AN INVESTIGATION INTO THE POSSIBLE RELATIONSHIP OF ADENOSINE TRIPHOSPHATE TO SENSORY SYNAPTIC TRANSMITTER SUBSTANCES

by

Christopher Robert Muirhead
B.A., University of British Columbia, 1955

A Thesis Submitted in Partial Fulfilment of
The Requirements for the Degree of
Master of Science

in the Department of
Physiology

We accept this thesis as conforming to
the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
April, 1962

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Physiology

The University of British Columbia,
Vancouver 8, Canada.

Date 26th April, 1962.

ABSTRACT

An attempt has been made to determine the relationship, if any, between adenosine triphosphate and the transmitter substance responsible for antidromic vasodilatation. Extracts of various areas of the central nervous system have been made by dialyzing boiled, ground brain tissue against distilled water. These extracts were analyzed for; labile phosphate content by the method of Berenblum and Chain (66), adenosine triphosphate content by paper chromatography and by the luciferin-luciferase enzyme method of Strehler and Totter (69).

The content of vasodilator activity of extracts from the same areas was determined by the method of Holton (35). The extracts to be tested were injected into the facial artery of a rabbit and allowed to flow into the auricular artery and through the ear. The changes produced in the ear were detected by means of a photoelectric cell which measured differences in the amount of light passing through the ear. Vasodilatation appeared as a decrease in the amount of light transmitted through the ear and vasoconstriction as an increase.

It was thought that if there was a relationship between adenozine triphosphate and the transmitter material the areas containing the most ATP should also contain the most vasodilator activity. A comparison of the location and concentration of the two substances revealed no such correlation.

The two most important questions to be answered were: 1. is ATP the substance responsible for antidromic vasodilatation? and 2. if so, is it also a sensory synaptic transmitter

substance? It was concluded that ATP was unlikely to be the substance responsible for antidromic vasodilatation. If one accepts Dale's hypothesis (48) that a neurone may employ the same transmitter substance at all branches of the axon, then it would also seem to rule out ATP as a sensory synaptic transmitter agent.

....

Hugh McLennan, Ph.D.

ACKNOWLEDGMENT

I wish to acknowledge with gratitude the guidance and encouragement given me throughout the course of these investigations by Dr. H. McLennan.

I would like to thank Dr. D.H. Copp for his kind assistance during my course of studies. My thanks are due also to Mr. R. Walker for his help with the rabbit ear preparations and to Mr. K. Henze for making the illustrations.

TABLE OF CONTENTS

Page

LIST OF TABLES

I. INTRODUCTION:

A form of vascular reflex phenomenon, which has since been named "antidromic vasodilatation" was first described by Lovén (1), who showed that when the central end of the great auricular nerve in the rabbit's ear was stimulated, vasoconstriction occurred in other organs although a marked vasodilatation was initiated in the ear itself. This same phenomenon was also described by Goltz (2), who observed that stimulation of certain fibres of the sciatic nerve would induce a peripheral vasodilatation in a localized area of the skin. In 1876 Stricker (3) confirmed this finding, and further reported that it could similarly be brought about by stimulation of the distal portion of a sectioned posterior root, a finding which seemed to be in direct opposition to the Bell-Magendie law. The Bell-Magendie law states that posterior roots contain sensory fibres only, and therefore do not conduct impulses towards the periphery. Because of the apparent contradiction to the Bell-Magendie law, Stricker's work was not generally accepted until Bayliss (4-8) confirmed and extended the studies. Bayliss showed that the fibres concerned seemed indistinguishable from ordinary sensory fibres, and he suggested that there might be a peripheral nerve network around the arterioles common to both sensory and vasodilator fibres. He named the phenomenon "antidromic vasodilatation" because the impulses passed in a direction opposite to that which was usually the case. Bruce (9) was able to confirm Bayliss' theory with some experiments in which he paralyzed sensory fibres with cocaine and found that no vasodilatation was produced on

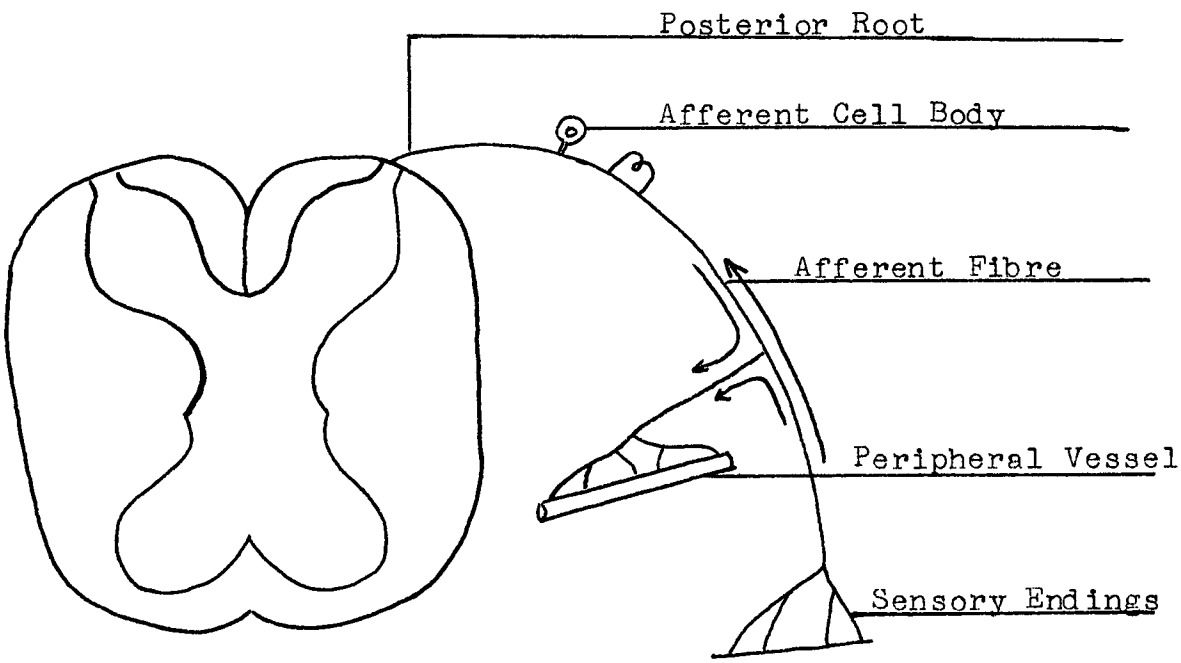


Figure 1. Diagram of Axon Reflex

applying oil of mustard to the area of the skin supplied by the sensory fibres. However, sectioning the nerve trunk to the area without anaesthetizing it did not prevent inflammation from occurring. He also found that when the nerve was allowed to degenerate no inflammatory reaction to the application of mustard oil occurred. He concluded that the vasodilatation required an intact nerve supply and that the sensory nerve concerned contained vasodilator fibres to the arterioles. When the skin was stimulated nerve impulses could pass in any direction over the various branches of a neurone and therefore could pass to a vasodilator branch and give rise to a dilatation of the arterioles innervated.

Langley (10,11,12) also studied antidromic vasodilatation and demonstrated that stimulation of posterior roots in the cat caused flushing in the skin of the foot. In experiments in which the abdominal aorta was clamped off he allowed the feet to become quite pale and then stimulated the lumbar posterior roots on one side. He observed a combination of flushing of the pads and toes of the limb on the stimulated side, which he concluded was a result of capillary dilatation, while there was a pallor on the opposite side. He reasoned that under the conditions of the experiment, dilatation of the arteries might take blood from the capillaries. As this did not occur, he considered the flushing to have been due to a decrease of capillary tone which allowed them to be slightly distended by the venous pressure. He suggested that the vasodilatation might be the result either of a special connection of afferent fibres with the capillaries, or that the stimulated afferent fibres set free metabolites which

secondarily gave rise to the dilatation. Langley favoured the latter theory chiefly because of the relatively long delay between stimulation and the start of the dilatation (2 to 8 seconds) which Bayliss had also noticed. Langley called the pathway involved an "axon reflex". Antidromic vasodilatation has since been shown to occur in the frog (13), the rabbit (14) and man (15).

Krogh (16,17) was able to show that the calibre of capillaries is not a function of the volume of blood coming from the arterioles, and that in the resting state the capillaries are in a state of contraction and therefore closed to the passage of blood, but that they can be opened by stimulation of posterior root fibres. He concluded that local reactions of skin vessels to mechanical and chemical stimuli were due to axon reflexes, and that there must be some mechanism for regulating the calibre of the capillaries which would be mainly situated in the capillaries themselves. Krogh's conclusion that a vasodilator mechanism must be found in the immediate area of the capillaries lends credance to Langley's theory that metabolites are set free by the afferent fibres in the capillaries, as any substance released to regulate the calibre of the capillaries would logically be released at the site of its action.

Gaskell (18), in 1916, first made the suggestion as to the possible nature of the mediator substance causing vasodilation by postulating that acid metabolites were released from skin cells. Special attention has been focused on humoral transmission since Loewi's experiments in 1921 (19) in which he demonstrated humoral control of the heart, several pieces of evidence since have been offered to strengthen the theory that

humoral transmission elicits antidromic vasodilatation. As mentioned above the most obvious evidence for humoral control was the long delay which occurred between stimulation and the onset of dilatation.

Before any particular compound can be considered as a possible humoral substance for antidromic vasodilatation certain criteria must be met:

(a) can the substance be found in the nerves or in the endings,

(b) are the nerve fibres concerned capable of synthesizing the substance,

(c) is there any enzyme capable of destroying the substance,

(d) can the presence of the substance be localized to any special area, such as nerve endings in the capillaries,

(e) if a specialized area can be found, can application of the substance produce an effect identical to the one obtained through stimulation of the nerve fibres involved?

Lewis and Marvin (20,21) noted the delay between stimulation and dilatation and went on to do some experiments which seemed unequivocally to show that the dilatation was produced by a humoral agent. They occluded the circulation to an area of skin and found that the flush produced by antidromic stimulation was prolonged, which they interpreted to mean that the "vasodilator substance" continued to act during circulatory arrest

and was only removed when the circulation was restored, as the subsidence of the flush seemed to depend on irrigation of the tissues. They also pointed out that while the decline of vasodilatation was delayed by circulatory arrest, the time of onset of the vasodilatation was not altered; they found that the peripheral vessels relaxed a few seconds after the nerve had been stimulated even though the circulation was stopped. They also offered more evidence for humoral transmission in experiments which related the length of stimulation with the duration of the flush. Lewis and Marvin claimed this indicated that during stimulation a vasodilator substance was released into the tissue spaces and that if stimulation continued, the concentration tended to rise. With longer occlusion of the circulation the reaction was prolonged, but only up to a point; a balance was eventually attained between liberation and removal of the substance so that a longer reaction was not induced by lengthening the stimulation.

Kibjakow (22) presented evidence which also strengthened the humoral transmission theory when he observed a vasodilator activity in the rabbit ear preparation present in blood collected from a region of antidromic vasodilatation in the cat.

