatory factor and choline acetylase in some areas of the nervous system

Tables 10 and 11 summarize the figures obtained for the concentration of active factor found in each region of the nervous system studied. In areas where no C.E.F. was detected,

the extracts had been tested in doses of up to ten times that normally required to excite cerebellar activity. For purposes of comparison, the distribution of choline acetylase activity in the nervous system of the dog (Feldberg & Vogt, 1948) has been included in Table 10.

	Horse	Cat	Rabbit
Cerebellum (whole)	-	100, 100	100 (12 extracts)
Dorsal roots	50	100, 100	100, 100
Ventral roots	50	100, 100	100, 100
Optic nerves	130, 130	100, 100	100, 100

Table 11 Distribution of C.E.F. in the horse, cat and rabbit

Except in the dorsal columns, sensory nerve, lateral geniculate body and ventral roots, the distribution of the cerebellar factor appears to bear an approximately inverse relationship to the distribution of choline acetylase. The significance of these results is discussed in the next chapter.

CHAPTER 7

DISCUSSION

In 1926, Loewi & Navratil provided the first experimental evidence for a chemical mechanism subserving the transmission of nerve impulses across nervous junctions. They found that substances were released into the fluid perfusing a frog heart during stimulation of the vagus-sympathetic nerve and that these substances, when applied to the heart, usually mimicked the effects of stimulation.

Since these experiments were performed, considerable evidence has accumulated in support of a hypothesis of chemical transmission and it is now generally agreed that, at least across all the synapses where a detailed study has been possible, transmission must be mediated chemically. At the neuromuscular junction, for example, it seems certain that transmission is chemical in nature and is mediated by acetylcholine. This junction is anatomically simple in the sense that there are relatively few nerve fibres impinging on the muscle end-plate. This is in contrast to the large numbers of nerve endings found in contact with nerve cells in most other parts of the nervous

system. It is possible that at the synapses of greater anatomical complexity the simultaneous arrival of impulses at many nerve terminals all lying in close proximity to a common post-synaptic membrane would result in a sufficient number of small areas of depolarization appearing on the membrane to cause a general depolarization; but all the evidence now available, including that provided by workers who previously upheld an electrical hypothesis, suggests that transmission is not mediated electrically.

With the general acceptance of a chemical hypothesis of transmission at some synapses, the important role of ACh has also been recognized, but it is agreed that this substance cannot be the only transmitter agent and that at least one other substance must be postulated. In the course of this investigation, an attempt has been made to study the substance, or substances, which might be responsible for non-cholinergic transmission.

The experiments described in Chapter 2 have demonstrated the existence of a cerebellar excitatory substance. Its existence independently of most of the other pharmacologically-active substances previously demonstrated in extracts of nervous tissue has been established by a variety of experiments reported in

	CEF	ACh	Histamine	Adrenaline	5-HT	Subs. P	ATP	ADP	Encephalin	Na^+, K^+	Thiamine	Subs. I	Subs. E	8-amino butyric	Hellauer's Substance	Holton's Substance
Excites ECbG (p. 27)	+	+	+	−	−	−	−	−		−	−			−		
Excites EEG (p. 126)	+	+		+			+	+		−		−				
Acid stable (p. 49)	−	+	+	+	±	−	−				+	+	+	+	+	−
Alkali stable (p. 49)	+	−	±	−	−	−	±				−	+		+	−	±
Acetone soluble (p. 44)	−	+	+	−	+	−	−					−		−		−
Adsorbed by alumina (p. 53)	−			+		+									+	
Active on smooth muscle (p. 130)	−	+	+	+	+	+	+	+			−					
Active on B.P. (p. 89)	−	+	+	+	+	+	+		+							
Pptd. by barium (p. 53)	−		+	+												
Conc. in cerebellum (p. 135)	High	Low (1)	Low (2) High (3)	Low (4)	Low (5)	Low (5,6)	As in Cerebrum	Low (8)	As in Cerebrum (9)	As in Cerebrum	As in Cerebrum (10)					
Other properties				Pptd. by 10% TCA		Dest. by trypsin						Insol. abs. alcohol			Soluble ethyl alc.	Insol. abs. alcohol

+, Is ..... −, Is not ..... ±, Is partially .....

Numbers shown in the left hand column indicate the page in this thesis in which the relevent property of C.E.F. and the techniques employed are reported in detail.

(1) MacIntosh, 1941
(2) Harris, Jacobsohn & Kahlson, 1952
(3) Kwiatkowski, 1943
(4) Vogt, 1954
(5) Amin, Crawford & Gaudum, 1954
(6) Pernow, 1953
(7) McIlwain, 1955
(8) Kratzing & Narayanaswami, 1953
(9) Raab & Gigee, 1951
(10) Ferrebee, Weissman, Parker & Owen, 1943

Table 12. A comparison of the properties of C.E.F. and previously demonstrated active constituents of nervous tissue

earlier chapters and conveniently summarized in Table 12. In this table, the more important properties of the cerebellar factor are compared with those of other active brain constituents. These properties have only been compared when they have been studied under similar experimental conditions.

Most of the substances mentioned in Table 12 have been shown not to be identical with C.E.F. Histamine, Holton's vasodilator substance and Hellauer & Umrath's excitatory substance, however, require a more detailed consideration.

Histamine Apart from acetylcholine and the cerebellar factor, histamine was the only constituent of nervous tissue which was found to enhance cerebellar electrical activity. The histamine response, however, differed from that of ACh and C.E.F. in the length of the time interval before its appearance and in its duration. The prior administration of an anti-histamine completely abolished the histamine response but caused no dimunition in the action of the cerebellar factor. This, together with the evidence summarized in Table 12, suggested that the cerebellar factor and histamine were not the same substance. It did not eliminate the possibility that the cerebellar substance was a breakdown product of histamine

or a histamine conjugate, especially as the long delay between the injection of histamine and the appearance of a cerebellar response was suggestive of some change occurring in the injected solution.

The possibility that the cerebellar response was caused by breakdown products of histamine was tested by injecting a solution of histamine boiled with strong alkali in the presence of inactivated brain extracts. This failed to produce a response. Furthermore, if histamine breakdown products were active, an antihistamine might not be expected to abolish the histamine response.

