J. Physiol. (1967), **188**, *pp.* 159–176
With 11 *text-figures*
Printed in Great Britain

THE SIGNIFICANCE OF ADENOSINE CYCLIC 3′,5′-MONOPHOSPHATE FOR THE CONTRACTION OF SMOOTH MUSCLE

BY H. J. BARTELSTONE, P. A. NASMYTH AND J. M. TELFORD

From the Departments of Pharmacology of The College of Physicians and Surgeons, Columbia University, New York City, U.S.A. and of St Mary's Hospital Medical School, London, W. 2

(*Received* 2 *August* 1965)

SUMMARY

1. The influence of adenosine cyclic 3′,5′-monophosphate (3′,5′-AMP) and of drugs believed to increase or decrease its concentration in the tissues has been determined on the response of vascular and uterine smooth muscles to catecholamines. Generally, drugs believed to increase tissue content of 3′,5′-AMP potentiated the responses and those believed to decrease it depressed them.

2. The cardiovascular responses of dogs (with major vessels occluded in the chest) to carotid occlusion were potentiated by infusions of theophylline and sodium fluoride. Infusion of theophylline also potentiated the response of the occluded abdominal vessels to noradrenaline.

3. Intravenous infusions of theophylline and sodium fluoride potentiated pressor responses to catecholamines in the pithed rat. Infusions of iminazole depressed the responses in two animals and was without effect in two others.

4. In spinal cats intravenous infusions of theophylline potentiated pressor responses to catecholamines, but sodium fluoride was without effect.

5. Contractions of the isolated rat aortic strip to noradrenaline were always potentiated by sodium fluoride and by theophylline, and depressed by iminazole, when they were recorded isometrically. Theophylline always potentiated the contractions, when they were recorded isotonically but sodium fluoride was mostly, and iminazole always, ineffective.

6. 3′,5′-AMP in concentrations from 0·1 to 20 μg/ml. potentiated the responses of the isolated rat aortic strip to noradrenaline in thirty-eight experiments out of hundred. Concentrations from 10 to 500 μg/ml. sometimes depressed contractions recorded isometrically. In five experiments,

exposure to low concentrations for 3 hr increased the resting tension of the preparation.

7. Responses to noradrenaline of uteri from oestradiol-treated rabbits were potentiated by vasopressin and by sodium fluoride, but not by theophylline or iminazole. In progesterone-treated animals the responses were unaffected by vasopressin and sodium fluoride, but potentiated by theophylline and depressed by iminazole. 3′,5′-AMP was without effect on the uterine responses.

8. It is concluded that the results support the view that an increase in the tissue content of 3′,5′-AMP potentiates the contraction of vascular and uterine smooth muscle in response to catecholamine. This view is supported by the observation that the nucleotide itself can potentiate the responses of the rat aortic strip to noradrenaline.

INTRODUCTION

When arginine vasopressin is infused intravenously in rats, cats and dogs at rates approximately equal to the estimated normal secretory rates, pressor responses to catecholamines are potentiated (Nasmyth & Bartelstone, 1962; Bartelstone & Nasmyth 1965). The mechanism by which this potentiation is produced is not clear, but there have been a number of observations which suggest a possible explanation. Orloff, Handler & Preston (1961) showed that the influence of vasopressin on water movement through the toad bladder could be imitated by 3′,5′-AMP. Later, Brown, Clarke, Roux & Sherman (1963) demonstrated that vasopressin increases the production of 3′,5′-AMP in the adrenal and in the cortex of the dog's kidney. Rall & West (1963) suggested that the potentiation of the inotropic response of the isolated rabbit auricles to noradrenaline by theophylline might be due to the accumulation of 3′,5′-AMP in the tissue. Consequently, Bartelstone & Nasmyth (1965) have discussed the possibility that the potentiation of pressor responses to noradrenaline by vasopressin might be explained in the same way.

A number of substances affect the enzymes responsible for the production and destruction of 3′,5′-AMP. Thus, cyclase, which produces the nucleotide from ATP, is activated by sodium fluoride and inhibited by zinc and acetylcholine (Sutherland, Rall & Menon, 1962; Rall & Sutherland, 1962; Murad, Chi, Rall & Sutherland, 1962). Phosphodiesterase, which destroys 3′,5′-AMP, is activated by iminazole and inhibited by theophylline (Butcher & Sutherland, 1962). Thus, if vasopressin potentiates pressor responses to noradrenaline by causing 3′,5′-AMP to accumulate in the tissues, then the substances mentioned above which are known to affect the production and destruction of 3′,5′-AMP should have predictable effects on the response of smooth muscles to noradrenaline.