It has been found that when skin is injured by scratching with a needle, by application of burning heat, by freezing or by other physical means, the vascular reaction is threefold. The vessels dilate locally, the skin blisters, and the surrounding skin shows a bright red flare. The first two reactions are independent of a nerve supply, and the third reaction is known to be reflex in origin. Lewis (23) has shown that the surrounding

red flare which can be produced by puncture of histamine into normal skin depends on an intact nerve supply; the flare was found to disappear when the nerve supply to the region had been allowed to degenerate. In view of these findings as well as the observations of Dale (24,25) that histamine shock was related to a general loss of tone in the capillaries, histamine was suggested as the substance likely to be responsible for antidromic vasodilatation. Lewis and Grant (26) were able to show that a histamine-like substance was released in injured skin, while Kwiatkowski (27) found histamine in the distal parts of sensory nerves from the skin and in the nerves which produce antidromic vasodilatation but little in motor fibres. He also found that stimulation of cut posterior roots in the cat liberated a histamine-like substance into venous blood, and claimed that this strengthened the theory that histamine is the vasodilator substance.

Ibrahim, Stella, and Talaat (28) have also concluded that histamine causes the vasodilatation produced by posterior root stimulation. They found that histamine was released from the skin but not from the muscles after antidromic stimulation of the nerve fibres supplying the area, but concluded that histamine was released from the tissues supplied by the nerves rather than from the nerve fibres themselves. They also have found that antihistamine compounds reduced antidromic vasodilatation but did not affect the dilatation produced by intra-arterial injection of acetylcholine, which they claimed to be evidence that the antihistamine compounds were producing their effect by acting directly on histamine itself.

Evidence has also been offered by Unger (29,30,31) to

support the theory that histamine release causes antidromic vasodilatation. He has shown that a histamine-like substance is released from peripheral ends of afferent fibres during antidromic activity. He called the substance histamine-like because although it has not been definitely identified its chemical properties suggest it to be histamine. As a biological test he has shown that the substance liberated was capable of augmenting gastric secretion, in a manner duplicated only by comparably small doses of histamine. Von Euler and Åstrom (32), studying segments of isolated peripheral nerve from cattle, have found that under certain conditions a histamine-like substance was released from one end of the segment following electrical stimulation of the other, which they claimed to indicate that histamine was the transmitter substance involved in antidromic vasodilatation.

On the other hand, considerable doubt has been cast upon the likelihood of histamine acting as a vasodilator substance in the body. Unlike Ibrahim et al. (28), Parrot and Lefebvre (33); Frumin, Ngai, and Wang (34) and Holton and Perry (35) have all been unable to show that antihistamine compounds block antidromic vasodilatation produced by nerve stimulation, which would be expected to happen if histamine were the transmitter substance. Further, Chauchard (36) has been able to show that histamine actually inhibits transmission in sensory neurones which would not be expected if histamine were the compound in question. Parrot (37) found that stimulation of lumbar posterior roots caused the release of a compound whose properties were similar to those of a derivative of adrenaline.

He also showed that after irritation of the skin, histamine was released by the injured cells, and this was able to stimulate the peripheral endings of the vasodilator fibres and elicit a vasodilator axon reflex. He suggested that histamine may not act as a mediator at the end of the axon reflex but as the stimulus at its origin. More recent investigations (38) have not supported the histamine transmitter theory and the functional significance of histamine present in the nervous system remains obscure.

Dale and Feldberg demonstrated the release of acetylcholine on stimulation of motor fibres to perfused voluntary muscle (39,40,41) and it has been suggested that this compound might also be the mediator of antidromic vasodilatation (42). Experimental evidence has been offered to support the theory that acetylcholine is released during antidromic vasodilatation by Wybauw (43,44,45,46). In a series of experiments on the perfusate of the hind limb of a cat collected during antidromic stimulation of posterior roots, he was able to demonstrate the presence of a substance which had the same pharmacological effects as acetylcholine on the isolated frog's heart, the eserinized leech muscle and the blood pressure of a chloralosed cat.

However, other observations have been made which indicate that acetylcholine is unlikely to be the chemical mediator in antidromic vasodilatation. Holton (35) found that the vasodilatation was not inhibited by atropine nor was it enhanced by eserine, so that although the release of acetylcholine peripherally on stimulation of transected posterior roots is not denied it seems unlikely that this substance itself could be responsible for antidromic vasodilatation (47).

Dale (48) reasoned that a sensory neurone would likely release the same transmitted substance peripherally and centrally, and if this were true it would be expected that the posterior roots but not the anterior roots would contain the transmitter substance. Hellauer and Umrath (49,50) have found posterior root extracts to contain a highly active vasodilator material which was absent in anterior root extracts. They also found that the vasodilator activity of the extracts was reduced by incubation with fresh brain tissue and that this destruction was inhibited by the addition of strychnine to the mixture. They suggested that the vasodilator substance was identical to the central sensory transmitter and that strychnine inhibited its normal enzymic breakdown, the presence of a destroying enzyme being one of the criteria for a transmitter substance. If this were so, it would be expected that strychnine should potentiate antidromic vasodilatation, by analogy with the action of eserine in cholinergic nerve transmission, if the enzyme is present at the peripheral ends of the neurone. This has not been found to be the case (35).

In order to clarify the problem further Holton (35) has attempted to measure the effects of specific antagonists and synergists of acetylcholine and histamine, as well as the effect of strychnine on antidromic vasodilatation. The effect of acetylcholine itself was measured and was found to produce a vasodilatation of less than a minute's duration, while antidromic stimulation produced a dilatation lasting from 3 to 4 minutes. Injection of 10μg histamine produced a vasoconstriction, while smaller doses tended to give a mild dilatation. Atropine, which inhibits

most peripheral actions of acetylcholine, was found to abolish the effects of injected acetylcholine but not those of antidromic nerve stimulation. Eserine, an anticholinesterase, was found to diminish the effects of antidromic stimulation rather than potentiate them, while the action of injected acetylcholine was enhanced. Mepyramine, an antihistamine, was found to abolish the effects of injected histamine completely but not the effects of stimulation.

From these findings Holton suggested that antidromic vasodilatation is brought about by the liberation of a chemical transmitter but the long latency and duration as well as the pharmacological evidence, make it unlikely that the transmitter is either acetylcholine or histamine. Two other facts make it unlikely that histamine is the transmitter, (a) histamine does not disappear from sensory nerves of the rabbit's ear on degeneration and (b) histamine appears to inhibit transmission in sensory neurones (36). As strychnine did not potentiate the response to antidromic stimulation it seems unlikely that the transmitter liberated is destroyed by a strychnine-sensitive enzyme. Holton suggested, however, that this last observation did not exclude the possibility that the transmitter substance is identical with the central synaptic transmitter, and that the enzyme responsible for its destruction may be present in the spinal cord but not in the peripheral nerve endings.

In another paper Holton (51) found that boiled saline extracts of both posterior and anterior roots contained a substance which causes vasodilatation very similar to antidromic vasodilatation when injected arterially into the rabbit's ear.

A vasodilator effect could also be obtained from extracts of acetone dried powders of both roots and there appeared to be no difference in the content of the active substance when extracts from the two sets of roots were compared. The active substance was heat stable at pH7 and dialysable, but was destroyed by boiling mineral acid although not by alkali. The chemical properties of this vasodilator substance rule out various compounds which upon injection cause a dilatation and which therefore might be the transmitter involved. Thus substance P is soluble in acetone (52), kallikrein is heat labile (53), necrosin is not dialysable (54), and bradykinin is resistant to boiling acid (55). The acetone dried powders of both posterior and anterior roots lost their activity enzymically when incubated in saline. Fresh posterior roots were found to retain their activity when mashed and incubated with saline while fresh anterior roots lost their activity as rapidly as acetone-dried powders.

Holton (56) has tried various vasodilator compounds to determine whether any could produce a dilatation resembling that resulting from antidromic stimulation. She found that injection of solutions of adenosine triphosphate (ATP) satisfied this criterion and fulfilled the chemical specifications given above. These properties were shown to be shared by adenosine diphosphate (ADP), but not adenosine monophosphate (AMP) or adenosine. In experiments on the perfused, isolated rabbit's ear Holton was able to show an increase in absorption of ultra-violet light in the 255-265mμ range, characteristic of purine compounds, on stimulation of the auricular nerve. She suggested these results

indicated that ATP or a similar compound was released on stimulation of the sensory fibres.

Holton (57) has made an effort to determine the content of vasodilator material in various areas of the central nervous system, but has not compared these with the ATP contents of these areas. She found that although the absolute vasodilator activity varied from brain to brain, there was a definite pattern of activity within each brain, with highest concentrations in the caudate nucleus and nucleus cuneatus. However, she admits that it was unlikely that all the vasodilator activity in any one extract could be due to a single chemical substance since the methods used could not discriminate between substances other than acetylcholine and histamine. It was maintained that the results showed no clear relationship inverse or direct between the vasodilator activity of incubated extracts from different regions of the central nervous system and the distribution of cholinergic neurones.

Holton thought that the type of response of the vasodilatation to injection of extracts could give some information to the site of action (58). For example, a substance which dilates the large vessels, the capillaries as well as opening the arterio-venous anastomoses, should give a quick response as the material would be rapidly swept away by the blood. If the substance dilated only the capillaries a prolonged response should result as the injected dilator substance would have to flow through the whole capillary bed before the response would be over. Also, one would expect a delay while the substance reaches the capillaries. If the larger vessels were already dilated and

the arterio-venous anastomoses were open before the injection the response should be short lived.

Holton's experiments demonstrated that the capillaries were the only vessels to respond to antidromic stimulation, provided the stimulation was short enough. In testing possible transmitters she found that adenosine, which dilates the large vessels, produced a quick response whether the rabbit's ear vessels were dilated or not. Spinal root extracts, however, were found to give a prolonged response as well as a degree of latency in preparations with constricted vessels and closed arterio-venous anastomoses, if the larger vessels were dilated and the arterio-venous anastomoses were open the response was over quickly. Holton concluded that the vasodilator substance may be liberated in the capillary bed at some distance from the larger vessels and that the capillaries may be more sensitive to the substance. Furthermore, the vasodilator substance of the spinal root extracts acted primarily on the capillaries in the same way as does antidromic stimulation. The prolonged nature of the vasodilatation indicates that the dilator substance is not quickly destroyed in the blood.

Holton has done various biochemical analyses in an effort to identify more closely the substance causing antidromic vasodilatation (59). Extracts of acetone powders of posterior and anterior roots were chromatographed, intense ultraviolet absorption occurred in positions corresponding to ATP and ADP. These spots were eluted and assayed for vasodilator activity, 16% of the activity was recovered from the ATP portion and 4% of the activity was recovered from the ADP portion with respect to the

original extract. A series of determinations were done in which the active substance was compared against pure ATP, as well as enzymic determinations of labile phosphate content of the extracts. Ultraviolet absorption was also done to obtain a rough estimation of the adenine nucleotide present in the extracts. Holton concluded that the three separate determinations agreed closely enough to be able to say that the vasodilator activity in the extracts was due to a mixture of ATP and ADP and small amounts of further breakdown products.

Holton has used the firefly luminescence method, which is highly specific for ATP, on perfusates from the stimulated rabbit's ear (60,61). She observed that ATP was released into the perfusate on antidromic stimulation of the sensory nerve only after the nerve had been sectioned but not after it had degenerated; as the amount liberated could not be related to the amount of hemolyzed erythrocytes present she concluded the source of ATP to be intracellular and from the nerve. However, the amount recovered was only 0.16% of the amount of ATP which had to be injected arterially to give a comparable dilatation. This introduces one of the most puzzling aspects of the problem, how to account for the large discrepancy of 10^5 order, between the amount of ATP which must be injected to duplicate the vasodilatation effects of stimulation and the maximum amount of ATP recovered from the perfusate after stimulation. Brown, Dale and Feldberg (62) have been able to recover 1/100 of the amount of acetylcholine injected necessary to elicit a response comparable to stimulation of cholinergic nerves. In light of this research a discrepancy of 10^5 order in recovery of injected ATP seems too large

to indicate that it is the substance responsible for the vasodilatation.

