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THE CAPILLARY DILATOR SUBSTANCES IN DRY POWDERS OF SPINAL ROOTS; A POSSIBLE ROLE OF ADENOSINE TRIPHOSPHATE IN CHEMICAL TRANSMISSION FROM NERVE ENDINGS

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The vasodilatation which results from antidromic stimulation of sensory nerves has suggested to several authors (Dale, 1935; Hellauer & Umrath, 1948), that the transmission of impulses from both peripheral and central endings of sensory fibres may be mediated by a chemical substance possessing a vasodilator action. In searching for such a substance in nervous tissue, Hellauer & Umrath (1947, 1948) found that potent vasodilator activity was present in extracts of dorsal but not of ventral roots. In contrast, we observed vasodilator activity in extracts of both dorsal and ventral roots (Holton & Holton, 1952). This paper describes an investigation into the properties of the active substance or substances, and suggests that the vasodilator activity of our extracts is due to adenosine triphosphate (ATP) and its breakdown products.

METHODS

Materials

The following substances were used for injection into the rabbit's ear. They were freshly diluted with, or dissolved in, saline (0·9% NaCl in distilled water) and the pH was adjusted to 7·4 before injection.

Adenosine triphosphate (*ATP*). The barium salt of ATP was prepared from rabbit muscle following the procedure of LePage (1949), omitting the magnesium anaesthesia but including precipitation with mercury. Neutral solutions of the potassium salt were obtained from this material either by the method described by Slater (1953), or by precipitating the barium from acid solution with a small excess of potassium sulphate, followed by neutralization with potassium hydroxide. The solutions were standardized by enzymic analysis (Slater, 1953), and contained negligible amounts of ADP and AMP.

Adenosine diphosphate (*ADP*) was prepared enzymically from ATP as described by Slater (1953), and standardized by enzymic analysis. It contained negligible amounts of ATP and AMP.

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Adenosine monophosphate (*AMP*) was a commercial preparation of muscle adenylic acid (Light), containing negligible amounts of energy-rich phosphate ($\sim$P).

Inosine triphosphate (*ITP*). The barium salt of ITP, which was kindly given by Prof. H. A. Krebs, was dissolved in acid. The barium was precipitated with sodium sulphate and the supernatant neutralized with sodium hydroxide.

Creatine phosphate (*CP*) was kindly given by the late Mr E. J. Morgan of the Biochemical Laboratory, Cambridge.

Yeast uridylic acid, yeast guanylic acid and muscle inosinic acid (*IMP*) were kindly given by Dr J. D. Smith.

Adenine (*Light*), *adenosine* (*Light*), *diphosphothiamine* (*Roche*) *and thiamine* (*B.D.H.*) were obtained commercially and used without further purification.

Substance P. Two preparations were used. They were kindly given by Dr W. Feldberg (*PF*) and Prof. J. H. Gaddum (P_4).

Preparation of biological material. Acetone-dried powders were prepared from the dorsal and ventral roots and caudate nucleus of horses as described previously (Holton & Holton, 1952) They were then extracted according to the procedure shown in Table 1 (first three stages), which increased the stability of the preparations without removing any appreciable vasodilator activity. The resulting 'solvent-extracted powders' (Table 1), were stored in dark bottles over phosphorus pentoxide and retained their full activity for at least 10 months. The powders are referred to as $D_{15}P$, $V_{15}P$, CN_3P, etc., indicating that they were prepared from the fifteenth batch of dorsal and ventral roots and the third batch of caudate nucleus respectively. Immediately before use an extract was prepared by boiling the powder for 1 min with distilled water or saline, centrifuging and discarding the residue. The results described in this paper were obtained with such extracts prepared from dorsal and ventral root powder and no difference was detected between them, apart from a slightly greater activity in the dorsal root preparation already reported. The term spinal root powder or extract is used in this paper to refer to powder or extract from either dorsal or ventral roots, and does not imply that the dorsal and ventral roots were mixed.

Barium precipitation of aqueous extracts

Two aliquots of 2 ml. were taken from a sample of a boiled saline extract of spinal root powder. To one was added 0·05 ml. of 0·9 M-barium acetate with thorough mixing, followed by 0·02 ml. of 1·0 M-Na_2SO_4. Both samples were then stored at 2° C for 2 days. 0·07 ml. of 1·0 M-Na_2SO_4 was next added to the untreated sample, both were centrifuged, and the clear supernatants tested for vasodilator activity.

Paper chromatography

A large amount of starting material was needed for the paper chromatography to allow for losses during manipulation. For this experiment we therefore used a solvent-extracted powder prepared from horse caudate nucleus. This tissue is easier to obtain in bulk and contains the slow dilator substance typical of the spinal roots (Harris & Holton, 1953; unpublished). The vasodilator activity was concentrated further by stages 4, 5 and 7 of the procedure in Table 1 and the resultant gum was taken up in a small volume of water and chromatographed on paper.