An active, alkali-stable histamine conjugate would be more likely to be the active factor present in extracts of cerebellum and, in this connection, it is relevant to point out a discrepancy which occurs in the literature relating to the distribution of histamine in the nervous system. Kwiatkowski (1943) reported the presence of histamine in the cerebellum of the dog and cat in amounts of 1.5-2.0 μg/g tissue, in contrast with the rest of the central nervous system, in no part of which he detected more than 0.3 μg/g. Harris, Jacobsohn & Kahlson (1952) found less than 0.2 μg/g of histamine in the cerebellum

of the dog and cat, though their results for the histamine content of other parts of the nervous system agree closely with those reported by Kwiatkowski. It is possible that the high figures obtained by Kwiatkowski were largely due to the presence of the cerebellar excitatory factor in his extracts. Although the final test solutions used by him could not have contained the active cerebellar substance itself, because his extraction process was based on that of Barsoum & Gaddum (1935) which involves boiling the extract in acid solution, the possibility that the cerebellar factor is a histamine conjugate from which histamine is liberated during the acid treatment must be considered.

Control solutions of cerebellar extracts which had been alkali-boiled in order to remove their contained ACh and histamine and which were then acid-boiled to break down any histamine conjugate and tested, failed to produce the response that might have been expected if histamine had been released from a conjugated form. It was also found that the histamine content of cerebellar extracts which had been boiled in strong acid for five minutes was not increased (Garven, unpublished experiments).

Kwiatkowski, unlike Harris et al., did not use an ether stage in his extraction procedure. His final solutions must therefore have contained considerable amounts of trichloracetate, but this would be expected to affect equally the results he obtained for all parts of the nervous system and not just those for the cerebellum.

The cerebellar factor has been shown not to be extracted from a TCA extract by the process of ether extraction. It is therefore unlikely that Harris et al. obtained their low figures for the cerebellum as a consequence of removing the cerebellar factor with ether. The use of an ether extraction stage, however, might explain the difference between the results of Kwiatkowski and Harris et al. for the cerebellum, since it has been shown that when an aqueous solution of histamine comes into contact with lecithin or cephalin in ether, part of the histamine moves into the ether phase and may be recovered only as a phosphatide-histamine complex from the ether phase (Lindahl, 1955). This process is influenced by the surrounding pH. Since the cerebellum has a higher cell density than the cerebrum, extracts of the cerebellum might be expected to contain higher concentrations of cephalin which

might result in a greater loss of histamine from the cerebellum following ether extraction.

Although no completely satisfactory explanation may be given for the difference between the histamine contents of the cerebellum as reported by Kwiatkowski and by Harris et al., the discrepancy cannot easily be attributed to the presence of the 'free' cerebellar excitatory factor or to the presence of an active, alkali-stable histamine conjugate.

Holton's vasodilator substance In an attempt to identify the substance or substances responsible for peripheral antidromic vasodilatation, Holton and her colleagues have detected in extracts of spinal roots a substance which causes vasodilatation when introduced into the rabbit's ear preparation. Holton's substance is of interest in this discussion because of the many properties it has in common with the cerebellar factor. Of the chemical and physical properties found for C.E.F., it solubility in absolute alcohol and its failure to be precipitated by barium are the only ones to differ from those reported for Holton's substance. These tests alone cannot be said to differentiate finally between the two

substances, for, if they are two distinct substances, they would certainly be expected to have other dissimilar properties or actions in the body.

Holton & Holton (1954) have suggested that the vasodilator substance of the spinal roots is ATP. This suggestion is supported by the demonstration that ATP, unlike ACh, histamine and substance P, gives the prolonged response typical of antidromic vasodilatation in the rabbit's ear. Further convincing support for this suggestion is provided by the estimation of ATP present in root extracts by an enzymatic determination of 'high energy' phosphates and by ultraviolet adsorption measurements. These experiments alone do not help in identifying the vasodilator substance, but the figures for the ATP concentrations are in remarkable agreement with those obtained from a series of assays of the vasodilator substance against ATP on the denervated rabbit's ear. These assays showed that all the vasodilator activity of the root extracts could be attributed to the action of a mixture of ATP, ADP and small amounts of further breakdown products.

In living nervous tissue, no ADP has been detected (Kratzing & Narayanaswami, 1953) but this substance has a

vasodilator activity equal to that of ATP. The breakdown products of ATP were probably present in the spinal roots at the time of assay and, consequently, some of the vasodilator activity found in these extracts would be conferred by substances not present in the living tissue. If post-mortem changes were avoided, it is possible that different assay results might be obtained. Although Holton's work does suggest that ATP is the vasodilator substance, there are several important considerations which do not altogether justify such a conclusion.

First, a study of the concentration of the vasodilator factor in saline extracts of nervous tissue after incubation showed an uneven distribution (Harris & Holton, 1953). ATP, on the other hand, would be expected to be evenly distributed throughout the nervous system. More recently, Holton (1956) has found that, when spinal root extracts are incubated with chymotrypsin, some of the vasodilator activity in the dorsal roots, but not in the ventral roots, is lost which suggests that some of the activity may be due to the presence of a polypeptide. This result is interesting in view of the work of Hellauer & Umrath on spinal root extracts and also since Holton's vasodilator substance and substance P appear to have a similar distribution in many parts

of the nervous system.

Secondly, it has been found by Florey & McLennan (1955) that sensory denervation of the rabbit's ear increases its sensitivity to the vasodilator activity of dorsal root extracts but has no effect on its sensitivity to ATP. If this is confirmed, it suggests that the two substances are acting in different ways to cause vasodilatation.

It has been shown that the vasodilator activity of ATP, like that of Holton's substance, is destroyed on incubation with acetone dried powders of dorsal or ventral roots (Holton & Holton, 1954). If ATP were incubated with a saline extract of dorsal and ventral roots at room temperature, important evidence for or against ATP being Holton's substance should be forthcoming. If the two substances are identical, the ATP should be rapidly destroyed on incubation with ventral root extracts, but should remain almost unaffected in contact with dorsal root extracts. This assumes that there is present in the dorsal roots an acetone-soluble inhibitor of the enzyme which destroys the vasodilator substance and not, as has also been suggested, a more powerful synthesising system in the dorsal roots than there is in the ventral roots.

Some of the evidence against C.E.F. and Holton's substance being identical has been discussed already, but perhaps the most important difference is that the enzymic destruction of C.E.F. may be inhibited by strychnine whereas the destruction of Holton's substance may not. When it is also considered that strychnine has no potentiating effect in the rabbit's ear, either on injected root extracts or on antidromic vasodilatation (Holton & Perry, 1951), it seems unlikely that, at least, the inactivating enzymes are identical.

The distribution experiments of Harris & Holton (1953), performed as they were on incubated saline extracts of nervous tissue, led to an identification of those parts of the brain with a low capacity for destroying vasodilator activity and in which the vasodilator substance might have had a transmitter role. The distribution in the nervous system was found to be uneven and the distribution of the cerebellar factor, reported in this thesis, bears no relation to that found for the vasodilator substance.