Preliminary reports of experiments of this kind have already been published (Bartelstone & Nasmyth, 1963; Bartelstone, Nasmyth & Telford, 1963). The present paper extends the work already reported and includes some similar observations on the smooth muscle of the uterus.

METHODS

Major vessel occlusion (M.V.O.) in dogs. The method of Bartelstone (1960) was used. Mongrel dogs weighing 9–16 kg were initially anaesthetized with an intravenous injection of 10 mg/kg of thiamylal. A tracheal cannula was inserted and anaesthesia maintained with nitrous oxide 80 % and oxygen 20 % delivered by a Bird respirator pump with CO_2 absorber in a closed system. The average respiratory rate was 10/min and the tidal volume was 300–400 ml. delivered at 12–19 cm water pressure.

The vagi were cut, the chest opened along the sternum and spread wide. The azygos vein, internal mammary vessels, and costocervical arteries were permanently tied. After these ties had been made, the circulatory system could be divided into two parts: a normally perfused cephalad zone and an isolated caudal zone by the simultaneous occlusion of the descending aorta and the inferior vena cava, just distal to the left subclavian artery (M.V.O.). During M.V.O., experimentally induced changes in the cephalad circulation (e.g. carotid occlusion) could affect the vessels in the caudal zone only through neuronal pathways. M.V.O. was maintained for 50–75 sec with an interval of 10–15 min between occlusions.

Polythene tubes were inserted into the central ends of the left and right femoral arteries and pushed in until their ends rested well inside the abdominal aorta. One of these cannulae was closed with a three way metal tap with a 50 ml. syringe attached to one branch and a 1 ml. syringe attached to the other. The other cannula was attached to a Statham strain gauge (P 23 A-arterial) which recorded pressure changes in the caudal arterial system. The central end of one femoral vein was similarly cannulated and the cannula was connected to a Statham strain gauge (P 23 B-venous) to measure pressure changes in the caudal venous system. Arterial pressure changes cephalad of the clamps in M.V.O. were measured with a Statham strain gauge (P 23 A-arterial) connected to a polythene cannula in the central end of the previously tied right internal mammary artery. Drug infusions were given through a cannula in the jugular vein.

To study the action of noradrenaline in the caudal vascular bed during M.V.O., the dose of noradrenaline, contained in a volume of saline less than the volume of the polythene cannula, was placed in the 1 ml. syringe. Depending on the size of the dog, 15–20 ml. of blood was withdrawn into the 50 ml. syringe. The clamps were then applied to the major vessels in the chest (M.V.O.) and a further 10–20 ml. of blood was withdrawn into the same syringe. The three-way tap was opened to the 1 ml. syringe and the dose of noradrenaline injected into the polythene cannula. The noradrenaline was then pushed into the occluded caudal vascular bed by the blood previously withdrawn. This volume of blood was sufficient to push the noradrenaline into the abdominal aorta, the abdominal arterial vessels, and across to the venous side. The reactions of the occluded arteries and veins were recorded by the appropriate strain gauges for the remaining period of the occlusion.

Pithed rats. Sherman or Wistar rats (weights 300–427 g) were anaesthetized with ether 15 min after an intraperitoneal injection of 10 mg/kg of atropine sulphate and pithed by the method of Shipley & Tilden (1947). Blood pressure was recorded from the carotid artery either via a Statham P 23 A transducer and a Grass polygraph or via a glass cannula connected to a Condon Rat B.P. Manometer (Palmer) arranged to write on a kymograph. Drugs were infused through one femoral vein while catecholamines were injected into the other.

Spinal cats. These were prepared under ether; they weighed from 1·9 to 3·3 kg. Blood pressure was recorded from a carotid artery with a mercury manometer, and drugs were infused through one femoral vein, while catecholamines were injected into the other.

Drug infusions. These were made either with a Harvard infusion pump (600–900) or a Palmer Continuous Slow Injector (standard model). The volume of infusions was not allowed to exceed 0·1 ml./min in rats or 0·4 ml./min in cats and dogs.

Rat aortic strips. Sherman or Wistar rats (weights 200–400 g) were killed by a blow on the head. Spiral strips were cut from that part of the aorta lying between the heart and the renal vessels. The strips were mounted in a 10 ml. bath of Krebs solution at 37° C gassed with 95 % O_2 and 5 % CO_2. Contractions were recorded either istonically with a frontal writing lever on a kymograph, or isometrically on a Grass polygraph using a Statham force-displacement transducer (FT-O3 springs removed). An initial tension of 1 or 2 g was applied to the strips in both methods. One hour was allowed before beginning the experiment; relaxation occurred during this period, and when isometric records were made it was necessary to readjust the tension periodically during this time. Doses of noradrenaline were added to the bath at 15 min intervals and allowed to remain for 100 sec.