The paper chromatography was kindly carried out by Dr J. D. Smith using a descending chromatogram and solvent 3 of Markham & Smith (1952). This consists of 70% (v/v) isopropanol and 30% water in an atmosphere of ammonia. The nucleotide spots were detected by ultraviolet photography (Markham & Smith, 1949), using samples of pure nucleotides as markers. They were cut out separately and eluted by mashing the paper with water and centrifuging. The eluates were tested for vasodilator activity after concentration by freeze-drying and adjustment of tonicity by addition of NaCl.

Ultraviolet absorption spectra

These were measured in a Hilger Uvispek spectrophotometer using 1 cm cells. Two different procedures were followed: (*a*) Boiled aqueous extracts of spinal root powders ($V_{16}P$ and $D_{16}P$, 10 mg powder/ml.) were diluted 10 times with water and read at intervals of 5 mμ against a reference

cell containing water. (*b*) A boiled-aqueous extract of spinal root powder ($D_{15}P$, 20 mg powder/ml.) was deproteinized by the addition of 40% (w/v) trichloroacetic acid (final concentration 5% w/v) and the clear supernatant was read against a reference cell containing 5% trichloroacetic acid at the wavelengths indicated in Fig. 1.

Assays of vasodilator activity

These were carried out by the rabbit's ear method already described (Holton & Holton, 1952), using rabbits whose ears were denervated by section of the great auricular nerve and removal of the superior cervical ganglion at the beginning of the experiment. The vessels of the ear were made insensitive to acetylcholine and histamine by giving atropine (5 mg/kg) and mepyramine (2·5 mg/kg) intravenously every 3 hr.

Investigation of prolonged vasodilator responses

Rabbits were specially prepared for these experiments by removal of the stellate and superior cervical sympathetic ganglia under aseptic conditions 2–3 weeks previously. This interval allowed the sympathetic fibres to degenerate and the vessels of the ear usually regained constrictor tone. Only those animals whose ear vessels had developed good constrictor tone were suitable.

Enzymic determination of ATP and ADP

This estimation was carried out by the enzymic method of Slater (1953). The method uses glycolytic enzymes to couple the dephosphorylation of ATP and ADP to the oxidation of reduced cozymase. The amount of reduced cozymase oxidized is measured spectrophotometrically and 0·05 μmole of ATP (approx. 25 μg) may be determined with an error of less than 5%.

The total ~P (ATP, ADP and CP) in extracts of three different spinal root powders was determined using Slater's analysis $B(a)$. This estimate includes the following interfering substances when present: pyruvate, phosphopyruvate, phosphoglycerate, oxaloacetate and hexosediphosphate. In order to determine the maximum possible contribution of these substances to analysis $B(a)$, one of the extracts was analysed by Slater's procedure $B(b)$, which estimates these five substances together with hexosemonophosphate. The amount of reacting material (moles) found in analysis $B(b)$ was 20% of that found in analysis $B(a)$. Since it was unnecessary that the estimation of ~P should be of great accuracy for comparison with the vasodilator assays, the substances contributing to analysis $B(b)$ were not investigated further. The difference $B(a) - B(b)$ was used to calculate the minimum amount of ~P in the extract, on the assumption that the substances reacting in analysis $B(b)$ consisted entirely of those which produce maximum interference in analysis $B(a)$ (that is, phosphopyruvate and phosphoglycerate; see Slater, 1953, for full discussion). Two other extracts were analysed by procedure $B(a)$ only, and the same percentage correction was applied to the result to give minimum values of ~P present. Maximum values of ~P were calculated directly from analysis $B(a)$, on the assumption that the only substance reacting in analysis $B(b)$ was hexosemonophosphate.

Inorganic phosphate determinations. These were carried out by the method of Lohmann & Jendrassik (1926).

RESULTS

Preliminary concentration

In attempting to concentrate the vasodilator activity of spinal root extracts, it was convenient to start with fairly large samples of acetone-dried powders. The course of purification was followed by comparing the vasodilator activity of the purified fractions obtained at each stage with a sample of the starting material. Table 1 summarizes the concentration procedure adopted. Extraction with organic solvents (stages 1–3) removed about 70% of the original weight of the powder without loss of activity. The activity was then extracted with boiling water (stage 4) and remained in the supernatant after protein

precipitation (stage 5). Precipitation with barium at neutral pH removed most of the active substance, however, and this suggested that it might be a nucleotide or a nucleotide derivative.

The concentration procedure up to this stage was carried out with samples of four powders prepared from two batches of dorsal roots and one batch each of ventral roots and caudate nucleus.