Although the evidence described suggests that the cerebellar factor and Holton's substance are not identical, the final solution must await the result of further investigation.

Hellauer & Umrath's excitatory substance Saline extracts of spinal roots have been reported by Hellauer & Umrath (1947, 1948) to cause vasodilatation when injected into the rabbit's ear. After denervation of the ear, the response to injections of dorsal root extracts was increased, but the response to ventral root extracts, ACh and histamine was roduced. It has been suggested that the ACh contained in the ventral roots was sufficient to account for the vasodilatation caused by these extracts and Umrath (1955) has further suggested that, in the living animal, ACh and substance P exist as a complex in the ventral roots and that the factor responsible for vasodilatation in the dorsal roots is combined with substance P. This hypothesis is supported by evidence that the substance P of the dorsal and ventral roots differs since its assay varies with the amount of atropine and the type of anti-histamine used (Umrath, 1956). The presence of an enzyme in dorsal roots which releases the vasodilator factor from its combination with substance P has also been reported (Umrath, 1955).

Although Hellauer's substance and C.E.F. have one important property in common, enzymic destruction by an enzyme which appears to be inhibited by small amounts of strychnine,

it is unlikely that the two substances are identical for the reasons already summarized in Table 12.

The inhibition of the destruction of C.E.F. and Hellauer's substance by small amounts of strychnine is of interest because the only other substance which appears to be affected by the presence of strychnine is substance I (Florey & McLennan, 1955), and none of these active substances have yet been finally identified.

Attempts to study the mode of action of strychnine have been made by an investigation of its effect on direct inhibition and on Renshaw cells (Eccles, Fatt & Koketsu, 1954). Eccles has suggested that strychnine might act as the curare of the inhibitory system. This may be so, especially since strychnine appears to act in opposition to Florey's inhibitory substance, but other possibilities which could also explain many of the actions of strychnine on the nervous system must also be considered.

Strychnine might cause the accumulation of an excitatory transmitter substance by the inhibition of its enzymic destruction. The application of strychnine to the cerebral hemispheres, the cerebellum and spinal cord causes an increase in nervous activity

at these sites and it has been demonstrated that C.E.F. causes similar increases in activity. That the action of strychnine might be attributable to its causing an accumulation of the cerebellar factor is suggested by the experiments reported in Chapter 5.11 in which it was shown that minute amounts of strychnine inhibited the destruction of an active amount of C.E.F. in an incubation tube. Further, in experiments on the perfused spinal cord of the frog (Chapter 5.17), it was shown that small doses of strychnine alone failed to affect the flexor reflex. Small doses of the cerebellar factor alone also had no effect. When these two substances were injected together, in the same small doses, C.E.F. had an action on the flexor reflex. This suggests that, in the absence of strychnine, the cerebellar factor was destroyed by enzymes present in the cord before it could exert any effect, but the addition of strychnine allowed the C.E.F. to accumulate.

This hypothesis is not entirely inconsistent with that suggested by Eccles for the action of strychnine on inhibitory synapses. There is no direct evidence to show that the cerebellar factor cannot act as an inhibitory transmitter or that strychnine has only one action. Should it transpire, for

instance, that substance I is an inhibitory transmitter and C.E.F. an excitatory transmitter in the spinal cord, then strychnine would be expected to exert its apparent antagonism towards substance I and, at the same time, allow the accumulation of C.E.F.

The opposing actions of strychnine and substance I suggest a further possible way in which strychnine might be involved. Substance I, when injected into mice which had been treated previously with a convulsive dose of strychnine, might oppose the protective action of strychnine on the cerebellar factor and so prevent the development of convulsions. If the accumulation of C.E.F. was responsible for strychnine convulsions, then an explanation for the anti-convulsive activity of substance I would become apparent. The injection of C.E.F. into the cerebrum or cerebellum produces an increase of electrical activity similar to, but of shorter duration than, the response elicited by strychnine. This supports the possibility that C.E.F. might be connected with strychnine convulsions and therefore with the anti-convulsive action of substance I. If substance I is found to be a transmitter of inhibitory impulses in the nervous system, confirmation of its

antagonism to strychnine in the manner described would certainly lend support to the view that strychnine acts as the curare of the inhibitory system.

If it is eventually found that C.E.F. acts as an excitatory transmitter and if the suggestion that strychnine acts on the nervous system by causing an accumulation of C.E.F. is confirmed, then a dual role for strychnine in which it affects both excitatory and inhibitory transmission may have to be postulated.

The role of the cerebellar factor in the nervous system

The demonstration of a cerebellar excitatory factor in extracts of cerebellum should be taken into account in any consideration of non-cholinergic transmission, since the properties which have been reported in this thesis for the cerebellar factor are not inconsistent with it being a transmitter substance. Moreover, many of its pharmacological actions parallel those of ACh whose transmitter role at some synapses is generally accepted.

Like ACh, the cerebellar factor seems to be bound to protein or to lipo-protein and appears to be protected from destruction in the excised brain. Freezing of excised brain tissue in liquid air, followed by rapid thawing causes the

destruction of both substances. These properties are probably common to many other constituents of nervous tissue, but a more striking parallel between ACh and the cerebellar factor is provided by their actions on the electrical activity of the cerebellum and cerebral cortex. The ACh content of 20 mg of cerebral tissue and the C.E.F. content of 20 mg of cerebellar tissue both caused similar increases in cerebellar activity. Equal amounts of cerebral or cerebellar extracts also increased the electrical activity of the cerebral cortex, although the doses required were greater than those which caused cerebellar excitation.

The release of ACh during stimulation has been shown to occur at motor nerve endings (Dale, Feldberg & Vogt, 1936), the sympathetic ganglion (Brown & Feldberg, 1936), the spinal cord (Bülbring & Burn, 1941) and the cerebral cortex (MacIntosh & Oborin, 1953). In the course of the present investigation, C.E.F. has been found to be released from the cerebellum during stimulation in amounts approximately proportional to the state of excitation of this structure. This finding is paralleled by the work of MacIntosh & Oborin who found a similar connection between acetylcholine release and the state of

excitation of the cerebral cortex. Most of the other actions of the cerebellar factor reported in this thesis are not paralleled by the actions of ACh but, nevertheless, have provided a basis for suggestions as to the possible role of the active factor in the nervous system.

From a study of physical and chemical properties, it appears that the active cerebellar factor is a small molecular weight compound of a basic nature. This basicity, in common with that of many pharmacologically-active substances, may be conferred by an amino nitrogen. The active factor is more stable to boiling in alkali than acid solution and cannot therefore be an aliphatic ester or a compound susceptible to hydrolysis by OH^- ions.