Holton (61) has suggested an explanation for the discrepancy. In perfusion experiments the ATP collected after stimulation had to pass through at least one layer of cells which would contain adenosine triphosphatase before reaching the lumen of the blood vessels, and that there is no convenient method of protecting ATP against enzymic breakdown.

In addition to the discrepancy mentioned, one also cannot ignore the well known role ATP plays in metabolism throughout the body. Since ATP is present in all nerve cells and is so closely associated with the metabolism of the neurone it is not surprising that ATP would fit some of the criteria for a transmitter substance. Thus ATP is present in all cells, including neurones. Secondly, as the neurones must have some control over their metabolism, they must be capable of synthesizing and destroying ATP. It seems unlikely, therefore, that such a compound would also be a transmitter substance. This becomes more apparent when one considers the distribution of acetylcholine throughout the central nervous system. It has been shown that not all neurones are capable of synthesizing acetylcholine, and that there is a tendency for neurones with high choline acetylase (the enzyme which synthesizes acetylcholine) content to alternate with cells in which the enzyme is absent. Sensory impulses reach the brain by a three neurone pathway, the first and third of which are deficient in choline acetylase while the second has a high acetylcholine synthesizing power. It seems reasonable to

believe that this alteration of neurones with different enzyme activities corresponds to an alteration of cholinergic and non-cholinergic elements which suggests that acetylcholine is probably the mediator of transmission across a large number of synapses in the central nervous system (63). Just such a distribution of ATP, in contrast, has not been shown to exist, as stated above, ATP is present in all body cells.

Although ATP does cause vasodilatation, there is still doubt that it is the transmitter substance in question because no suitable blocking agents are known for ATP so its effects cannot be ruled out as are the effects of acetylcholine and histamine for which blocking agents are known.

Florey and McLennan (64) have produced some evidence which indicates that ATP is not the transmitter substance. It is known that when sensory fibres have degenerated the area formerly supplied by them becomes sensitized to their transmitter substance. Basing their work on this phenomenon, Florey and McLennan cut segments out of the great auricular nerves of rabbits and allowed the nerves to degenerate. If ATP were the transmitter substance responsible then the reaction to ATP in the denervated preparations should be potentiated. However, this was not found to be the case. When extracts of posterior spinal roots were injected into the denervated area the vasodilatation was very marked while the responses to ventral roots extract and solutions of ATP were decreased.

The question which naturally arises is what substance is responsible for antidromic vasodilatation? This question assumes great importance when one considers Dale's (48) remark

that since the bipolar afferent neurones involved in cutaneous flare do not synapse until they are within the central nervous system, the vasodilator substance released in the periphery during antidromic vasodilatation may also be the transmitter at the central synapse of the afferent neurones, which would further suggest that sensory neurones only and not motor neurones, would contain the humoral agent. Feldberg (65) also expresses the same idea when he says that different branches of the same axon are unlikely to have different transmitter substances. In this case any information made available on the substance responsible for antidromic vasodilatation could also perhaps give a valuable insight into the function of the central nervous system itself.

It has been proposed that ATP is the substance responsible for antidromic vasodilatation (56) and evidence has been produced to support this suggestion. However, by the same token, evidence has also come to light which appears to contradict this proposal (61,64), consequently the problem remains unsolved. Specifically it was in an effort to resolve these apparent contradictions and to determine just what role ATP had, if any, in antidromic vasodilatation that the following research was undertaken.

II. MATERIALS AND METHODS:

1. The Distribution of "vasodilator" activity in the Central Nervous System.

(a) Method for Preparing Dialysates of Brain

Beef brain tissue was obtained from freshly killed cattle, known weights of tissue were taken from the areas of cerebral cortex, caudate nucleaus, thalamus, sub-cortical white matter, reticular formation, floor of the fourth ventricle, cerebellar cortex, optic chiasma, lentiform nucleus, nuclei cuneatus et gracilis, superior corpora quadrigemina, inferior corpora quadrigemina, dorsal roots, ventral roots, dorsal columns of spinal cord, and deep cerebellar structures.

As quickly as possible the fresh tissue was boiled for five minutes in a volume of distilled water equal to two times the weight of the fresh tissue. This was necessary in order to destroy any inactivating enzymes present in the tissue. The boiled material was then homogenized by grinding in a mortar with fine sand or in a Waring blendor, depending on the volume involved. The homogenate was placed in cellophane dialysis tubing and dialyzed against distilled water twenty times the volume of the homogenate for forty-eight hours at 5°C.

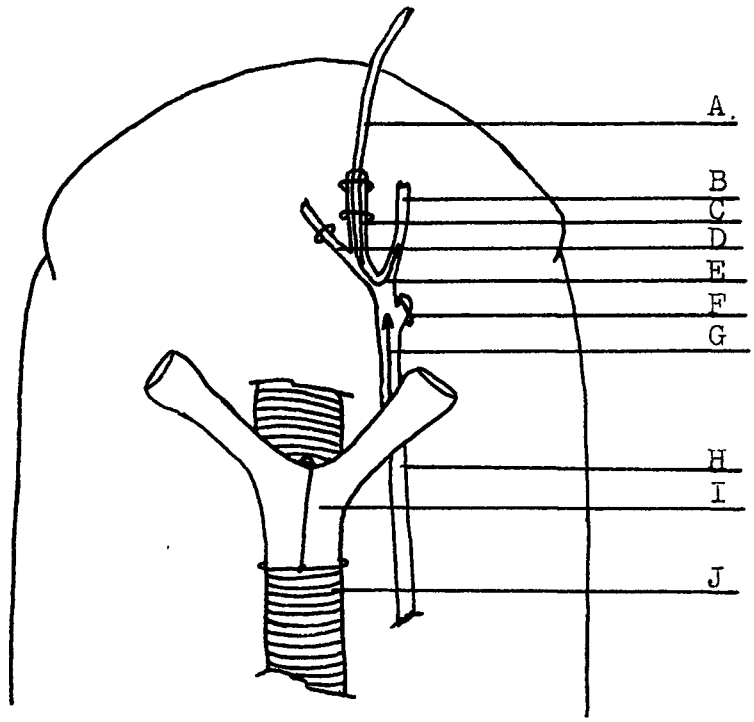
The dialysates were evaporated to dryness in a flash evaporator and redissolved in physiological saline which was one-third the volume of the fresh tissue. These extracts were stored at -5°C. until used. Certain extracts were treated to remove the majority of potassium present, and this was done by adding one-tenth the extract volume of 11.6 N. perchloric acid, filtering out the resultant precipitate, and neutralizing to

pH7 by adding 1 N. sodium hydroxide. These solutions were stored at -5°C. also.

(b) Method for Preparing the Rabbit Ear

The vasodilator activity of the extracts was determined by injecting 0.125 cc. of the material to be tested into the rabbit's ear and measuring the amount of vasodilatation produced in the ear. The rabbit was first anaesthetized with nembutal (50 mg./ml.). A tracheal cannula was inserted first to ensure adequate ventilation, a cannula was then inserted into the femoral vein of one of the hind legs so more anaesthetic could be given if necessary. A third cannula (a small polyethylene tube) was inserted into the facial artery, pointing towards the heart, on the same side of the rabbit as the ear to be tested. All the major branches of the carotid artery including the internal carotid artery were tied off, with the exception of the auricular artery. This allowed free passage to the ear of the injected extract. On injection the extract was forced to flow backward down the facial artery into the external carotid artery where it was then swept into the auricular artery by the blood flow and thereby carried to the ear itself. To avoid interference from extraneous nerve impulses the auricular nerve of the ear to be tested was sectioned before the experiments began.

The rabbit was also given an injection of atropine (1 mg./Kg.), a blocking agent for acetylcholine, and benadryl (10 mg./Kg.), a blocking agent for histamine, before experimentation in order to rule out any effects of acetylcholine and histamine.



Key

A. Polyethylene Cannula
B. Auricular Artery
C. Facial Artery Cannulated
D. Superior Thyroid Artery Tied Off
E. Direction of Injection Flow
F. Internal Carotid Tied Off
G. Direction of Blood Flow
H. Common Carotid
I. Tracheal Cannula
J. Trachea

Figure 2. Cannulation for Rabbit's Ear Preparation

(c) Method for Measuring the Amount of Vasodilatation

Vasodilatation in the rabbit's ear was measured electrophotometrically; the white rabbit's ear was shaved and made more translucent by spreading paraffin oil over it. A light with a green filter over it was shone through the ear, which was taped to a plastic box; beneath the ear was a clear window in the box which allowed the light passing through the ear to fall onto a photoelectric cell. Changes in the intensity of light passing through the ear were detected as changes in intensity of a current produced by the photocell. These changes were amplified and recorded on a moving strip of film which passed in front of an oscilloscope screen. A vasodilatation, therefore, was shown as a curve rising from a predetermined base line on the film strip and similarly, a vasoconstriction was shown as a fall below this base line (see Figure 6.). Before each injection of extract, a photograph of the stabilized base line was taken to enable any change in base line to be measured.

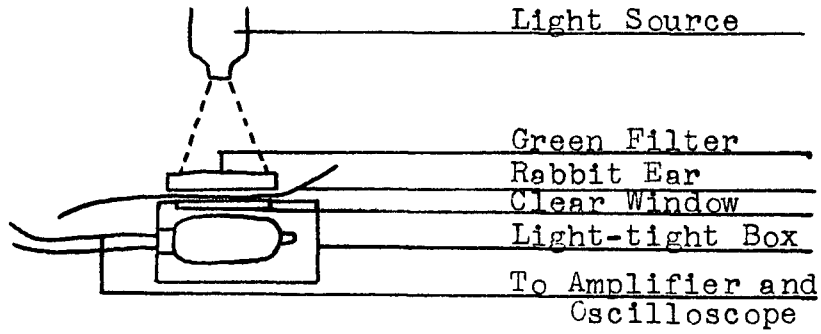


Figure 3. System for Measuring Vasodilatation in Rabbit's Ear

2. Distribution of Adenosine Triphosphate in the Central Nervous System.

(a) Methods for Determining ATP in Central Nervous System

(i) Phosphomolybdic acid method (66). This method is based on the solubility of reducible phosphomolybdic acid in isobutyl alcohol. It is essentially the reduction of colourless phosphomolybdic acid to the blue reduced form by shaking the alcoholic extract with an acidified aqueous solution of stannous chloride.

The amount of inorganic phosphate present in the dialysates was determined first, then the dialysates were hydrolyzed in hot 1 N. hydrochloride acid for seven minutes and the phosphate again determined. The difference between the two measurements was taken to be the acid labile phosphate which was assumed to be adenosine triphosphate.