TABLE 1. Concentration and purification of vasodilator substance present in acetone-dried powders of spinal roots and of caudate nucleus

Stage	Starting material	Procedure	Resulting material	Activity recovered (%)
1	Acetone-dried powder	6 hr extraction with dry ether at 25° C in Soxhlet apparatus	Ether-extracted powder	100
2	Ether-extracted powder	6 hr extraction with dry acetone at 25° C in Soxhlet apparatus	Ether/acetone-extracted powder	100
3	Ether/acetone-extracted powder	6 hr extraction with absolute ethanol at 25° C in Soxhlet apparatus	Solvent-extracted powder	100
4	Solvent-extracted powder	Boiled with distilled water for 2 min. Cooled, centrifuged, residue discarded	Boiled-water extract	100
5	Boiled-water extract	Treated with trichloroacetic acid, centrifuged, residue discarded	Deproteinized extract	100
6	Deproteinized extract	Treated with equivalent amounts of barium acetate and sodium sulphate. Cooled 2 days, centrifuged, residue discarded	Barium-precipitated extract	10
7	Deproteinized extract	Concentrated by freeze-drying	Gum used for paper chromatography	Not tested

Paper chromatography

The developed chromatogram showed intense ultraviolet absorption in the positions corresponding to ATP and ADP. The paper was cut into three portions corresponding to the ATP spot, the ADP spot and the remainder; each portion was eluted with water and the eluate assayed for vasodilator activity. Approximately 16% of the activity was recovered from the ATP spot, about 4% from the ADP spot and only a trace from the remainder of the paper. The freeze-drying and chromatographic procedure had resulted in a loss of 80% of the total activity. It was concluded that at least some of the vasodilator activity was due to a mixture of ATP and ADP, since both of these substances have a potent dilator action. Since the total recovery of activity was poor, other methods were used to investigate what proportion of the total activity could be attributed to these substances.

Ultraviolet absorption spectrum

The ultraviolet absorption spectrum of boiled-water extracts of spinal root powders was determined on three different preparations. All the extracts showed a marked absorption band between 260 and 265 mμ. In one experi-

ment the extract was deproteinized before being examined and this band was then more sharply defined. This spectrum is shown in Fig. 1. An intense absorption band at 260 mμ is shown by many naturally occurring substances besides ATP, including all those which contain a purine or a pyrimidine nucleus. Thus the magnitude of the specific absorption in this region of the spectrum can only be used to calculate an upper limit to the amount of ATP present. The concentrations of adenine nucleotide corresponding to the observed optical densities were roughly estimated by measuring the height of

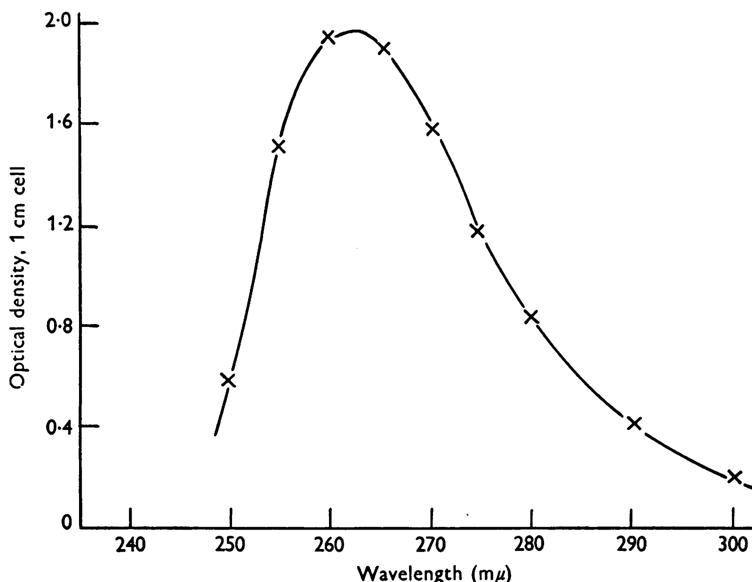

Fig. 1. Ultraviolet absorption spectrum of a deproteinized extract of spinal roots (20 mg solvent-extracted powder per ml.).

the absorption band at 260–265 mμ above the level of non-specific absorption. The absorption coefficient for ATP given by Gulland & Holiday (1936) was used for the deproteinized extract (pH approx. 2), and that given by Kalckar (1947) for the neutral extracts (pH 7). The values obtained in this way for the three different preparations were 6·6, 9·2 and 8·2 mμmole adenine nucleotide per mg. The figures given in Table 2, line 2, (3·3, 4·6 and 4·1 μg ATP per mg) have been calculated for ATP (mol.wt. 503) and would be rather smaller for substances of lower molecular weight such as ADP and AMP.

Assays of vasodilator activity against ATP

A useful step in identifying a biologically active substance in tissue extracts is to make a series of determinations of the activity of a standard extract in terms of the pure substance. If the results do not agree it is unlikely that the unknown and pure substances are identical. In line 1 of Table 2 the results

are given for such a series of assays. Extracts of a standard acetone-dried powder ($D_{15}P$) were compared with ATP on seven different preparations of the rabbit's ear. The relative activity of the extracts varied between 1·8 and 4·5 μg ATP/mg of powder. Since assays by this method are not very precise this represents sufficiently close agreement to suggest that the whole of the vasodilator activity of the extract could be due to ATP or substances with closely similar properties.