The final identification of C.E.F. would require first of all a simple, speedy and reliable method of quantitative assay, though clues to its identity may be obtained by comparing the properties of C.E.F. with those of the amino acid constituents of brain which have recently been found to have actions on the central nervous system (Hayashi & Nagai, 1956). With a good quantitative assay it might also be possible to produce very pure samples of the cerebellar factor which might then be

chemically analysed.

Investigations into the action of C.E.F. on the spinal cord have produced conflicting results. C.E.F. was found to inhibit the pure monosynaptic activity studied in the isolated frog spinal cord, but to increase the activity in the nervous pathways subserving the knee-jerk reflex. An explanation for these different actions on basically the same reflex arcs must await the results of further investigation into the nervous pathways involved.

A study of the literature concerning the action of drugs, especially the action of ACh, on spinal reflexes, reveals numerous conflicting results. If, however, it is assumed that the administration of a large dose of a drug to the cord will probably not have the same effect as a small dose of that same drug, then the previous discrepancies in the experimental evidence can be largely resolved.

When ACh was injected into the spinal cord by a direct arterial route, large amounts of the injected material, unless hindered by a blood-nervous tissue barrier, would be expected to reach the cord without suffering great loss. Under these conditions, ACh was always found to depress the monosynaptic

extensor reflex (Bülbring & Burn, 1941; Holmstedt & Skoglund, 1953; Taverner, 1954) and, under the same conditions, C.E.F. was found to have an excitatory effect. When ACh was given intrathecally or intravenously, the monosynaptic reflex was usually potentiated (Schweitzer & Wright, 1937; Calma & Wright, 1947). Under conditions of intravenous injection, a large amount of material would be expected to be lost in the body before it reached the spinal cord and, by applying ACh intrathecally, it is possible that the c.s.f.-nervous tissue barrier impedes its action on nervous elements, or else it becomes diluted in the c.s.f. before it has diffused to its site of action.

It is interesting to note in this respect that substance I, which has been found to inhibit monosynaptic reflexes when applied topically to the cord, has no action when given by an intra-arterial route (Florey & McLennan, 1955), the suggestion being that the blood-c.s.f. barrier is impermeable to substance I. Using a variety of techniques, most workers, including Angelucci (1956) who used the perfused frog spinal cord <u>in situ</u>, have found that ACh potentiates the flexor reflex. In the present investigation, C.E.F. was found to inhibit this

reflex in a preparation similar to that used by Angelucci. Because of the rather indirect approach of these experiments to the study of drugs on single reflexes and in view of the large range of doses of drugs used, it is dangerous to make any detailed comparison of the actions of ACh, substance I and C.E.F. It can be concluded, however, that C.E.F. appears to affect the flexor and monosynaptic extensor reflex in an opposite way to these other substances. Until further evidence on the mode and site of action of these substances in the reflex arcs becomes available, a detailed interpretation of these results cannot be attempted.

One peculiarity of the cerebellar factor which has become apparent during the course of this investigation is that, while it has an action on several sites in the nervous system, it has not been found to affect smooth muscle preparations. No previously postulated transmitter substance shares this property and, in fact, most of these substances have first been detected by the action of nervous extracts on smooth muscle. It is, of course, no criterion of a transmitter substance that it should have an action on smooth muscle. Indeed, those parts of the nervous system having a very low pharmacological activity

of this kind could hardly be expected to contain transmitter substances with striking smooth muscle actions.

The search for non-cholinergic transmitter agents may well profit by a shift of emphasis from an investigation of the pharmacological to the nervous actions of the substances.

The distribution of the cerebellar excitatory factor (Chapter 6) has provided encouraging evidence that this substance might play a part in nervous transmission at some non-cholinergic synapses. It might reasonably be expected that a non-cholinergic transmitter substance would be found to predominate in tracts of the nervous system poor in acetylcholine and choline acetylase activity. A comparison of the choline acetylase activity and the C.E.F. content of the nervous links in the visual pathways reveals an approximately inverse relationship (Table 13).

In the optic nerves a clear inverse relationship exists, but in the optic tracts C.E.F. is slightly reduced and choline acetylase increased. This may be explained by the presence in the tracts of cholinergic supraoptic commissural fibres. The lateral geniculate body has a high concentration of both substances which might be expected since the synaptic

	C.E.F. % Cb. (whole)	Choline acetylase µg ACh/g powder/hr.
Optic nerve	100	16
Optic tract	80	100
Lat. geniculate body	100	325
Visual cortex	<10	148

Table 13 The distribution of choline acetylase activity and C.E.F. in the visual pathways of the dog

connections between cholinergic and non-cholinergic fibres are present in this region. In the visual cortex both cholinergic and non-cholinergic neurones would be expected to occur, but the relationship that might therefore have been predicted to exist between the concentrations of C.E.F. and choline acetylase was not found. It may be that the fifth neurone in the visual pathway, like the fourth, is cholinergic. Alternatively, the fifth neurone may contain a non-cholinergic transmitter other than C.E.F. or contain C.E.F. only in amounts which could not be detected by the assay.

A convincing demonstration of a transmitter action of the cerebellar factor might be provided by an investigation of

its action on synapses in the lateral geniculate body. This region is particularly suited to a study of this kind, since it is comprised almost entirely of sensory fibres and their nervous junctions and, as far as is known, contains no short interneurones or inhibitory synapses. If the electrical events occurring at synapses in this region were recorded in the manner described by Bishop & McLeod (1954) during stimulation of the optic nerve, the application of C.E.F. either topically, arterially or by micro-injection might demonstrate an effect of this substance on synaptic transmission. This experiment could be performed more simply by recording evoked potentials from the visual cortex during the application of C.E.F. to the geniculate body.

The figures obtained for the distribution of C.E.F. in the other sensory pathway studied in this investigation require some comment. In sensory nerves and in the dorsal roots large amounts of C.E.F. have been detected, but none has been found in the gracile and cuneate fasciculi. No instance is known in which an uninterrupted tract changes its chemical constituents during its course and it has, moreover, always been tacitly assumed that the fibres comprising a sensory nerve are chemically

homogeneous. Crossland (1956) has recently stressed the lack of evidence to support this latter view and, if it is false, a logical explanation for the distribution of the cerebellar factor in this region may be suggested. Since C.E.F. is found in sensory nerves but not in the dorsal columns, it may be that it is not present in all the fibres comprising a sensory nerve. It would not be expected to occur in those fibres which ascend the dorsal columns without interruption. In some, or even all the sensory nerve fibres which make synaptic connections with their secondary neurones soon after entering the cord, the cerebellar factor might be expected to be found. Apart from the dorsal columns, there is little information available concerning the chemical constituents of the ascending tracts of the cord, but it may well be that some of them contain the cerebellar factor. The high C.E.F. content of the cerebellar peduncles which contain some of these fibres makes this possibility more likely.