Five ml. of the dialysate to be determined for phosphate content was put into a separatory funnel and the following were added: 0.5 ml. of 10 N. sulphuric acid, 2 ml. distilled water, 2.5 ml. of an 85% solution of ammonium molybdate, and 10 ml. of isobutyl alcohol. This mixture was shaken for two minutes and the aqueous layer discarded. The alcoholic solution was washed with two portions of 5 ml. each of 1 N. sulphuric acid and then shaken with 15 ml. of a solution of 0.4 gm. stannous chloride/ ml. concentrated hydrochloric acid which had been diluted two hundred times with 1 N. sulphuric acid for thirty seconds, and the aqueous layer discarded. The resulting blue solution was poured into a 10 ml. volumetric flask, the

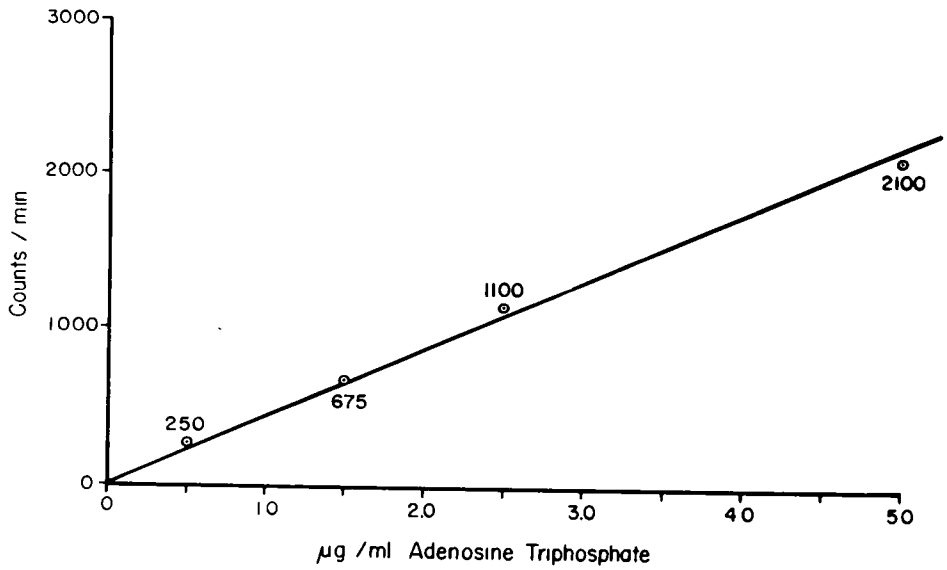
separatory funnel washed with ethyl alcohol and the solution made up to 10 ml. with the washings. The solutions were read in a Klett colorimeter and the amount of phosphate determined from a standard curve which was prepared with each experiment.

(ii) The firefly luminescence enzyme method for determining ATP in dialysates of areas of the CNS. Strehler and Totter (68,69) have reported that light emission by living organisms was a product of a series of chemical reactions and that it was possible under certain conditions to extract from luminous organs a heat stable substance, luciferin, and a heat labile substance, luciferase, which when mixed together would emit light. The light emitting step depended upon the oxidation of the luciferin molecule in the presence of oxygen and the enzyme, luciferase. It was shown that a partially purified Cypridina luciferin (from a species of luminous fish) contained labile phosphate groups which were removed during the light emitting reaction and it was postulated that the energy derived from the breakdown of the sidechain was conserved as phosphate energy bonds.

Support for this suggestion that labile phosphate groups are concerned in the luminescent reaction was obtained with extracts from firefly material. It was possible to restore light emission in extracts which had ceased to luminesce by adding ATP and a divalent ion Mg^{++}.

Implicit in the finding that firefly luminous organ extracts which had become dark would respond to added ATP by the production of light was the possibility of using this phenomenon for the assay of ATP in living material under various

Standard Curve of Luciferine - Luciferate Enzyme Method



conditions. This has been found to be the case using both standard and highly refined light-measuring devices. ATP can be determined directly in mixture with other compounds by making use of a linear relation between light output and ATP added to an extract of firefly lanterns.

The method used in this research was based on the method developed by Strehler and Totter with some modifications by Holton. Fifty mg. of dried firefly lanterns were extracted with 5 ml. of 0.1 M. arsenate buffer at pH 7.4, the mixture was centrifuged and 20 mg./ml. of magnesium sulphate was added to the supernatant liquid. For each determination 1.0 ml. of this enzyme mixture was placed in a tube, 1.0 ml. of dialysate was added, the solution mixed, a stopwatch started, and exactly 15 seconds later the luminescence was measured. Standard solutions of known concentrations of ATP were run with each set of dilutions of the unknown dialysates.

The light emitted by the luminescent reaction was measured by means of a commercial scintillation counter from which the phosphor had been removed and replaced by a clear window of perspex $\frac{1}{2}$ inch thick. A hole the size of the reaction tubes was drilled into the thickness of the window and a mirror, silvered side facing in, was attached to the window on the side opposite the photomultiplier tube so that most of the light emitted would be reflected back into the counter. The whole device was enclosed in a light-proof enclosure.

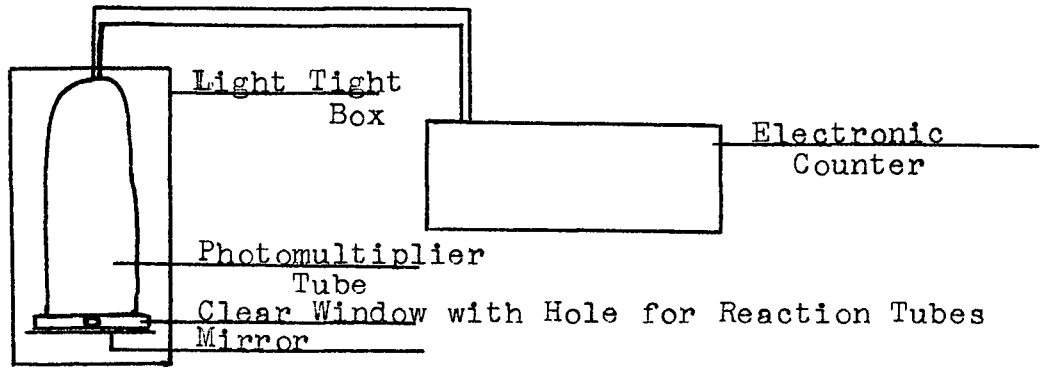


Figure 5. System for Measuring Firefly Luminescence

3. Chromatography.

The basic technique was the same in all cases; varying amounts of ATP were applied to the papers and generally the papers were developed by the two dimensional descending method, the differences being in the solvents used and the methods of detecting the ATP in the developed chromatograms.

(a) 5, 10, 20 and 30 lambda amounts (0.005, 0.010, 0.020, 0.030 ml.) of a 1 mg./ml. solution of ATP were spotted on four full size sheets of Whatman #4 chromatography paper. The papers were developed using the two dimensional descending solvent run technique. The first dimension solvent system was n. propanol, ammonium hydroxide, water (60:30:10) and was run for 8 hours. The second dimension solvent system was tert: butanol, picric acid, water (80 ml., 4 gm., 20 ml.) which was run for 5 hours. The papers were then sprayed with a colourizing solution which contained 5 ml. of 60% perchloric acid, 25 ml. of 4% ammonium molybdate solution, 10 ml. of 1 N. hydrochloric acid, and 60 ml. of water. The papers were then heated for 15 minutes at 85°C.

(b) 25, 50, 75 and 100 lambda amounts of 1 mg./ml. solution of ATP were spotted on sheets of Whatman #4 chromatogram paper as in (a), the solvent systems and techniques were the same as in (a) except the second dimension solvent system was allowed to run for 20 hours instead of 5. The colourizing solution used was the same as (a) except the sprayed papers were heated after being allowed to dry completely first.

(c) 20, 50, 75 and 100 lambdas of 1 mg./ml. solution of ATP were spotted on Whatman #4 papers, and the papers were developed as above. The colourizing solution used this time contained 1 gm. of ammonium molybdate in 8 ml. of water, 3 ml. of concentrated hydrochloric acid, 3 ml. of 70% perchloric acid, and this mixture then diluted to 100 ml. with acetone. The developed papers were dipped in this solution, allowed to dry, and exposed to ultraviolet light for 30 minutes to bring out the colour.

(d) Four papers of Whatman #4 grade, each having the equivalent of 100 mg. of ATP spotted on them, were run in two dimensions using the descending solvent run technique. The two solvent systems were n-propanol, ammonium hydroxide, water (60:30:10) and isopropanol, saturated ammonium sulphate solution, and water (2:79:19); the first system was run for 18 hours, the second for $4\frac{1}{2}$ hours (70). The same colourizing solution was used as above.

(e) Four papers were spotted with 500 lambdas (0.5 ml.) of a water solution of acetone powders made from the caudate nucleus, 50 mg. of ATP were also spotted to serve as a marker

compound; a second 50 mg. portion was also put on after the first solvent system was run, and this served as a marker compound during the second solvent run. The developed papers were scanned first with ultraviolet light and then dipped in the colourizing solution used in (c).

(f) Five hundred lambdas (0.5 ml.) of the dialysate from the thalamus region was spotted on each of 4 papers, and 50 mg. of ATP was also spotted as a marker compound. The chromatograms were developed and the spots coinciding with the marker spots were eluted with distilled water and analyzed for acid labile phosphate as mentioned above by the method of Berenblum and Chain.

(g) One hundred lambda amounts (0.1 ml.) of the dialysates of the caudate nucleus and the cerebral cortex were spotted on 3 MM. chromatogram paper, the papers were developed by the descending technique in n-propanol, ammonium hydroxide, water (60:30:10) for 16 hours. The length of the developed chromatogram from origin to solvent front was divided into ten sections, each of these sections was eluted with distilled water, evaporated to dryness, and redissolved in physiological saline of a volume approximately equal to the original dialysate sample volume. These samples, representing the ten areas on the chromatogram, were then tested on the rabbit ear preparation described above for vasodilator activity.

4. Chemical properties of the vasodilator substance and ATP.

(a) Acid-base stability.

One milliliter portion of the dialysate from the caudate nucleus was boiled with an equal volume of 2 N. hydrochloric

acid and neutralized on cooling, another portion was boiled with an equal amount of 1 N. sodium hydroxide and also neutralized on cooling, a third portion was boiled for the same time but no acid or base was added to it. The same procedure was followed with portions of the dialysate from the crebral cortex area. These samples were then tested on the rabbit ear preparation for vasodilator activity. The same thing was also done with a 1 mg./ml. solution of ATP.

(b) Dialysis of the vasodilator substance and ATP

Experiments were done to try and determine how much ATP was dialysed by the method used for dialyzing the areas of brain for vasodilator material.

Five milliliters of a 1 mg./ml. solution of ATP were placed in a dialysis bag and dialyzed against distilled water in the cold for 48 hours. At the end of this time the dialysate and the contents of the bag were both analyzed for ATP content by the firefly luminescence enzyme method mentioned above.

(c) Treatment of dialysates with exchange resins

Two active dialysates were treated with ion exchange resins in an effort to determine some basic chemical properties of the substance responsible for vasodilatation.

Two milliliter portions of the dialysates from the caudate nucleus and the cerebral cortex were treated with portions of Dowex-50 exchange resin, separate portions were similarly treated with Dowex-1 exchange resin. After filtering the mixtures to separate out the resins, the dialysates were then tested on the rabbit ear preparation for vasodilator activity.

Further 1 milliliter portions of the same dialysates were treated with resin IR-45, a weak anion exchanger, and IRC-50, a weak cation exchanger. Before treatment both resins were washed thoroughly; IR-45 in 1 N. hydrochloric acid to obtain the chloride form, and then twice in distilled water, IRC-50 with 1 N. sodium hydroxide to obtain the hydroxide form, and then twice in distilled water.