Isolated rabbit uteri. Adult rabbits were killed by a blow on the head and exsanguinated. The uteri were removed and a section about 2·0 cm in length was cut from each horn and mounted in an isolated organ bath of 35 ml. capacity containing Krebs solution at 37° C. The tissue was connected to an isometric lever (Palmer's student's isometric lever) 1·0 cm from the fulcrum and 115 cm from the writing point. The tissue did not shorten by more than 5 % of its length, which is similar to the conditions recommended by Csapo (1954). Doses of noradrenaline were added to the bath at 5 min intervals and allowed to remain until the contraction was complete. The time varied from 20 to 90 sec for different tissues, but once established for any one tissue it remained constant throughout the experiment.

Oestradiol treatment. Intact adult rabbits treated with oestradiol before isolation of the uterus were given total doses varying from 150 to 300 i.u. intramuscularly in arachis oil over periods ranging from 3 days for the smaller dose to 7 days for the larger dose. All the animals were used within 18–24 hr of the last dose.

Progesterone treatment. Intact adult rabbits treated with progesterone before isolation of the uterus were primed with a daily intramuscular dose of 30 i.u. of oestradiol in arachis oil for 5 days. Forty-eight hours after the last dose of oestradiol they were given total intramuscular doses of progesterone in arachis oil ranging from 0·8 to 50 mg over 3 days. All the animals were used between 18 and 24 hr after the last dose of progesterone.

Krebs bicarbonate solution. This solution had the following composition: NaCl 0·69 %, KCl 0·035 %, $CaCl_2.6H_2O$ 0·06 %, $MgSO_4.7H_2O$ 0·029 %, $NaH_2PO_4.2H_2O$, 0·016 %, $NaHCO_3$, 0·21 %, Glucose 0·2 %.

Drugs. Adrenaline and noradrenaline bitartrates and acetylcholine chloride were used. They were dissolved in normal saline, when given by intravenous injection, and in Krebs solution, when injected into isolated organ baths. Iminazole, 3′,5′-AMP and theophylline were employed as bases, but theophylline glycinate was also used in some experiments. Vasopressin was a mixture of arginine and lysine vasopressin ('Pitressin', Parke Davis and Co.). Sodium fluoride was of 'Analar' quality. All doses in the text are expressed as base unless otherwise stated. Solutions for infusion into whole animals were dissolved in normal saline. When the effects of drugs other than catecholamines were tested on isolated organs, they were dissolved in Krebs solution and usually incorporated in a reservoir of the bathing fluid, so that their influence persisted even during the periods when the bath was washed out. Occasionally they were injected into the bath immediately after washing, but this usually produced less reliable results, and was most commonly used in order to conserve the expensive 3′,5′-AMP.

RESULTS

Pressor responses to noradrenaline and to carotid occlusion in dogs during M.V.O.

Influence of theophylline. When the major vessels in the chest were clamped (M.V.O.), pressure changes occurred in the cephalad circulation and in the occluded arteries and veins of the caudal vascular bed. The pressures stabilized 25–30 sec after M.V.O. and both carotid arteries were occluded for 30–35 sec. The clamps on the carotid arteries and on the major thoracic vessels were then removed simultaneously. The occlusion of the carotid arteries produced reflex rises in pressure in the cephalad arteries and in the occluded arteries and veins of the caudal vascular bed, which were remarkably constant when the procedure was repeated at 10–15 min intervals during the control period. Constant pressor effects in the occluded vessels of the caudal vascular bed were induced by the injection of 2 μg of noradrenaline in the abdominal aorta during M.V.O.

An intravenous infusion of 10 $\mu g\ kg^{-1}\ min^{-1}$ of theophylline glycinate potentiated the responses to carotid occlusion in two of five dogs. An infusion of 20 $\mu g\ kg^{-1}\ min^{-1}$ potentiated both the responses to carotid occlusion and those to the injection of noradrenaline in all five dogs. The potentiation was maximal 30 min after starting the infusion. An infusion of 40 $\mu g\ kg^{-1}\ min^{-1}$ produced the greatest potentiation of the response to carotid occlusion and increased the response to noradrenaline by 100%. The responses to carotid occlusion returned to control levels 25–30 min after stopping the infusion, but those to noradrenaline sometimes required 60 min.