If this hypothesis is correct, then the distribution so far determined for the cerebellar factor in these pathways would be consistent with the suggestion that C.E.F., when it is present, is found in highest concentrations in tracts having a

low choline acetylase activity. It does, however, require the assumption that sensory nerves are not always chemically homogeneous. This possibility has not been investigated by workers who have followed the suggestion by Dale (1935) that the substance responsible for peripheral antidromic vasodilatation might also act as a central transmitter and, since dorsal root extracts are known to contain many active substances, the possibility of more than one sensory transmitter substance being present in sensory pathways should be considered in any study of non-cholinergic transmission.

The conclusions to be drawn from the distribution found for the cerebellar factor are not entirely in accordance with the tentative suggestion made by Feldberg & Vogt (1948) that a system of alternating cholinergic and non-cholinergic neurones might exist in some nervous pathways. The sensory nerve fibres which synapse in the posterior horn cells have been shown to contain C.E.F. but little choline acetylase. The secondary neurones arising from these fibres are unlikely to be all cholinergic, since the inferior cerebellar peduncles, which contain many of these fibres, are low in choline acetylase activity. The choline acetylase that is present in the inferior

peduncles could be accounted for by the cholinergic fibres which enter from the gracile and cuneate nuclei. It has also been found that the regions of the spinal cord which contain, amongst other fibres, those from the spino-cerebellar tract have only intermediate values for choline acetylase activity (Feldberg, Harris & Lin, 1949).

The pyramidal tracts whose fibres make synaptic connections with cholinergic motor nerves have a low choline acetylase activity and also contain little C.E.F., but if C.E.F. is the only non-cholinergic transmitter present in these fibres, then at least some of them might be expected to be cholinergic. Moreover, the pyramidal fibres converge on the anterior horn cells which, besides containing large amounts of choline acetylase, are also apparently sensitive to the application of acetylcholine (Kennard, 1951) and might therefore be expected to be impinged upon by cholinergic neurones.

In the visual pathway a clear alternation of fibres containing C.E.F. and choline acetylase exists, except in the visual cortex where only choline acetylase is found, but where at least two types of neurone would be expected. It has already been suggested that more than one non-cholinergic transmitter

substance may have to be postulated and, if this is so, some of the cortical neurones might well contain a transmitter substance other than C.E.F. or ACh.

In this investigation, no property of the active cerebellar factor has been found which excludes the possibility that the substance mediates transmission across some non-cholinergic synapses. This does not, however, constitute evidence for the transmitter nature of the substance. This can only be obtained as a result of experiments of a more direct nature. The most convincing evidence for the participation of C.E.F. in nervous transmission would be the demonstration of a direct action on a post-synaptic cell membrane. With the introduction of intracellular recording techniques, this type of experiment has become possible and has already been applied to a study of transmission at the motor end-plate (Fatt & Katz, 1951), the sympathetic ganglion (Paton & Perry, 1953) and to spinal neurones (Eccles, Fatt & Koketsu, 1954).

Since C.E.F. has been found to have an action on reflexes, it is possible that it has a transmitter action at the spinal motoneurone. Using the methods described by Eccles and his colleagues for the introduction of microelectrodes into

motoneurones (Brock, Coombs & Eccles, 1952) and by injecting purified C.E.F. into the cord by the arterial route described by Holmstedt & Skoglund (1953), it should be possible to demonstrate any action that the cerebellar factor might have on the motoneurone. If it was found that C.E.F. caused a general depolarization of the cell membrane, convincing evidence for the transmitter nature of the substance would be provided.

The direct action of the cerebellar factor on the cerebellum has been demonstrated, but it would be of interest to study the mechanism of this action in more detail than has been possible in this investigation. It is unlikely that work of this nature would provide direct evidence for any transmitter role of C.E.F., but it might provide answers to some of the problems peculiar to the cerebellum. The cerebellum, despite a high cell density, is low in acetylcholine and choline acetylase but has a high concentration of 'true' cholinesterase. Possibly some nerve cells are extremely sensitive to the action of ACh, but the full consequences of this sensitivity are reduced by the presence of the cholinesterase. It is interesting in this respect to note that eserine, perhaps by inhibition of the

cholinesterase, produces a striking increase in cerebellar activity. A study of the actions of C.E.F. and ACh on the electrical activity of individual nerve cells in the cerebellum would therefore be of interest. This could best be done by employing the microelectrode techniques used by Granit & Phillips (1956) in their study of the activity of a single Purkinje cell. C.E.F. and ACh could be applied to the cerebellum, either topically or by the arterial injection technique used in the present investigation, while the electrical activity of single nerve cells was recorded.

It is not possible to say whether the active cerebellar factor will eventually be shown to be a non-cholinergic transmitter substance. None of its properties eliminate this possibility and its distribution in the nervous system, particularly in the visual pathways, lends support to the suggestion.

It is suggested that, of all the substances so far postulated as mediators of non-cholinergic transmission, the cerebellar factor has the strongest claims to this position and should certainly be taken into account when considering the problem of central transmission by substances other than acetylcholine.

SUMMARY

1. A technique for the introduction of substances into the cerebellum of the decerebrate rabbit was developed and the effects of drugs and brain extracts on the electrical activity of the cerebellum were studied using this method.

2. Extracts of cerebellar tissue were found to increase the electrical activity of the cerebellum and this action could not be attributed to ACh, histamine or to any other identified pharmacologically-active constituent of the nervous system, nor could it be attributed to a vascular action. The activity was not detected in similar concentrations in extracts of cerebral hemispheres or upper brain-stem.

3. The physical and chemical properties of the cerebellar excitatory substance were studied and attempts to purify it from crude brain extracts were made.

4. The enzymic destruction of the active factor was demonstrated and this destruction was shown to be inhibited by small amounts of strychnine. The active substance was found to be released from the cerebellum under various conditions of stimulation.

5. The active substance had no apparent effect on any of the common pharmacological preparations, nor did it affect transmission at the neuromuscular junction and superior cervical ganglion.

6. The cerebellar factor was found to affect spinal reflexes and the electrical activity of the cerebral cortex.

7. Nervous tissue from six animal species was found to contain the active substance, but it was not detected in similar concentrations in tissues outside the nervous system. Its distribution in various regions of the nervous system of the dog was shown to bear an approximately inverse relationship to the distribution of choline acetylase activity and acetylcholine.