TABLE 2. The amounts of ATP or ADP in extracts of solvent-extracted powders of spinal roots, estimated by three different methods

Method of estimation	Specificity of method of estimation		μg ATP or ADP present in 1 mg powder Preparation $D_{15}P$	$D_{16}P$	$V_{16}P$
(1) Assay of vasodilator activity against ATP	*Includes:* AMP, adenosine. *Excludes:* other substances listed in Table 3, acetylcholine, histamine		4·5		
			2·2		
			4·5		
			1·8	6·0	5·1
			1·8		
			4·5	6·5	5·0
			1·8		
	Mean		3·01	6·25	5·05
(2) Intensity of absorption band at 260 mμ	*Includes:* AMP, other nucleotides, purines, pyrimidines and other substances with specific absorption at 260 mμ. *Excludes:* acetylcholine, histamine, substance P		3·3	4·6	4·1
(3) Enzymic analysis for ~P	*Includes:* creatine phosphate, phosphopyruvate, phosphoglycerate. *Excludes:* AMP, other nucleotides, purines, pyrimidines, acetylcholine, histamine, substance P	If all as ATP	1·35–1·45	3·5–4·2	2·5–3·0
		If all as ADP	2·1–2·5	5·9–7·1	4·2–5·1

Enzymic determination of labile phosphate (~P) in extracts

A more definite indication that adenosine phosphates were wholly responsible for the vasodilator activity of the extracts was obtained by comparing the results of the biological assays with direct estimation of the ~P in the extracts, using Slater's enzymic method. The results are given as two ranges in line 3 of Table 2. The upper figure of each range is that obtained from the uncorrected analysis ($B(a)$) for ~P, whereas the lower figure was obtained by subtracting the estimated maximum contribution of interfering substances (see Methods). Two ranges are given since 1 mole of ATP contains twice as much ~P as 1 mole of ADP. If our extracts contain a mixture of ATP and ADP, as suggested by paper chromatography, the true value (ATP + ADP) would lie between the extreme limits of the two ranges, i.e. 1·35–2·5, 3·5–7·1 and 2·5–5·1 μg/mg respectively, for each of the three extracts analysed. These intermediate values agree well with the estimates of ATP obtained by assay of vasodilator activity and by ultraviolet absorption. The possibility that other

phosphate compounds (creatine phosphate, phosphopyruvate and phosphoglycerate) contribute to the ~P analyses is discussed below and is shown to be unlikely.

Vasodilator activity of substances related to ATP

In order to determine the specificity of the vasodilator test, various substances were assayed against ATP or AMP in different preparations of the rabbit's ear. The results are given in Table 3. It can be seen that ADP, adenosine and, in some experiments, AMP had activity of the same order as ATP but that the other substances were much less active.

TABLE 3. Vasodilator activity of various substances compared with ATP

Substance	% activity of 1 mg (1 mg ATP = 100)
ADP	100, 100, 50, 100
AMP (muscle adenylic acid free from ATP and ADP)	100, 5000, 1000, 30, 1, 100, 5, 3
Adenosine	25, 100
Adenine	<10
ITP (free from ATP)	5, 3, 10
IMP	<1, <1, 0·1
Creatine phosphate	<1
Thiamine	0·1, <1
Diphosphothiamine	1, <1

The ratio of activity of ATP to AMP varied over a wide range in different experiments and sometimes changed even during the course of a few hours in the same preparation. When this happened it was usually because the vessels became relatively more sensitive to ATP; at the same time the sensitivity to spinal root extracts also increased. This suggested that little of the vasodilator activity of spinal root extracts was due to AMP, a result supported by the observation that only 10% of the activity resembled AMP in being barium-soluble (see Table 1).

Table 3 also includes some observations on thiamine, suggested as a possible chemical nerve transmitter by Muralt (1946). The absence of vasodilator activity confirms an earlier observation (Holton & Perry, 1951). Diphosphothiamine (cocarboxylase), which might be expected to occur in tissue extracts, was also without appreciable vasodilator activity. Two other nucleotides, yeast uridylic acid and yeast guanylic acid, were tested against AMP but were found to have less than 0·1% of its activity.

Comparison of vasodilatation produced by ATP with that produced by antidromic stimulation of the sensory nerve

In earlier papers it was shown that certain distinctive attributes of antidromic vasodilatation were also shown by the vasodilator responses to injected spinal root extract. In suitable preparations of the rabbit's ear, the vasodilator responses were prolonged (Holton & Holton, 1952) and they were also reduced

after injection of cholinesterase inhibitors (Holton, 1953). These two observations, together with the fact that the vasodilator activity disappears from incubated spinal root extracts by enzymic action (Holton & Holton, 1952), make the study of these extracts relevant to the investigation of the chemical transmitter responsible for antidromic vasodilatation. It was therefore of interest to see whether ATP and related substances also possessed these characteristics.