Influence of sodium fluoride. The responses of the blood vessels to carotid occlusion caudal and cephalad of M.V.O. were potentiated by infusions of 2–10 $\mu g\ kg^{-1}\ min^{-1}$ of sodium fluoride. The effect was most marked with an infusion of 4 $\mu g\ kg^{-1}\ min^{-1}$ (Fig. 1). Potentiation was evident within 8 min and was maximal after 80–100 min. Responses returned to control values within 10 min, if the infusion was stopped within a few minutes of first observing an effect. When the infusion was continued for 80–100 min, the potentiation persisted for periods up to 3 hr after stopping the perfusion, beyond which time observations were not continued. When 200 $\mu g\ kg^{-1}\ min^{-1}$ was infused, the responses were potentiated within 2 min, but depressed if the infusion was continued.

Pressor responses to catecholamines in pithed rats and spinal cats

Pithed rats. The effects of intravenous infusion of various drugs are summarized in Table 1. Potentiation of pressor responses to noradrenaline by an infusion of theophylline is illustrated in Fig 2.

The degree of potentiation produced by sodium fluoride varied from

animal to animal and bore no relation to the dose of fluoride. In one rat an infusion of 40 μg kg^{-1} min^{-1} of theophylline further increased the response already apparently maximally potentiated by sodium fluoride. When the potentiation caused by the theophylline reached a steady state, 300 μ-u. kg^{-1} min^{-1} of vasopressin still further increased the response.

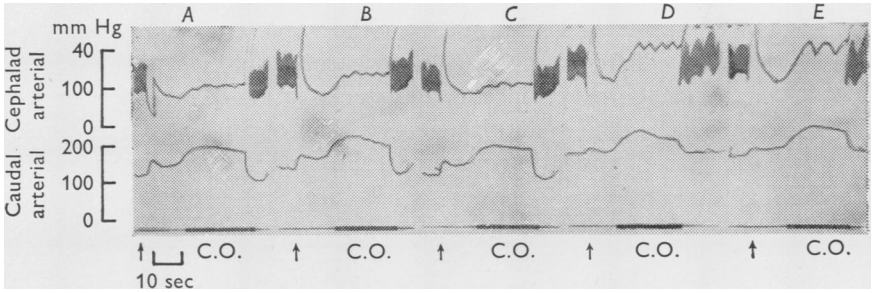

Fig. 1. Dog (male, 10 kg). Anaesthesia: N_2O 80 %, O_2 20 %. Artificial respiration. Upper record: caudal arterial pressure. Lower record: cephalad arterial pressure. At each arrow the thoracic aorta and the inferior vena cava were clamped simultaneously (M.V.O.) and the caudal pressure scale was changed from 0–200 mm Hg to 0–40 mm Hg. At the beginning of each bar both carotid arteries were clamped. The clamps on the carotid arteries and the major thoracic vessels were removed simultaneously at the end of the bar, and the caudal arterial pressure scale was changed to 0–200 mm Hg. *A*: control response to carotid occlusion (C.O.). *B*: 8 min after beginning an intravenous infusion of 2 μg kg^{-1} min^{1} of sodium fluoride. Note potentiation of responses. *C*: 10 min later and 8 min after stopping the infusion. Responses have returned to control values. *D*: 124 min after the continuous infusion of 4 μg kg^{-1} min^{-1} of sodium fluoride. Note the much greater potentiation of the responses. *E*: 64 min after stopping the infusion. The potentiation of the responses to carotid occlusion does not decay after long periods of infusion of sodium fluoride.

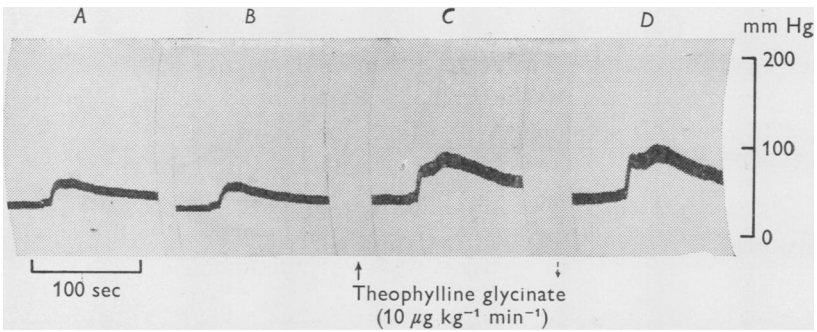

Fig. 2. Pithed atropinized rat (female, 400 g); blood pressure. *A* and *B*: responses to intravenous injections of 40 ng of noradrenaline during the control period. *C*: response to the same dose of noradrenaline 50 min after start of a continuous infusion of 10 μg kg^{-1} min^{-1} of theophylline glycinate. *D*: response to the same dose of noradrenaline 45 min after stopping the infusion.