8. It has been suggested that the cerebellar factor has some claims to be considered as a non-cholinergic transmitter of nerve impulses and experiments which might eventually confirm this hypothesis have been considered.

APPENDIX

THE ELECTRICAL ACTIVITY OF THE CEREBELLUM

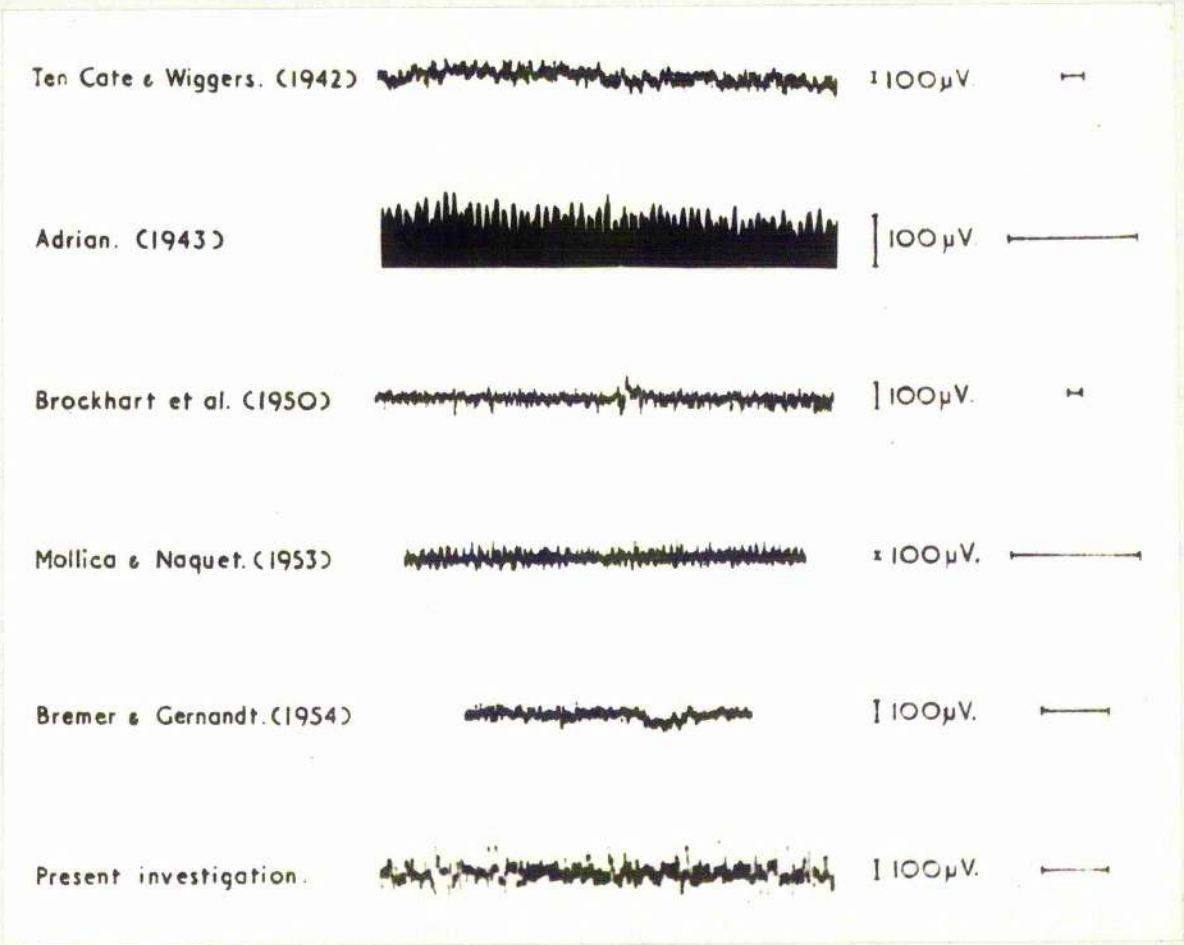
Throughout the present investigation, an assay technique has been used in which increases in the spontaneous electrical activity of the cerebellum have been taken to signify the presence of an excitatory substance in solutions injected into the cerebellum. Cerebellar electrical activity has usually been recorded with a direct-writing oscillograph, the pens of which had a maximum frequency response of about 70 c/s. Since the most characteristic rhythm of the cerebellum is about 150-300 c/s, the records produced by the pens must either be of a slow frequency component of cerebellar activity or else be the most faithful reproduction of the fast rhythms that the pens can provide. In the past there has been some controversy as to the existence of a true cerebellar slow rhythm and it is therefore worth reviewing briefly the previous work relevant to this subject.

The earliest recordings of the electrical activity of the cerebellum were made by Ferrier (1886), Sherrington (1898)

and Beck & Bickeles (1912), all using string galvanometers as their recording instruments. In 1935, Adrian made the first detailed study of cerebellar activity using modern recording techniques and he recognized the high frequency of the rhythm.

The first mention of a slow cerebellar rhythm was made by Spiegel (1937) in a comparative study of thalamic, cerebral and cerebellar potentials. He found that, besides fast activity (200 c/s), there appeared at the end of some experiments a slow rhythm (10 c/s). Both these rhythms persisted after decerebration and even after section of the cerebellar peduncles, although the amplitude of both was then reduced. As the very slow rhythms appeared only at the end of an experiment, it is possible that they resulted from pathological changes. Dow, in an extended study of the electrical activity of the cerebellum (1938), found, besides a characteristic fast activity (150-250 c/s), a slow rhythm which appeared only after decerebration. He attributed this to pathological changes since, unlike the fast activity, it remained unaffected by a fall in blood pressure and by anoxia.

Most evidence now suggests that the slow rhythms sometimes recorded from the cerebellum are dependent upon, and



All the records were taken from decerebrate cats except those of the present investigation in which decerebrate rabbits were used. The decerebrate cat used by Adrian was lightly anaesthetized with 'Dial' and the record illustrated has been retouched for the purposes of reproduction.

Figure 19 A comparison of oscilloscope records of the electrical activity of the cerebellum of decerebrate animals

probably radiated from, the cerebral cortex and disappear following decerebration. This does not include any abnormal rhythms arising from pathological changes (Ten Cate & Wiggers, 1942; Adrian, 1943; Brockhart, Moruzzi & Snider, 1950; Mollica & Naquet, 1953; Bremer & Germandt, 1954). A study of the oscilloscope records of cerebellar activity obtained by various workers using decerebrate preparations under conditions similar to those used in this investigation shows no obvious slow rhythms (Figure 19).