Duration of the response. These experiments were carried out with preparations of the rabbit's ear in which the larger vessels were constricted (see Methods). In such preparations prolonged vasodilatation was observed when ATP or ADP was injected. These responses were similar to the response to injection of extract of spinal roots or caudate nucleus and to antidromic vasodilatation, and they are probably due to capillary dilatation (Hilton & Holton, 1954). The results are illustrated in Fig. 2, which shows also that AMP

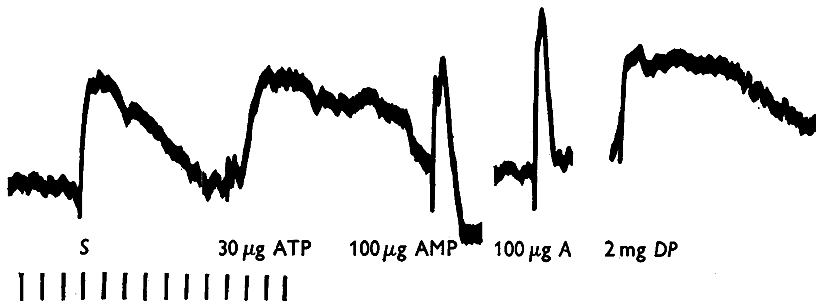

Fig. 2. Prolonged vasodilatation in the rabbit's ear produced by stimulation of the great auricular nerve at *S* by three shocks and arterial injection of ATP and spinal root extract (*DP*) in contrast to the short-lived responses to arterial injection of AMP and adenosine (A). Time in min.

and adenosine cause a short-lived vasodilatation suggesting that these substances act on the larger blood vessels. The contrast in the responses to ATP and ADP on the one hand, and AMP and adenosine on the other, is in accordance with observations reported in the literature. Anschütz & Schroeder (1950) found that ATP dilates capillaries, while Drury (1936) records that AMP and adenosine cause arteriolar dilatation.

Effect of cholinesterase inhibitors. The arterial injection of small doses of prostigmine or eserine decreased the vasodilator response to ATP and to ADP, but the response to adenosine was unaffected. This is illustrated in Fig. 3, which shows the effect of 50 μg prostigmine on the vasodilator responses to ATP, ADP, spinal root extract (*DP*) and adenosine. This antagonistic effect of cholinesterase inhibitors can thus be observed with spinal root extracts, ATP, ADP and the transmitter responsible for antidromic vasodilatation.

Incubation of ATP with acetone-dried spinal roots. The untreated acetone-dried powders of dorsal and ventral roots were used in these experiments since

it was found that the solvent extraction procedure shown in Table 1 destroyed the enzymic activity under observation. When ATP was incubated in saline at 37° C with either dorsal root powder or ventral root powder it lost more than 90% of its vasodilator activity in 30 min. At the same time the ~P fell by more than 80% and inorganic phosphate was liberated. The ultraviolet absorption at 260 mμ remained unchanged. These preliminary observations indicate that ATP and the vasodilator substance in spinal roots have similar biochemical, as well as pharmacological, properties.

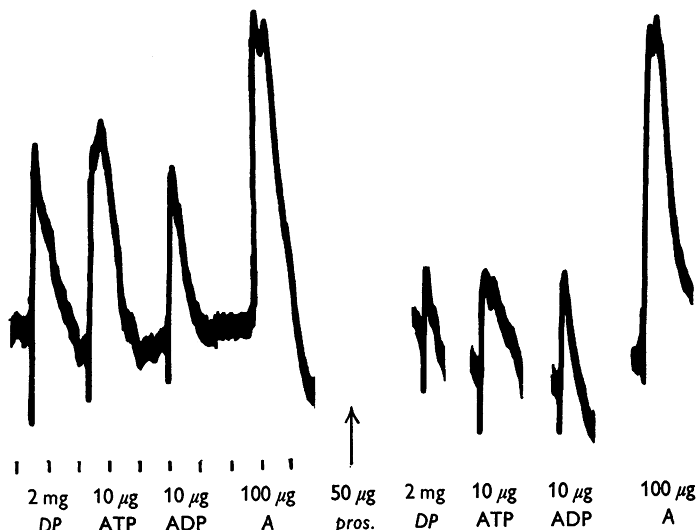

Fig. 3. Dilator responses of the rabbit's ear vessels to arterial injection of spinal root extract (*DP*), ATP, ADP and adenosine (A) 50 μg prostigmine (*pros.*) was injected arterially where indicated. Time in min.

Comparison of the spinal root dilator substance with substance P

Several authors have recently reported that substance P occurs in dorsal roots in greater concentration than in ventral roots (Pernow, 1953; Lembeck, 1953; Gaddum, personal communication). Since substance P causes vasodilatation, these findings have led Lembeck to suggest that it is responsible for the vasodilator activity found by Hellauer & Umrath (1948) in extracts of dorsal roots and that it is a transmitter at sensory nerve endings. Hellauer (1953) has also found that dorsal root extracts have many properties in common with substance P. It was thus of interest to compare the properties of the vasodilator substance of our extracts with substance P as well as with ATP.