After shaking the dialysate with the resins, the liquid was saved. The remaining resin IR-45, was eluted with 1 N. and 3 N. ammonium hydroxide, the eluate was evaporated to dryness and redissolved in physiological saline and neutralized. Similarly, IRC-50 was eluted with 3 N. acetic acid, this eluate being also evaporated to dryness and redissolved in physiological saline and neutralized. These samples were all also tested for vasodilator activity.

III. RESULTS:

Holton (57) has tested various areas of the central nervous system for vasodilator activity and has reported that a definite pattern of distribution existed, the most activity being found in caudate nucleus and nuclear cuneatus. The following groups of experiments were undertaken in an effort to determine the distribution of the vasodilator activity and to see if the distribution paralleled that claimed by Holton. Also, it was felt that if ATP were the transmitter substance or related to the substance in question, the distribution of ATP in the central nervous system should be the same as, or closely similar to, the distribution of vasodilator activity, therefore the distribution and concentration of ATP in the brain was also determined.

1. The distribution of vasodilator activity in the central nervous system

Table I shows the relative amounts of the substance responsible for producing vasodilatation in the rabbit's ear found in the areas of the central nervous system mentioned. The degree of vasodilatation produced by dialysates of the areas are graded from the strongest effect to the weakest by a number of +'s; ++++being the strongest effect and + being the weakest. Where no vasodilatation was obtained an 0 is indicated.

Holton found caudate nucleus and nucleus cuneatus to contain the most vasodilator activity followed by thalamus and hypothalamus. The results here agree generally with those of Holton in that caudate nucleus contains the greatest amount of vasodilator material, while the thalamus also contains much

TABLE I: DISTRIBUTION OF VASODILATOR ACTIVITY IN EXTRACTS FROM VARIOUS AREAS OF THE BRAIN OF THE COW

Area of CNS. Tested	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Inferior colliculus	-	-	++++	-
IVth ventricle floor (mixed)	0	-	-	0
Lenticular nucleus (cellular)	+++	+++	0	-
Thalamus (cellular)	++	++	++	++
Caudate nucleus (cellular)	+++	++++	++	++
Reticular formation (mixed)	0	-	+++	-
Cerebral cortex (precentral areas)	+++	+++	+	+++
Superior colliculus	-	0	0	-
Subcortical white matter	+	++	0	-
Hypothalamus (cellular)	-	-	0	0
Cerebellar cortex (cellular)	++++	-	+	++++
Anterior roots (white)	++	+++	-	-
Nucleic cuneatus et gracilio (cellular)	0	-	-	++
Optic chiasma (white)	0	-	-	-
Deep cerebellum (mixed)	0	0	-	-
Dorsal roots	-	-	-	+++
Ventral roots	-	-	-	+++
Posterior columns (white)	+++	+++	-	-

activity. There the agreement ends. Holton claims hypothalamus has much activity while these results indicate it has no activity; these results also show that cerebral cortex contains a great deal of activity but Holton reported little activity in the same region.

2. Distribution of adenosine triphosphate in the central nervous system

An effort was made to discover a correlation between the distribution of vasodilator activity and the distribution of ATP in the central nervous system. If ATP were in some way connected with antidromic vasodilatation, the distribution of vasodilator activity should be similar to that of ATP.

The method employed was the firefly luminescence enzyme method which is highly specific for ATP and was described in the methods and materials above. The determinations were carried out on water dialysates as well as acetone powders of areas of the central nervous system in order to determine whether the methods of isolation might be affecting the amount of ATP recovered.

Table II shows the amounts of ATP expressed in μg./gm. of fresh brain tissue in water dialysates as well as acetone powders from the areas indicated.

The water dialysate of the floor of the fourth ventricle contained the most ATP, followed by inferior and superior colliculus. The caudate nucleus which contained the most vasodilator activity (see Table I) did not contain the amounts of ATP which one would expect consistent with its vasodilator

TABLE 2: ADENOSINE TRIPHOSPHATE CONTENT OF AREAS OF COW BRAIN EXPRESSED IN μg./gm. FRESH BRAIN

Area of Brain Tested	Water 1	Dialysate 2	Acetone 1	Powder 2
IVth ventricle floor	25.3	76.0	0	0
Inferior colliculus	20.7	62.0	45.0	18.0
Superior colliculus	11.2	33.6	35.2	26.4
Caudate nucleus	4.1	12.2	26.7	20.0
Cerebral cortex	3.7	11.0	24.6	24.6
Thalamus	3.3	10.0	80.0	60.0
Subcortical white matter	2.5	7.4	48.0	48.0
Lenticular nucleus	2.0	6.0	39.5	29.6
Cerebellar cortex	1.7	5.0	16.0	24.6
Reticular formation	1.0	3.0	0	0
Hypothalamus	0	0	0	0

activity if ATP were the transmitter substance. The same can be said for cerebral cortex, thalamus, and cerebellar cortex, the areas which also contained large amounts of vasodilator activity.

The acetone powder of caudate nucleus also did not contain the amounts of ATP consistent with its vasodilator activity if ATP were the transmitter substance concerned. The acetone powder of the thalamus contained the greatest amount of ATP but none of the other areas which had vasodilator activity contained large amounts of ATP. Caudate nucleus, cerebral cortex, and cerebellar cortex all contained relatively small amounts of ATP by comparison with the other areas tested.

It is interesting to note that the amounts of ATP in the acetone powders were all considerably larger than the amounts found in the water dialysates and also that the order of decreasing amounts of ATP is not the same in both cases. This seems to indicate that the dialysis performed was not sufficient to extract all the ATP present in the brain homogenate inside the dialysis tubing. However, the dialysis was sufficient to extract the vasodilator activity present. This would tend to indicate that ATP and the vasodilator substance are two different compounds.

3. <u>A comparison of vasodilator activity with adenosine triphosphate content of the central nervous system.</u>

It was thought that the distribution of vasodilator activity could be best compared with the distribution of ATP in the central nervous system in tabular form. The areas of the central nervous system are listed in order of decreasing content

of the appropriate substances indicated.

Table 3 shows that caudate nucleus, cerebral cortex, and cerebellar cortex were the areas where the greatest amount of vasodilator activity was consistently found, but they were never found to contain the most ATP. It is apparent that there is little correlation, direct or inverse, between the ATP content of the water dialysates and acetone powders and the vasodilator substance content of areas in the central nervous system.

4. A comparison of the chemical properties of the vasodilator activity and ATP.

It was hoped that some indication of the chemical nature of the vasodilator substance as compared to ATP would aid in determining if any chemical relationship existed between the two compounds. The effect of several ion exchange resins was tested in order to see if the compounds had molecules which were charged and if so, whether they were anions or cations. The stability of the vasodilator activity to acid, alkali, and heat was also determined and compared to the stability of ATP to these agents. Another simple experiment was done which gave a good indication of the molecular size of the vasodilator agent. The homogenates of brain were dialyzed against distilled water to prepare the extracts for testing on the rabbit ear, so solutions of ATP were also dialyzed in the same manner to see if enough ATP passed through the membrane and would then indicate its molecule was of a similar size.

In these experiments only water dialysates of caudate nucleus and cerebral cortex were used as these were the two areas where vasodilator activity was consistently found.

TABLE 3: COMPARISON OF THE AMOUNTS OF ATP AND VASODILATOR SUBSTANCE

Water Dialysates of Areas of CNS Containing ATP	Areas of CNS Containing Vasodilator Substance	Acetone Powders of Areas of CNS Containing ATP
IVth ventricle floor	caudate nucleus	thalamus
inferior colliculus	cerebral cortex	subcortical white matter
superior colliculus	cerebellar cortex	inferior colliculus
caudate nucleus	thalamus	lenticular nucleus
cerebral cortex	lenticular nucleus	superior colliculus
thalamus	reticular formation	caudate nucleus
subcortical white matter	subcortical white matter	cerebral cortex
lenticular nucleus	inferior colliculus	cerebellar cortex
cerebellar cortex	superior colliculus	IVth ventricle floor
reticular formation	IVth ventricle floor	reticular formation
hypothalamus	hypothalamus	hypothalamus

(a) Table 4 shows the effect of strong and weak cation and anion exchange resins on dialysates containing vasodilator activity and on solutions of ATP. The vasodilator activity was tested on the rabbit ear preparation as described above, the amount of dilatation is shown by +'s, being the strongest dilatation effect and the weakest as recorded on a film strip moving past an oscilloscope screen.

The amount of ATP present was measured by the amount of light passing through a known solution path at a wavelength of 260 mμ. The concentration of the ATP can be calculated from the optical density of the solution, the molecular extinction coefficient at pH 7.0, and the measured path length. Thus, a solution having an optical density of 0.090, from the equation $A = kcl$; where A = optical density, k = molecular extinction coefficient, c = concentration in moles/litre, and l = length of light path in cms., will have a concentration of $\frac{0.090}{kl}$. Where k for ATP = 15.4×10^3 at pH 7.0 and l = 0.5 cm., the concentration of ATP will therefore be $\frac{0.090}{(15.4 \times 10^3)(0.5)}$ or 0.011×10^{-3} m/l or expressed in g./ml. 6.85.

It can be seen from Table 4 that ATP was completely taken out of solution by the two anion exchange resins Dowex-1 and Amberlite IR-45. The strong cation exchange resin Dowex-50, on the other hand, removed less than half the ATP present. The weak cation exchange resin, Amberlite IRC-50 removed less than one-tenth of the total ATP present. These data indicate that the ATP molecule under neutral conditions is an anion, and therefore under these conditions is similar to the vasodilator substance.

TABLE 4: THE EFFECT OF VARIOUS ION EXCHANGE RESINS ON ACTIVE DIALYSATES AND SOLUTIONS OF ATP

Sample (Water Dialysates)	Vasodilatation in Rabbit Ear
caudate nucleus treated with Dowex-1 (anion exchanger)	0
caudate nucleus treated with Dowex-50 (cation exchanger)	+++
cerebral cortex treated with Dowex-1 (anion exchanger)	0
cerebral cortex treated with Dowex-50 (cation exchanger)	+++
caudate nucleus treated with IR-45 (weak anion exchanger)	0
caudate nucleus treated with IRC-50 (weak cation exchanger)	++
cerebral cortex treated with IR-45 (weak anion exchanger)	0
cerebral cortex treated with IRC-50 (weak cation exchanger)	+

		Amount of ATP
10 γ/ml.)	ATP solution treated with Dowex-1 (anion exchanger)	0
"	ATP solution treated with Dowex-50 (cation exchanger)	6.85
"	ATP solution treated with IR-45 (weak anion exchanger)	0
"	ATP solution treated with IRC-50 (weak cation exchanger)	9.34

(b) A comparison of the stabilities of the vasodilator material and ATP to conditions of alkaline and acidic hydrolysis as well as heat was also thought to give an insight into a possible relationship between the two compounds. In these experiments, as mentioned above, successive portions of water dialysates from caudate nucleus and cerebral cortex were heated to boiling with 2N.HCl and 1N.NaOH as well as being heated to boiling with no hydrolyzing agent present. The same thing was also done with solutions of ATP. The method of determining the remaining vasodilator activity was to inject the neutralized solutions into the rabbit ear preparation, measuring any vasodilatation as mentioned before.