It is important to consider the site of origin of the fast rhythm since it has been found not to be a feature exclusive to the cerebellum but is also found in the internal capsule, thalamus, mesencephalic tegmentum, geniculate nuclei, brachium pontis, vestibular nuclei, inferior olive and reticular formation (Eldred & Snider, 1950). It may be that the fast cerebellar activity is 'driven' by one of these areas or that the activity said to originate from the cerebellum is merely a reflection of the fast activity in adjacent nervous tissue. If this were true, substances injected into the internal carotid artery and which appear to affect the electrical activity of the cerebellum might, in fact, be acting at some other site in the

nervous system. Evidence that the cerebellum does possess its own spontaneous fast activity is shown by the fact that activity may still be recorded from the cerebellum isolated *in situ* (Spiegel, 1937), or even for a short time from the cerebellum in complete isolation from the animal (Snider & Eldred, 1949). The evidence from a study of unitary activity in the cerebellum also lends support to the idea that the fast activity is an intrinsic property of this organ (Brockhart *et al.*, 1949, 1950, 1951; Crepax, Nigro & Parmoggiani, 1956). Evidence that drugs and brain extracts, when injected into the internal carotid artery, modify the fast electrical activity of the cerebellum by a direct action on the cerebellum is provided by experiments in which active drugs and brain extracts have been applied to the surface of the cerebellum and have produced an increase in electrical activity (Miller, 1937; Crepax & Infantellina, 1956).

Although the pen records of cerebellar activity made during the course of this investigation were always monitored on, and sometimes photographed from, an oscilloscope, the wide use of a pen-oscillograph for fast frequency recording must be further justified.

Since many of the experiments in this investigation

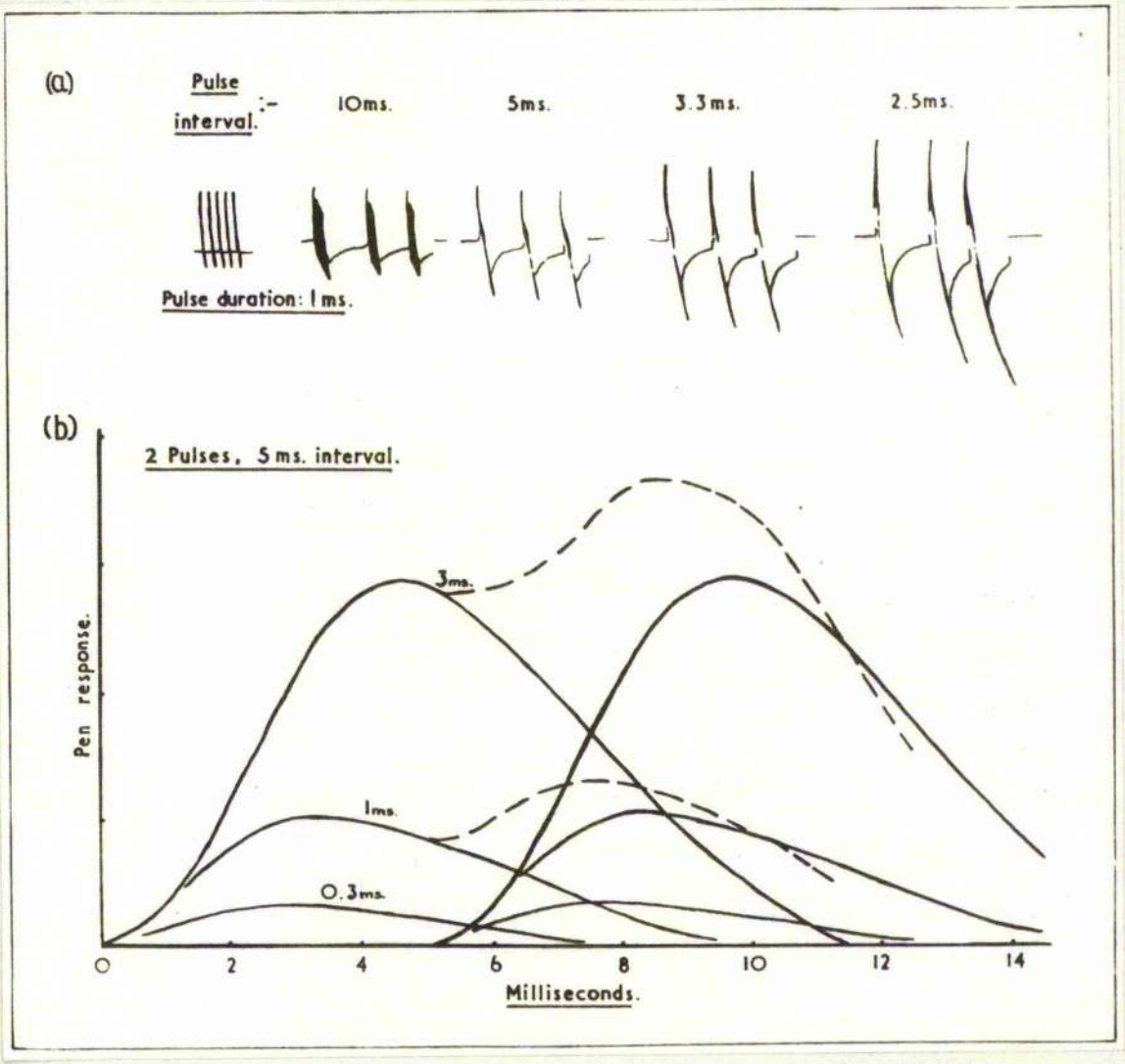
required the detailed study of very large numbers of electrical records, it was found impracticable always to employ photographs of oscilloscope traces as the permanent recording method because of the time necessary for processing the film and the prohibitive cost. Under the experimental conditions already described, the pen-writers were found to record the electrical activity of the cerebellum with an apparent frequency of 40-70 c/s. The oscilloscope pictures taken simultaneously showed the fast activity (150-300 c/s) characteristic of the ECbG with, occasionally, a very low amplitude slow wave corresponding to the 50 c/s mains fluctuation (Figure 21).

The physical characteristics of the pens used in this investigation have been studied by Stephens (1956) and it is worth mentioning some of the results he obtained since they explain the manner in which the pens may have interpreted the fast cerebellar activity. Any attempt to record at frequencies above the level of the natural frequency of the pens will simply result in a pen response of about 70 c/s. This observation seems to have been overlooked by some previous workers who have recorded the electrical activity of the cerebellum with pen-oscillographs and have then claimed the existence of slow cerebellar

rhythms (50-70 c/s) in the decerebrate preparation (Johnson, Browne, Markham & Walker, 1950). Other workers (Swank & Brendler, 1951; Cooke & Snider, 1954) have attempted to record the electrical activity of the cerebellum from non-decerebrate animals using pen-writing equipment. Inspection of their records shows a preponderance of slow (10-40 c/s) activity, presumably radiated from the cerebral cortex, together with a rhythm which appears to be of about 60-80 c/s. Again, it could easily be that the pens are interpreting the fast cerebellar activity (150-300 c/s) at their own natural frequency.