The effect of hot alkali and hot acid on the spinal root extracts was studied in a series of experiments, and the results are summarized in Table 4. Acid or alkali were added to the extracts which were then held in a boiling water-bath for 20 min as described previously (Holton & Holton, 1952). After neutralizing,

the extracts were tested on the rabbit's ear. It is clear that the vasodilator activity is readily destroyed by heating with acid, but that the lability in hot alkali varied on different occasions; in general, the extracts lost some but not all of their vasodilator activity in alkali. In Expt. 5, spinal root extracts were compared directly with ATP and substance P. Both dorsal and ventral root extracts as well as ATP lost a little vasodilator activity in alkaline solution in contrast with substance P which had no detectable activity remaining. Thus

TABLE 4. The vasodilator activity of extracts of acetone-dried powders of dorsal and ventral roots, compared with ATP and substance P, after 20 min in acid or alkali in a boiling water-bath

Expt.	Preparation	Medium	% vasodilator activity remaining after: Acid	Alkali	Notes
1	$D_{13}P$	0·2N-HCl	<10	—	Holton & Holton (1952, fig. 5)
	$V_{10}P$	0·2N-HCl	<10	—	
	$D_{13}P$	0·2N-KOH	—	20	
	$V_{10}P$	0·2N-KOH	—	20	
2	$V_{13}P$	0·2N-HCl	<10	—	—
	$V_{13}P$	0·2N-NaOH	—	50	—
3	$V_{14}P$	0·2N-HCl	<10	—	—
	$V_{14}P$	0·2N-NaOH	—	70	—
4	$V_{14}P$	0·2N-KOH	—	32	—
	$V_{14}P$	0·33N-KOH	—	24	—
	$V_{14}P$	0·2N-NaOH	—	50	—
	$V_{14}P$	0·33N-NaOH	—	38	—
5	$D_{16}P$	0·3N-HCl	<5	—	Solvent-extracted spinal root powders
	$V_{16}P$	0·3N-HCl	<5	—	
	ATP	0·3N-HCl	<5	—	
	Substance P (*PF*)	0·3N-HCl	10	—	
	$D_{16}P$	0·3N-NaOH	—	70	
	$V_{16}P$	0·3N-NaOH	—	70	
	ATP	0·3N-NaOH	—	70	
	Substance P (*PF*)	0·3N-NaOH	—	<10	

the vasodilator substance in our extracts resembles ATP and differs from substance P in its relative stability to hot alkali. These results confirm our previous finding that the vasodilator activity of spinal root extracts is destroyed by hot acid, but our statement that it is also destroyed by hot alkali must be modified. We now find that the substance is only partially destroyed by heating in an alkaline medium.

A few other experiments have been carried out with substance P, and the results all suggest that it cannot be responsible for the vasodilator activity of our extracts. When substance P was assayed against a standard spinal root extract on two preparations of the rabbit's ear the equivalent doses were 1 μg $P_4 = 3$ μg $D_{15}P$ and 1 μg $P_4 = 40$ μg $D_{15}P$. These ratios are so different that the vasodilatation cannot be due to the same chemical substance in P_4 and $D_{15}P$. Also in a single experiment P_4 was incubated with an enzyme preparation from caudate nucleus. After 30 min P_4 had lost no dilator activity while the vasodilator activity of caudate nucleus extract had been almost

abolished. In addition to these observations, it was shown previously that substance P (*PF*) did not produce the prolonged vasodilator response typical of spinal root extract (Holton & Holton, 1952, fig. 3*a*).

DISCUSSION

The substance responsible for the vasodilator action of spinal root extracts, prepared according to our methods, shares many properties with ATP. As well as having similar chemical characteristics, the vasodilator activity of spinal root extracts and of ATP is antagonized by cholinesterase inhibitors and destroyed by incubation with spinal root powders, and the vasodilator responses are prolonged in suitable preparations. Apart from ADP, we have found no other substance which so closely resembles the active factor of spinal root extracts and the transmitter responsible for antidromic vasodilatation. There is thus strong evidence that the vasodilator activity of spinal root extracts is due, at least in part, to ATP or ADP. The significance of the quantitative comparisons between ATP and the vasodilator factor must be considered in the light of the specificity and accuracy of the three different methods.

The vasodilator assay against an ATP standard is not highly specific, but it was possible to exclude interference by some of the other dilator substances likely to be present in nervous tissue. Acetylcholine and histamine would have been antagonized by atropine and mepyramine respectively and the acetone-soluble factor of Major, Nanninga & Weber (1932) would have been removed in the preparation of the extracts.

The alkaline stability and enzymic lability of the active material in our extracts showed that substance P was not present in sufficient amount to contribute appreciably to their vasodilator activity. This was unexpected since substance P is known to occur in nervous tissue (Amin, Crawford & Gaddum, 1953; Kopera & Lazarini, 1953; Lembeck, 1953; Pernow, 1953). Our method of preparation included exhaustive extraction of the tissue with ether, acetone and alcohol, and it is possible that substance P would have been removed at this stage, as it is slightly soluble even in dry solvents (Pernow, 1953). In addition, the ether extraction may have increased the ease of extraction of substance P by the other solvents. However, we have not excluded the possibility that substance P contributes a small part (less than 20%) of the total vasodilator activity.