The water dialysates which had been subjected to conditions of alkaline hydrolysis and to heat alone showed no loss of vasodilator activity. The dialysates which had been subjected to conditions of acidic hydrolysis, however, showed a complete absence of any vasodilator activity.

Similarly, a solution of ATP when subjected to alkaline hydrolysis or heat alone showed little decrease in vasodilator activity, while a solution of ATP subjected to acidic hydrolysis showed a marked decrease of vasodilator activity.

(c) To prepare the water dialysates homogenates of tissue from the areas of the brain indicated were dialyzed in the cold against distilled water for 48 hours. The resulting dialysates were concentrated down to a final volume which was one-third the volume of the fresh tissue.

A solution of ATP was also treated in the same manner

to determine whether the ATP molecule would behave in the same way against a concentration gradient as did the vasodilator substance. The amount of ATP which passed through the dialysis membrane was measured by the firefly luminescence enzyme technique (see materials & methods).

The amounts of ATP which were found to pass through the membrane were so small as to render it most unlikely that ATP was the vasodilator substance dialyzed out of the brain tissue homogenates. It would indicate also that the substance responsible for the vasodilatation had a smaller molecular diameter than ATP as it passed through the dialysis membrane almost completely after 48 hours.

5. Chromatography of active extracts for vasodilator material and ATP

An attempt was made to isolate the vasodilator substance as well as ATP chromatographically from the active water dialysates. It was thought that if the active vasodilator material and ATP could be isolated then these substances could be eluted from the chromatograms and injected into the rabbit ear preparation to determine whether any similarity could be found between the eluted material and the vasodilator effects of the water dialysates.

Chromatograms of water dialysates were developed in n-propanol, ammonia, water (60:30:10) for 18 hours and upon being dried were scanned under ultraviolet for light absorbing areas (which is characteristic of ATP). ATP was also run along one border as a marker compound. Several light absorbing areas

were found although none corresponded to ATP. Neither did any areas upon elution produce vasodilatation in the rabbit ear preparation. Similarly, water dialysates were chromatographed and developed in isopropanol, $(NH_4)_2SO_4$ saturated solution, water (2:79:19) for 4½ hours. Ultraviolet light absorbing areas were again tested unsuccessfully for vasodilator activity.

Strips of chromatogram paper were spotted with active water dialysates and developed in the two solvent systems mentioned above. On drying the strips were cut into ten sections between the origin and the solvent front and each section was eluted with isotonic saline. Each elution in turn was tested on the rabbit ear preparation for vasodilator activity without success.

It was hoped that a correlation could be established between an area whose eluate caused vasodilatation and a reaction of the same area to specific colourizing agents. Similar strips of paper were prepared at the same time and treated with various colourizing agents specific for certain species of compounds.

Ninhydrin solution which yields coloured compounds with molecules possessing free-NH_2 groups was used. Most dialysates yielded 4-6 ninhydrin positive areas, probably indicating the presence of amino acids which most certainly would be there.

Molybdic acid spray was used to indicate the presence of organic phosphorous compounds, of which most dialysates gave four distinct areas.

A Hg. containing reagent was used, which indicated the presence of thiouracil, uracil, uridine-5-PO_4, 3,4 dihydroxy-

phenylalanine, and L-adrenaline. Most water dialysates yielded four areas of positive reaction to this reagent.

Ehrlich's reagent, containing p-dimethylaminobenzaldehyde, whose positive reaction gives a yellow colour to dihydrouracil, thymine, phenylalanine, tyrosine, quanosine, and quanine ribotide was also used. The chromatograms of active water dialysates all yielded only one area of positive reaction to this reagent.

Sakaguchi reagent whose positive reaction yields an orange colour with quanidine type compounds was also tried. The water dialysates all had only one area which reacted with the reagent.

As no vasodilator activity could be found in any of the eluates, no correlation could be made with the areas of the chromatograms which had reacted with the various species' specific colouring reagent.

6. Recordings of vasodilatation produced in the rabbit ear preparation.

(a) This tracing is an example of the vasodilatation produced by stimulating the great auricular nerve antidromically. There is a lapse of 5 seconds between the time of nerve stimulation and the onset of vasodilatation. At the end of 55 seconds the vasodilatation is still very marked.

(b) This curve is a recording of the vasodilatation caused by injection of a solution of ATP (1 mg./ml.). At first there is a small drop in the curve caused by the entry of the clear solution into the blood vessels, this appears as a

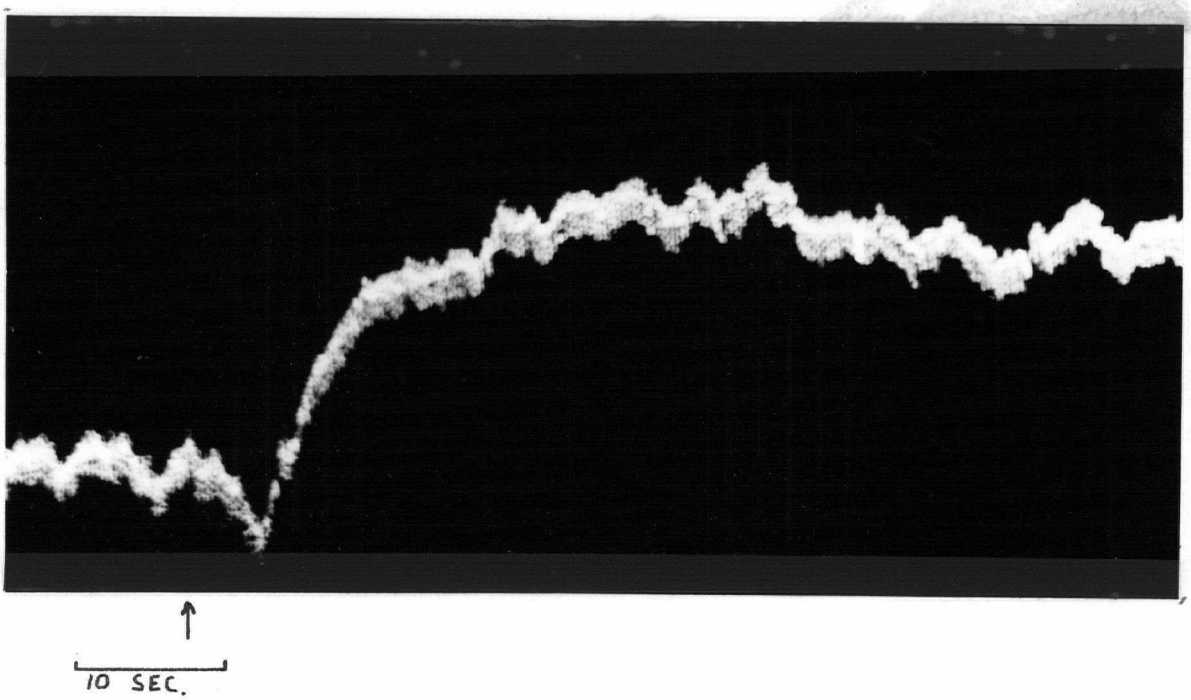


Figure 6A. This tracing is an example of the vasodilatation produced in the rabbit ear preparation by stimulation of the auricular nerve; the arrow indicates the point of stimulation.

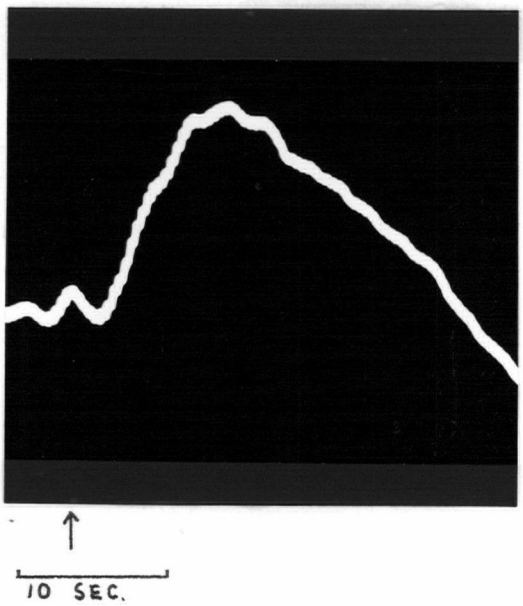


Figure 6B. This tracing is an example of vasodilatation produced in the rabbit ear preparation by injection into the facial artery of ATP solution (1 mg./ml.). The arrow indicates the point of injection.

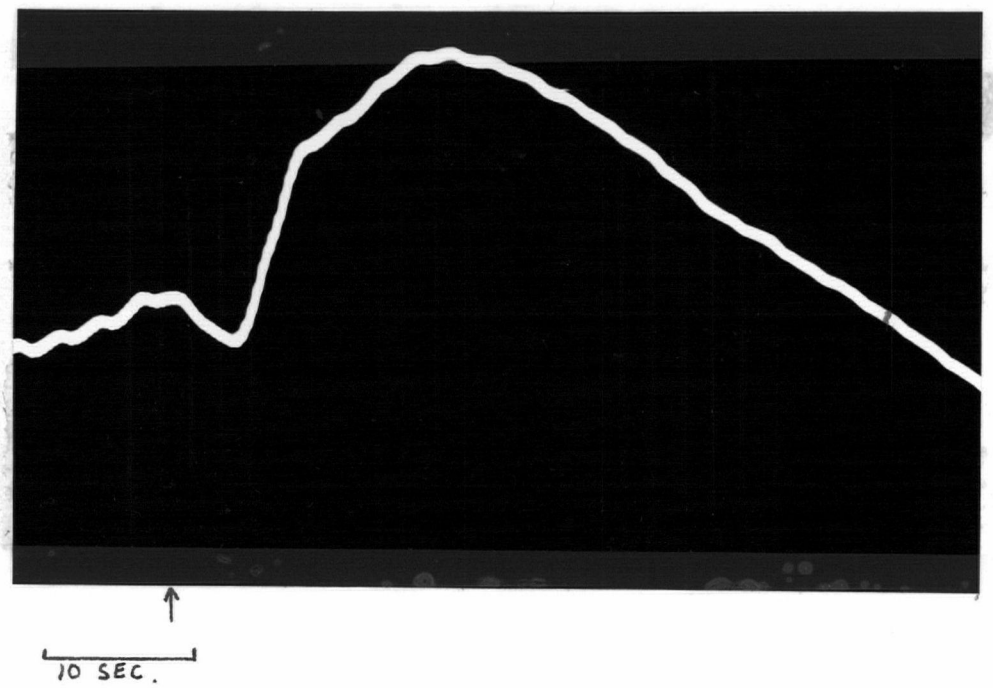


Figure 6C. This tracing is an example of the vasodilatation produced in the rabbit ear preparation by injection of extract of the caudate nucleus. The arrow indicates the point of injection.