Despite the fact that, while fast cerebellar activity is being recorded, the pens are only recording at their upper frequency limit, the records they produce will not be of a simple nature. One form of distortion will occur because the response of the pens becomes smaller as the duration of a potential change applied to them is reduced, even though the strength of the stimulus may remain constant (Figure 20 (b)).

Further distortion of the original record will occur because the pens give an apparent summation of discrete, consecutive stimuli when the interval between the stimuli falls below a critical value (Figure 20 (a) and (b)).



a) Pen records of 1 msec pulses separated by short time intervals.
b) Summation of paired pulses of 0.3, 1 and 3 msec duration when separated by 5 msec. Dotted line indicates summating effect of pen response.

Figure 20 Apparent summation of discrete, consecutive stimuli by a pen-writing oscillograph (modified from Stephens, 1956)

The electrical activity of the cerebellum, as recorded on the oscilloscope, shows rapid potential fluctuations of about 1 msec duration which occur irregularly about 250 times a second. This would mean that, on the average, the duration between spikes would not exceed 3 msec. Reference to Figure 20 shows that a train of 1 msec spikes separated by intervals of 3 msec would suffer summation by the pens. This type of integration of the fast, irregular activity of the cerebellum, together with the frequency response limitations imposed by the pens, could give rise to the slow rhythm recorded in this investigation. It must be emphasized that irregular biological potential fluctuations have been compared here with regular, electronically-applied pulses and any conclusions from this comparison can only be of a tentative nature.

Although no visible 'wave-to-wave' relationship exists between the records of normal cerebellar activity made by an oscilloscope and by a pen-oscillograph, directly a change of amplitude in the electrical activity of the cerebellum occurs, the amplitude of both types of recordings are modified in the same way (Figure 21 (a)-(d)). It is this fact which finally justifies the use of the pen-oscillograph.

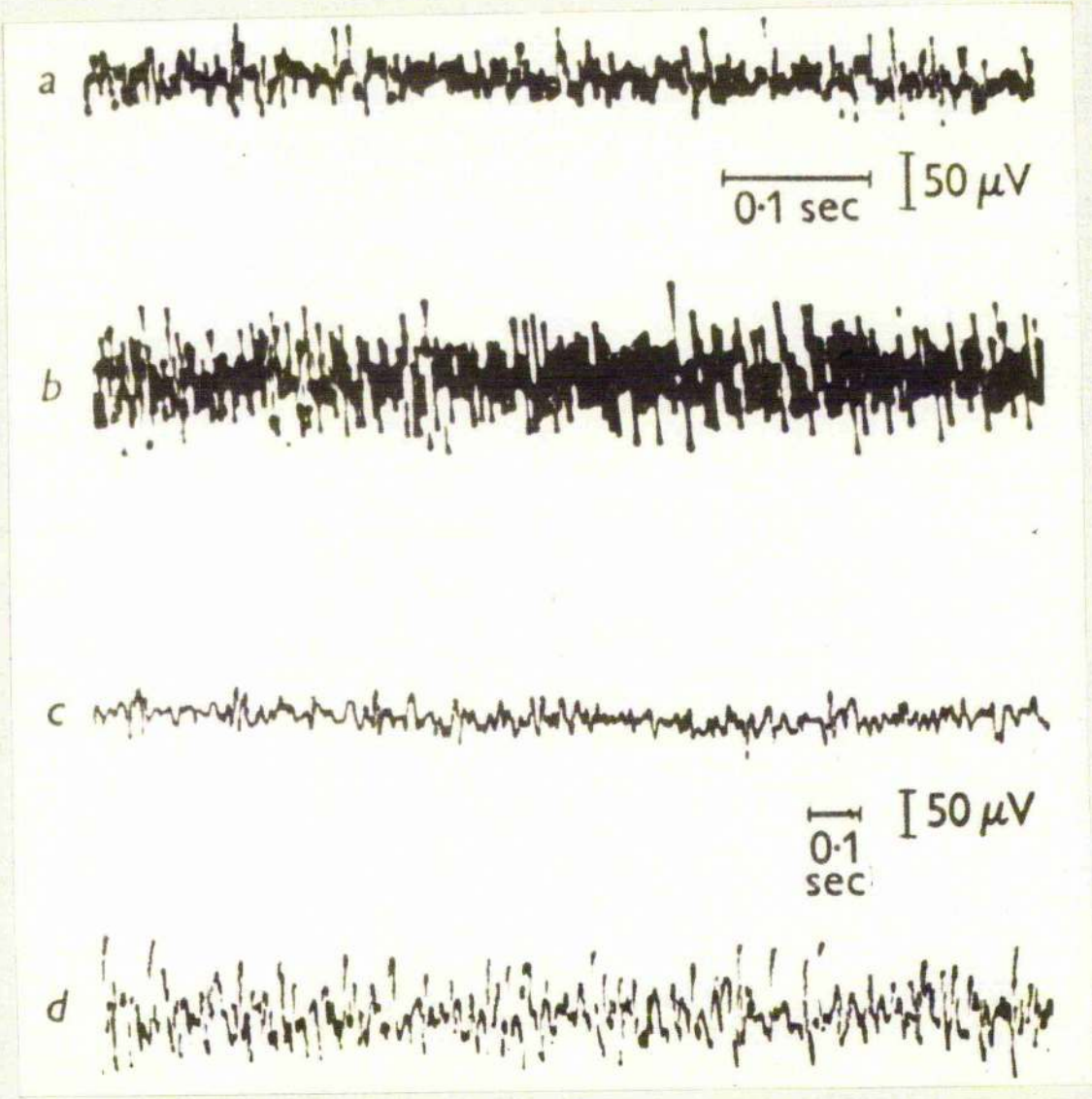


Figure 21 Oscilloscope (a and b) and pen-oscillograph (c and d) records of the rabbit ECbC before (a and c) and after (b and d) the injection of an extract of 20 f.b.e. of cerebellar extract

Further evidence that the pen and oscilloscope records follow each other in the same direction with regard to amplitude has been obtained on many occasions when it was observed that, as the blood pressure of a preparation deteriorated, the record on the oscilloscope and that produced by the pens both fell and eventually disappeared simultaneously.

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