Although the above substances probably did not interfere with the vasodilator assay, breakdown products of ATP (ADP, AMP and adenosine) would certainly affect the assay, since they sometimes show as potent an action as ATP itself. Since the relative activities of these adenosine compounds vary from rabbit to rabbit, the assay of extracts likely to contain a mixture of them cannot be absolutely reproducible. In addition to this uncertainty, the figures

for each assay are subject to an error of up to ± 30 %. The scatter of values for the vasodilator activity of spinal root extracts, shown in Table 2, is easily accounted for by these two factors.

The estimation of ATP in nervous tissue by its absorption at 260 mμ has the same degree of specificity as the vasodilator assay. Although many other substances possess a similar absorption spectrum it is probable that only adenosine and its phosphates contribute to the 260 mμ absorption band, since the amount of other nucleotides in nervous tissue is negligible (Kerr, 1942). Uncertainty is also introduced into the estimation by the unspecific absorption of the extracts. Thus a possible error of about ± 15 % must be allowed for in the measurement of the height of the 260 mμ absorption band.

The enzymic method of determining ~ P gives a figure which sets an upper limit to the amount of ATP or ADP in the extracts, but which includes an unknown contribution from various interfering substances. A lower limit may be calculated by assuming that the whole of the analysis *B*(*b*) represented phosphopyruvate and/or phosphoglycerate. If we also assume that creatine phosphate contributed 25 % of the remaining ~ P, which is the proportion found in freshly frozen brain (Kratzing & Narayanaswami, 1953), the lower limit of ATP and ADP in spinal root powders is 44 % of the uncorrected value. However, it is unlikely that these substances did in fact contribute significantly to the ~ P estimation since creatine phosphate in nervous tissue disappears rapidly after death (McIlwain, 1952) and there is likely to be very little phosphopyruvate or phosphoglycerate present (Kratzing & Narayanaswami, 1953). The enzymic analysis, unlike the other methods used in this work, is reproducible to within less than 5 %. It thus seems justifiable to take the figures for total ~ P obtained by the enzymic analysis as giving a close estimate of the amount of ATP and ADP present in the spinal root extracts.

The quantitative measurements obtained by these three different methods show good agreement when the respective accuracies of the methods are taken into account. It is therefore reasonable to conclude that our extracts contained a mixture of ATP and ADP in sufficient concentration to account for the whole of their observed vasodilator action.

This conclusion contradicts our earlier view (Holton & Holton, 1952) that the vasodilator substance was not a derivative of adenosine. At the time, only adenosine itself had been tested on the rabbit's ear and it was excluded on the grounds that it dilated the larger blood vessels and that it was stable to alkaline hydrolysis. It was wrongly assumed that these properties would be shared by all derivatives of adenosine. However, it has now been shown that ATP, like the spinal root extract and unlike adenosine, is a capillary dilator and that it loses some of its vasodilator activity when heated with alkali.

The presence of breakdown products of ATP in extracts of acetone-dried powders of spinal roots does not necessarily indicate that they are present in

appreciable amounts in the living tissue. After the creatine phosphate has completely disappeared after death, the concentration of ATP falls rapidly, while the concentrations of ADP and AMP rise (Kerr, 1942). Even if most of the adenosine phosphate were present as ATP in the living nerve, sufficient hydrolysis would occur before our extracts were prepared to give the sort of mixture that we found. This is a probable explanation of our results since other workers have shown that nervous tissue contains much more ATP than other adenosine phosphates (Kerr, 1942; Kratzing & Narayanaswami, 1953). For brevity we shall therefore refer to the active physiological substance present in our extracts as ATP.

The occurrence of high concentrations of ATP in nervous tissue is well known and can readily be explained by its function in cell metabolism. Nevertheless, it is worthwhile to speculate on the possibility that ATP has an additional function in nervous tissue, namely that of transmitting nervous impulses from certain nerve endings. Such a situation would be analogous with that in skeletal muscle, where ATP is considered to play a specialized part in the contraction of the muscle fibre, a function distinct from its general participation in cell metabolism.

Our investigation of dilator activity in spinal roots was originally prompted by the claim of Hellauer & Umrath (1948) to have demonstrated the transmitter substance of sensory neurones in dorsal root extracts. In a recent paper, Hellauer (1953) now reports that the substance in his extracts has much in common with substance P and that it differs from ATP particularly in being readily destroyed by hot alkali. It is clear that Hellauer and ourselves have been studying different substances, and indeed it is not surprising that tissue extracts should contain several substances with vasodilator activity. Any of these substances may be considered as a possible chemical transmitter at sensory nerve endings. In the central nervous system it is likely that there is more than one such transmitter. For instance, the existence of two different sensory transmitters had been suggested recently by the definitive observations of Brock, Coombs & Eccles (1952) on potential changes at the first synaptic junction.

Thus the two possibilities that ATP and substance P are transmitter substances in the central nervous system are not mutually exclusive. However, it is reasonable to assume that the vasodilator effect of stimulating dorsal root fibres antidromically is caused by a single substance, probably a capillary dilator whose action is antagonized by cholinesterase inhibitors. The results obtained by Lembeck (1953) and Hellauer (1953) with extracts of spinal roots tested on the guinea-pig's intestine thus need to be examined by sensitive and quantitative vasodilator tests before their relevance to the problem of the antidromic transmitter can be assessed. The photometric method used in our experiments is particularly useful for investigating antidromic vasodilatation.