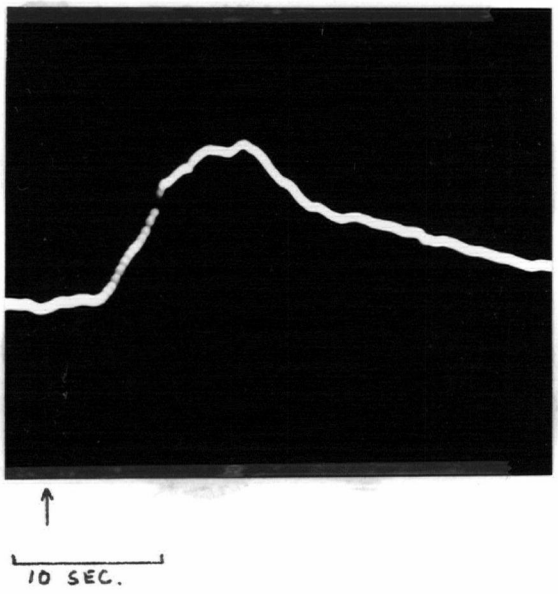


Figure 6D. This tracing is an example of the vasodilatation produced in the rabbit ear preparation by injection of extract of posterior roots of the spinal cord. The arrow indicates the point of injection.

constriction, the dilatation is delayed by 5 seconds. Unlike the dilatation produced by nerve stimulation, the dilatation produced by ATP starts to subside after 13 seconds.

(c) Shows the dilatation produced by injection of the extract of the caudate nucleus. Here there is a short constriction again caused by the entry of the clear solution, again the onset of the dilatation is delayed by 3-4 seconds. The peak vasodilatation is over after 13 seconds.

(d) This tracing illustrates the vasodilatation produced by injection of an extract of posterior roots. The onset of the dilatation is delayed by 5 seconds and the dilatation itself subsides after 8 seconds.

IV. DISCUSSION:

Dale (48) and Feldberg (65) have both put forward the same concept, that a neurone could be expected to release the same transmitter agent at all synapses formed by the different branches of the axon, and further, that a bipolar neurone would possess the same transmitter material at the central end as well as at the peripheral end of its processes. It would therefore be expected that any information obtained about the transmitter released at the peripheral terminals of a primary sensory neurone could also be applied to the transmitter at the central synapse.

Evidence has come to light recently which substantiates Dale's hypothesis. Among spinal neurones acetylcholine is known to be the transmitter substance elaborated for the propagation of a train of impulses from the motor neurones and liberated from their axonal endings to cause transmission across neuromuscular junctions leading to the activation of the muscle. Inhibitory interneurones (Renshaw cells), whose action inhibits the activity of the motor neurones, are present and are served by collateral fibres from the same motor neurones. Acetylcholine has been found to activate the muscle at the neuromuscular junction and it will also activate the Renshaw cells (75); this indicates that acetylcholine is acting as the transmitter substance at more than one branch of the axon of the motor neurone. It is but a short step to postulate that the bipolar sensory neurone with its branches going to the periphery and also synapsing centrally in the spinal cord, could probably also employ the

same transmitter substance at either end of what is essentially one continuous process.

Sensory information reaches the higher centres of the central nervous system by a three neurone pathway, the first synapse can either be within the segment at which the posterior root fibre enters the spinal cord, or higher up in the nuclei cuneatus et gracilis. The second synapse in the pathway is in the thalamus and the final synapse in the appropriate area of the cerebral cortex.

Evidence in cholinergic transmission in the central nervous system has shown that not all the neurones of the pathway are capable of synthesizing acetylcholine, the synthesis of which is taken to indicate a transmitter role for acetylcholine (63). A tendency appears to exist, in some cases at any rate, for neurones of high choline acetylase activity to alternate in a chain with neurones which contain no synthesizing enzyme (71,72). Of the three neurones involved in the primary sensory pathway, the first and third are deficient in choline acetylase while the second neurone has a high activity of this enzyme. An alternation here seems to exist, of neurones having different enzyme activities which is reasonable to believe corresponds with an alternation of cholinergic and non-cholinergic elements of transmission.

The distribution of vasodilator substance in beef brain was determined in the present experiments (see Table 1, results). The results agreed roughly with those reported by Holton (57), Table 5, in that the area found to contain the most activity was

the caudate nucleus, while the thalamus also contained a degree of activity, as did the dorsal and ventral roots. However, here the agreement ends. Holton found the nucleus cuneatus to have much vasodilator material while in these experiments the nuclei cuneatus et gracilis was found to possess almost no vasodilator material. Holton also reports little activity in the cerebral cortex whereas in the above experiments the cerebral cortex contained almost as much vasodilator substance as the caudate nucleus.

At this point it is of interest to compare the distribution of vasodilator material in areas of the central nervous system with the content of other neurologically active substances. Table 6 shows the distribution of some compounds likely to be of importance at central synapses. There does not appear to be any correlation between the distribution of the various substances given in Table 6 and the distribution of vasodilator material from the above experiments. This is not surprising in view of the fact that the substances mentioned have all been ruled out as possible vasodilator agents either because of their chemical or pharmacological properties. Hence, histamine could not be the vasodilator material because vasodilatation will still occur in the presence of antihistaminics compounds; or substance P cannot be the substance as it will not dialyze through a semi-permeable membrane while the active material will dialyze through.

It is tempting to postulate that the transmitter substance responsible for antidromic vasodilatation may also be responsible for synaptic transmission at the first and third neurones

TABLE 5: VASODILATOR ACTIVITY, NOT DUE TO ACETYLCHOLINE OR HISTAMINE, OF EXTRACTS FROM DIFFERENT REGIONS OF THE HORSE BRAIN[1]

	Exp. #1	#2	#3	#4	#5
caudate nucleus	100	100	100	100	100
nucleus cuneatus	300	-	-	-	400
nucleus gracilis	5	-	-	-	-
fasiculus gracilis	-	-	-	30	-
pyramidal tracts	2	-	-	4	-
dorsal root	-	10	-	-	-
optic tract	1	-	-	-	-
trapezoid	2	-	-	-	3
fimbria	3	-	-	4	-
thalamus	5	5	-	8	-
cerebral cortex	1	-	-	4	4
internal capsule, post. limb	2	-	-	4	-
anterior pituitary	-	<2	-	-	-
hypothalamus	-	20	-	20	-
medial eminence of tuber cinereum	-	-	constric-tor	20*	-
standard acetone-dried powder of dorsal roots	40	100	80	20	100

* after treatment which inactivated the posterior pituitary principle - extract of caudate nucleus taken as the standard for each brain, and the activity of the other samples was found by matching each with a dilution of the standard. The activity is therefore expressed as a percentage of that in the caudate nucleus. In addition, the caudate nucleus extract was assayed against a saturated acetone-dried preparation of dorsal roots

1 P. Hollin, G.W. Harris, Journal of Physiology, Vol. 120, page 254, (1953).

TABLE 6: DISTRIBUTION OF NEUROLOGICAL ACTIVE COMPOUNDS IN AREAS OF CENTRAL NERVOUS SYSTEM[2]

	Choline acetylase µg./gm. powder		Noradrenaline µg./gm.	5 HT µg./gm.	Histamine µg./gm.	Subst. P unit/gm.	Holton's vasodil. subst. %	CEF %
deep roots	-	<20	0.01	0	4-11	40	10	100
optic nerves	16	0	0.02	0	9	6	1	100
dorsal columns	33	-	-	0	0.3	27	30	0
internal capsule (posterior)	70	-	-	-	-	-	-	100
cerebellum	26	90	0.07	0.01	<0.1	1.6	1	100
pyramids	42	-	0.06	0	-	-	3	15
ventral roots	573	11000	0.06	0	6-9	6	0	100
sympathetic ganglia	-	-	6	0	61	-	-	0
lateral geniculate body	325	2600	0.07	-	-	-	-	100
midbrain	170	-	0.37	0.20	-	-	-	0
hypothalamus	-	2000	1.03	0.28	12	70	20	-
thalamus	823	3000	0.24 med. 0.28 lat.	0.07 med. 0 lat.	<0.4	12.5	6	100
area postrema	-	-	1.04	0.24	-	460	-	-
caudate nucleus	437	13300	0.10	0	<0.2	46	100	-

2 from Crossland, Journal of Pharmacy & Pharmacology, Vol. 12, page 10, (1960).

of the sensory pathway. Holton has indeed proposed such a role for ATP largely on the grounds that it gives rise to a capillary dilatation and no pharmacological agents are known which block its action. She has also produced some evidence, which has been discussed above (see page 15, Introduction), that ATP is released upon stimulation of sensory fibres.

Measurements of the amounts of ATP were carried out on various areas of the brain with the idea in mind that if ATP were the transmitter responsible for antidromic vasodilatation, or related to it, the areas containing the most vasodilator activity should also contain the most ATP. As can be seen from Table 3 in the results, no relationship is apparent between the location and concentration of the two substances.

In postulating the presence of the vasodilator material in the sensory pathways it is necessary to comment on the possible significance of distribution in the areas tested. If one accepts Dale's hypothesis that a neurone will utilize the same transmitter substance at all branches of the axon, and there is some evidence to support this, then one might well expect to find the active substance at the peripheral ends of the sensory neurones and at the central ends, in which case the vasodilator substance would be present in the posterior roots. In considering the three neurone sensory pathways the first neurone synapses with the second neurone in the thalamus, hence it would seem reasonable the vasodilator material be in the thalamus at the central synapse of the first sensory neurone. This has been shown (see Table 1) in the results. If the distribution of the vasodilator

substance alternated with the distribution of acetylcholine, then one would expect to find vasodilator material in the first and third neurone of the three neurone pathway; in which case the cerebral cortex should also contain vasodilator material. This has been demonstrated, in fact the cerebral cortex possesses a large amount of vasodilator material. However, one enigma still remains. Of all the areas tested for vasodilator material the caudate nucleus was consistently found to contain the greatest amount. As can be seen from Table 6. the caudate nucleus contains appreciable amounts of most of the active substances. The presence of the vasodilator material in the caudate nucleus is difficult to reconcile with the fact that it has been shown to date to have motor functions only. However, there is no reason why the material, if it is an excitatory transmitter, should only be in sensory pathways.

In view of the above evidence and from the fact that ATP has such an important role as a source of energy for the body in general, it seems unlikely that it can be the substance responsible for sensory nerve transmission. As ATP is present in all cells, including neurones, it is obvious that it would meet some of the criteria for a transmitter agent. Some of the criteria are; there must be some system for synthesizing the substance, and there must also be some mechanism for destroying the substance. Since neurones must be able to control their source of energy accurately they possess a system for synthesizing ATP and also a mechanism for destroying ATP thereby, in effect, fulfilling some of the criteria for a transmitter agent.

The two most important questions for which answers were sought in this research were: 1. is ATP the substance responsible for antidromic vasodilatation? and 2. if it is, is it also a sensory synaptic transmitter substance? It appears from the above research that ATP is unlikely to be the substance responsible for the vasodilatation, which would seem to rule it out as the sensory synaptic transmitter substance. However, although this has not been definitely demonstrated in the present work, if one accepts Dale's proposal, then the answer for both questions should be the same and the substance responsible for antidromic vasodilatation should also be the central synaptic transmitter substance.

BIBLIOGRAPHY

1. Lovén, C.: Uber die Erweiterung von Arterien in Folge einer Nervenerregung. Berichte über die Verhandlungen der Sächsischen Gesellschaft der Wissenschaften, Leipzig, 18: 85, 1866.

2. Goltz, F.: Uber gefässerweiternde Nerven. Pfleuger's Arch. ges. Physiol., 9: 185, 1874.

3. Stricker, S.: Untersuchung über die Geffässerwuzeln des Ischiadicus. Sitz. Kaiserl. Akad. Wissenchr. (Wien), 3: 173, 1876.

4. Bayliss, W.M.: On the origin from the spinal cord of the vasodilator fibres of the hind limb and on the nature of these fibres. J. Physiol., 26: 173, 1901.

5. Bayliss, W.M.: Principles of General Physiology (4th ed.) Longmans, Green & Co., (London, 1924).

6. Bayliss, W.M.: Further researches on antidromic nerve impulses. J. Physiol., 28: 276, 1902.

7. Bayliss, W.M.: The excitation of vasodilator fibres in depressor reflexes. J. Physiol., 37: 264, 1908.

8. Bayliss, W.M.: The Vasomotor System, Longmans, Green & Co., (London, 1923).

9. Bruce, A.N.: Uber die Berzichung der sensiblen Nerven digungen zum Entzundungsvorgang. Arch. Exp. Path. Pharmakol., 63: 424, 1910.

10. Langley, J.N.: The Autonomic Nervous System. Cambridge University Press, (London, 1921).

11. Langley, J.N.: I. Antidromic action. J. Physiol., 57: 428, 1923.

12. Langley, J.N.: II. Antidromic action. Stimulation of the peripheral nerves of the cat's hind foot. J. Physiol., 58: 49, 1923-24.

13. Doi, V.: On the existence of antidromic fibres in the frog and their influence on the capillaries. J. Physiol., 54: 227, 1920.

14. Feldberg, W.: The peripheral innervation of the vessels of the external ear of the rabbit. J. Physiol., 61: 518, 1926.

15. Foerster, O.: Uber die Vasodilatatoren in den peripheren Nerven und hinteren Rüchenmarkswruzeln beim Menschen. Dtsch. Z. Nervenheilk, 107: 41, 1928.

16. Krogh, A.: The supply of O_2 to the tissues and the regulation of the capillary circulation. J. Physiol., 52: 457, 1919.

17. Krogh, A., Harrop, G.A., Rehberg, P.B.: Studies on the physiology of capillaries. III. The innervation of the blood vessels in the hind legs of the frog. J. Physiol., 56: 179, 1922.

18. Gaskell, W.H.: Involuntary Nervous System, Longmans, Green & Co., (London, 1916).

19. Loewi, O.:. Uber humorale Ubertragbarkeit der Herznervenwirkung. Pfleuger's Arch. ges. Physiol., 189: 239, 1921.

20. Lewis, T., Marvin, H.M.: Herpes zoster and antidromic impulses. J. Physiol., 62: XIX, 1926.

21. Lewis, T., Marvin, H.M.: Observations relating to vasodilatation arising from antidromic impulses, to herpes zoster and trophic effects. Heart, 14: 27, 1927.

22. Kibjakow, A.W.: Zur Frage des Vasodilatations mechanismns bei der Reizung antidromes Nerven. I. Uber die gefässerweiternden Eigenschaften des Blutes beim Reiz der hinteren sensiblen Wurzeln. Pfluger's Arch. ges. Physiol., 228: 30, 1931.

23. Lewis, T., Harris, K.E.: Influence of the cutaneous nerves on various reactions of the cutaneous vessels. Heart, 14: 1, 1927.

24. Dale, H.H., Richards, A.N.: The vasodilator action of histamine and some other substances. J. Physiol., 52: 110, 1919.

25. Dale, H.H., Laidlaw, P.P.: Histamine shock. J. Physiol., 52: 355, 1919.

26. Lewis, T., Grant, R.T.: Vascular reactions of the skin to injury. The liberation of a histamine-like substance in injured skin; the underlying cause of factitious urticaria and of wheals produced by burning; observations upon the nervous control of certain skin reactions. Heart, 11:209, 1924.

27. Kwiatkowski, H.: Histamine in nervous tissue. J. Physiol., 102: 32, 1943.

28. Ibrahim, F.D., Stella, G., Talaat, M.: The mechanism of antidromic vasodilatation. Quart. J. Exp. Physiol., 36: 189, 1951.

29. Ungar, G.: Sur les rapports des appareils périphériques vasodilatateurs avec les terminasons nerveuses sensitives, d'après la conception de la transmission humorale histaminique. Ann. Anat. Path., 12: 586, 1935.

30. Ungar, G.: Effet de l'excitation du bout périphérique des nerfs sensitifs sur la sécrétion gastrique. Transmission neuro-humorale histaminique. C.R. Soc. Biol. (Par.), 118: 620, 1935.

31. Ungar, G., Parrot, J.L.: Sur la nature de la substance libérée au cours de la vasodilatation dite antidromique. C.R. Soc. Biol. (Par.), 129: 753, 1938.

32. von Euler, U.S., Åström, A.: Liberation of histamine and sympathin by stimulation of isolated splenic nerves of cattle. Acta Physiol. Scand., 16: 97, 1949.

33. Parrot, J.L., Lefebvre, J.: Analyse de la triple réaction cutanée au moyen d'un antagoniste de l'histamine. C.R. Soc. Biol. (Par.), 137: 316, 1943.

34. Frumin, N.J., Ngai, S.H., Wang, S.C.: Evaluation of vasodilator mechanisms in the canine hind leg. Question of dorsal root participation. Am. J. Physiol., 173: 428, 1953.

35. Holton, P., Perry, W.L.M.: On the transmitter responsible for antidromic vasodilatation in the rabbit's ear. J. Physiol., 114: 240, 1951.

36. Chauchard, P.: Recherches chromaximetrique sur l'histamine et ses antagonistes application au choc anaphylactique. Arch. int. Pharmacodyn., 79: 139, 1949.

37. Parrot, J.L.: The place of histamine in neurohumoral transmission. Pharmacol. Rev., 6: 119, 1954.

38. Chapman, L.F., Ramos, A.O., Goodell, H., Wolff, H.G.: Neurohumoral features of afferent fibres in man. Arch. Neurol., 4 (6): 617, 1961.

39. Dale, H.H., Feldberg, W.: The chemical transmission of secretory impulses to the sweat glands of the cat. J. Physiol., 82: 121, 1934.

40. Dale, H.H., Feldberg, W.: Chemical transmission at motor endings in voluntary muscle. J. Physiol., 81: 39P, 1934.

41. Dale, H.H., Feldberg, W.: Release of acetylcholine at voluntary motor nerve endings. J. Physiol., 86: 353, 1936.

42. Dale, H.H., Gaddum, J.H.: Reactions of denervated voluntary muscle and their bearing on the mode of action of parasympathetic and related nerves. J. Physiol., 10: 109, 1930.

43. Wybauw, L.: Etude, chez le chat, des réactions vasodilatatrices périphériques provoquées par l'excitation électrique des racines postérieures spinales. C.R. Soc. Biol. (Par.), 121: 1377, 1936.

44. Wybauw, L.: Transmission humorale de la vasodilatation provoquée par l'excitation du bout péripherique des racines postérieures lombaires chez le chat. C.R. Soc. Biol. (Par.), 123: 524, 1936.

45. Wybauw, L.: II. Mécanisme périphérique de la vasodilatation antidromique sa médiation chemique. Arch. int. Physiol., 46: 324, 1938.

46. Wybauw, L.: II. Les réflexes axoniques vasodilatateurs leur signification fonctionelle. Arch. int. Physiol., 46: 345, 1938.

47. Chapman, L.F.: Neurohumoral features of the axon reflex. Fed. Proc., 18: 25, 1959.

48. Dale, H.H.: Reizübertragung durch chemische Mittel im peripheren Nervensystem. (Nothnagelstiftung Vortrag) Vienna, Urban and Schwarzenberg, 1935.

49. Hellauer, H.F., Umrath, K.: The transmitter substance of sensory nerve fibres. J. Physiol., 106: 20P, 1947.

50. Hellauer, H.F., Umrath, K.: Uber die Aktionsubstanz der sensiblen Nerven. Pfluger's Arch. ges. Physiol., 249: 619, 1948.

51. Holton, P., Holton, F.A.: The vasodilator activity of spinal roots. J. Physiol., 118: 310, 1952.

52. Gaddum, J.H., Schild, H.O.: Depressor substances in extracts of intestines. J. Physiol., 83: 1, 1934.

53. Kraut, H.: Uber den Nachweis und das Vorkommen des Kallikreins in Blut. Hoppe-Seyl Z. 222: 73, 1933.

54. Menkin, V.: Chemical basis of injury in inflammation. Arch. Path. (Lab. Med.), 36: 269, 1943.

55. Rocha e Silva, M., Bernaldo, W.T., Rosenfeld, G.: Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venom and by trypsin. Am. J. Physiol., 156: 261, 1949.

56. Holton, P., Holton, F.A.: The possibility that ATP is a transmitter at sensory nerve endings. J. Physiol., 119: 50P, 1953.

57. Holton, P.: Vasodilator activity in extracts of various regions of the central nervous system. J. Physiol., 120: 254, 1953.

58. Holton, P., Holton, F.A.: Antidromic vasodilatation and blood flow in the rabbit's ear. J. Physiol., 125: 138, 1954.

59. Holton, P., Holton, F.A.: The capillary dilator substances in dry powders of spinal roots; a possible role of ATP in chemical transmission from nerve endings. J. Physiol., 126: 124, 1954.

60. Holton, P.: Liberation of ATP from perfused rabbit's ear on antidromic stimulation of the sensory nerve. J. Physiol., 141: 13P, 1958.

61. Holton, P.: Liberation of ATP on antidromic stimulation of sensory nerves. J. Physiol., 145: 494, 1959.

62. Brown, G.L., Dale, H.H., Feldberg, W.: Reactions of the normal mammalian muscle to acetylcholine and to eserine. J. Physiol., 87: 394, 1936.

63. Crossland, J.: Chemical transmission in the central nervous system. J. Pharm. & Pharmacol., 12: 1, 1960.

64. Florey, E., McLennan, H.: Is ATP the sensory transmitter? Naturwissenschaften, 42: , 1955.

65. Feldberg, W.: Central excitation and inhibition from the view point of chemical transmission. Proc. Roy. Soc. B., 140: 199, 1952.

66. Berenblum, J., Chain, E.: An improved method for the colorimetric determination of phosphate. Biochem. J., 32: 295, 1938.

67. Smith, J.: Chromatographic and Electrophoretic Techniques. Heinemann, (London, 1960).

68. Strehler, B.L., Totter, J.R.: Firefly luminescence in the study of energy transfer mechanisms. Substrate and enzyme determinations. Arch. Biochem. Biophys., 40: 28, 1952.

69. Strehler, B.L., Totter, J.R.: Determination of ATP and related compounds. Meth. Biochem. Anal., 1: 341, 1954.

70. Deutsch, A., Nilsson, R.: Separation of adenosine and inosine phosphates by paper chromatography. Acta Chem. Scand., 7: 858, 1953.

71. Feldberg, W., Vogt, M.: Acetylcholine synthesis in different regions of the central nervous system. J. Physiol., 107: 372, 1948.

72. Feldberg, W., Harris, G.W., Lin, R.C.Y.: Observations on the presence of cholinergic and non-cholinergic neurones in the central nervous system. J. Physiol., 112: 400, 1951.

73. Torda, C.: Effects of ATP on electrical activity of the brain during the preconvulsive and convulsive phases induced by various convulsants. Am. J. Physiol., 178: 123, 1954.

74. Robinson, F., Hughes, R.A.: Effects of adenine compounds on electrocortical activity. J. Neurophysiol., 14: 387, 1951.

75. Eccles, J.C., Fatt, P., Koketsu, K.: Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurones. J. Physiol., 126: 524, 1954.