Besides being at least a 100 times more sensitive than the method of direct inspection used by Hellauer (1953) it is readily adapted to quantitative assay and responds chiefly to capillary dilatation.

The experiments described in this paper show that ATP and ADP resemble the transmitter responsible for antidromic vasodilatation. Preliminary experiments also indicated that a substance resembling ATP was liberated into the perfusion fluid when the great auricular nerve to the rabbit's ear was stimulated antidromically (Holton & Holton, 1953). It seems possible that such stimulation releases ATP or a similar substance from the nerve endings, and that the substance acts on the blood vessels to produce antidromic vasodilatation.

It is not certain whether the neurones responsible for antidromic vasodilatation are sensory neurones or whether they belong to a special efferent system (Lewis, 1942), but it is reasonable to suppose that the chemical substance liberated at their peripheral endings might also be produced in the central nervous system by an afferent sensory impulse (Dale, 1935). It thus becomes of interest to consider the general hypothesis that ATP acts as a physiological transmitter of impulses at certain nerve endings in the central nervous system.

There are several observations in the literature which indicate that ATP stimulates activity in the central nervous system. Arterial injection of small amounts into the cervical region of the cat's spinal cord produces movements of the forelimbs which are thought to be due to direct stimulation of the ventral horn cells (Buchthal, Engbaek, Sten-Knudsen & Thomasen, 1947). Emmelin & Feldberg (1948) also found that ATP injected into the vertebral artery stimulated the medulla. Stimulating and depressing effects of ATP in the central nervous system have also been observed by Babskiĭ & Malkiman (1950), Feldberg & Sherwood (1954) and Göpfert & P. Holton (unpublished). These findings all indicate that if extracellular ATP were released in small amounts in the central nervous system it would produce a marked effect.

There is no direct evidence that ATP is released at nerve endings. However, the appearance of material with a 260 mμ absorption band in the perfusate from a rabbit's ear during stimulation of the great auricular nerve may be explained in this way. In this connexion, it is worth noting the observations of Hydén (1947) who showed that the concentration of nucleotide in the relevant nerve cells is greatly decreased in guinea-pigs subjected to intense auditory stimulation or to prolonged muscular activity. ATP, which is abundant in nervous tissue, may be the nucleotide involved in this effect.

Cholinergic and adrenergic neurones can be shown to contain large amounts of acetylcholine and noradrenaline respectively (Loewi & Hellauer, 1938; Euler, 1951). By analogy, it would be expected that a sensory transmitter substance would be present in dorsal roots but not in ventral roots. In postulating that ATP may be such a transmitter, it is necessary to account for

the fact that it is a constituent of motor as well as sensory neurones. There is however this difference; the vasodilator activity persists in homogenates of fresh dorsal roots but disappears from fresh ventral roots (Holton & Holton, 1952). Thus it may be that neurones whose transmitter is ATP differ from cholinergic and adrenergic neurones in the rate at which they destroy ATP rather than in their ATP content. This seems a reasonable supposition when it is recalled that, in contrast to acetylcholine and noradrenaline, ATP fulfils a general metabolic function in all cells.

The concept of a special physiological action of free ATP has a direct analogy in present theories of the way in which skeletal muscle is made to contract. The arrival of the action potential at each point of the muscle fibre is thought to liberate bound ATP in a form which acts directly on the contractile mechanism. It is tentatively suggested that in certain sensory nerves, ATP is similarly liberated by the arrival of the action potential at the nerve ending, and that it then acts either on a blood vessel (as in antidromic vasodilatation), or, in the central nervous system, as a synaptic transmitter.

SUMMARY

1. Dry powders prepared from dorsal and ventral roots of horses have been analysed for ATP and its breakdown products by three different methods:

(*a*) Assay of vasodilator activity on the rabbit's ear vessels, using a series of animals.

(*b*) Measurement of specific light absorption at 260 mμ.

(*c*) Analysis of labile phosphate by enzymic methods.

2. The results show sufficiently close agreement to justify the conclusion that all the vasodilator activity of these preparations is due to a mixture of ATP and ADP with small amounts of further breakdown products.

3. The vasodilator activity of ATP is destroyed and the labile phosphate disappears on incubation with an acetone-dried powder of dorsal or ventral roots.

4. The vasodilator activity of ATP and of spinal root extracts is destroyed by acid and is slightly decreased by alkaline treatment. The activity of substance P is destroyed by both acid and alkaline treatments under the same conditions.

5. Arterial injection of ATP and ADP resembles antidromic nerve stimulation both in causing a prolonged vasodilatation in suitable preparations of the rabbit's ear and in being antagonized by previous injection of cholinesterase inhibitors. These properties are not shared by various other vasodilator substances.

6. The possibility that ATP is a transmitter liberated at sensory nerve-endings is discussed.

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