TABLE 1. The effect of infusing various drugs (I.V.) on pressor responses to adrenaline and noradrenaline in pithed rats and spinal cats

	Drug	No. of animals	Dose (μg kg^{-1} min^{-1})	Time to appearance of effect (min)	Effect on response	Time to max. effect (min)	Time for recovery	Comments
Rats	Theophylline	7	5–40	6	Increase in 7	30–62	No recovery within 90 min	Usually slight further increase when infusion stopped
	NaF	4	2–10	14	Increase in 4	14–56	Within 32 min in 3 animals	No recovery within 99 min in one animal
	Iminazole	4	632–1280	15–30	Decrease in 3	30–62	More than 80 min	No decrease seen in 1 animal. One animal was anaesthetized (see text)
Cats	Theophylline	4	0·04–0·64	15–30	Increase in 4	40–75	No recovery within 90 min	Usually further increase when infusion stopped
	NaF	4	1–640	—	—	—	—	No effect

TABLE 2. The effect of drugs on contraction of the isolated rat aortic strip in response to noradrenaline

Drug	Concentration (g/ml.)	Type of record	No. of expts.	Effect on response	Time to max. effect (min)	Time for recovery (min)	Comments
NaF	1×10^{-15} to 1×10^{-9}	Isotonic	13	Increase in 3 No effect in 9 Depression in 1	15	30	Lowest doses potentiated Highest dose depressed (see text)
	1×10^{-15} to 1×10^{-8}	Isometric	5	Increase in 5	15	30	—
Theophylline	5×10^{-9} to 2×10^{-7}	Isotonic	6	Increase in 6	15	15	—
		Isometric	25	Increase in 25	15	15	—
Acetylcholine	5×10^{-10} to 1×10^{-8}	Isotonic	6	Decrease in 4 No effect in 2	15	Up to 90	—
Zinc sulphate	1×10^{-5} to 5×10^{-5}	Isotonic	3	Decrease in 3	Up to 80	Irreversible	Effective only at highest dose level
Iminazole	5×10^{-6} to 2×10^{-5}	Isotonic	4	None	—	—	—
		Isometric	8	Depressed in 8	10	Up to 60	—
3',5'-AMP	1×10^{-7} to 2×10^{-5}	Isotonic	23	Increase in 5 No effect in 18	15	15	—
	1×10^{-7} to 2×10^{-6}	Isometric	33	Increase in 33	15	15	For effects of low doses over period of time see text
	1×10^{-5} to 4×10^{-5}	Isometric	38	None	—	—	Occasionally very slight depression
	1×10^{-4} to 5×10^{-4}	Isometric	6	Depression in 6	1–8	15	—
5'-AMP	1×10^{-7} to 2×10^{-6}	Isometric	7	None	—	—	—

One of the rats treated with iminazole was not pithed but anaesthetized with urethane. In this animal, 100 μg of iminazole injected together with the adrenaline reduced the pressor response by 16%. In one of the two animals in which an infusion of iminazole depressed the responses to adrenaline there was some recovery 80 min after stopping the infusion, in the other there was no recovery within this period of time.

Spinal cats. As in the pithed rat, the increase in pressor responses caused by an infusion of theophylline did not disappear when the infusion was stopped, but reached a second maximum after 15–90 min (Fig. 3).

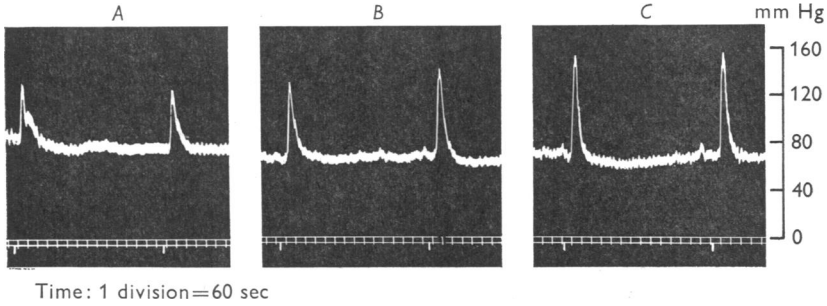

Fig. 3. Spinal cat (male, 3 kg): blood pressure. *A*: pressor responses to the intravenous injection of 2 μg of noradrenaline during the control period. *B*: pressor responses to the same dose of noradrenaline 59 and 74 min after the start of an infusion of 0·5 μg kg^{-1} min^{-1} of theophylline. *C*: further potentiation of the pressor responses to noradrenaline 30 and 45 min after stopping the infusion.

Contraction of the rat aortic strip in response to noradrenaline

Table 2 summarizes the effects of various drugs on the response of the rat aortic strip to noradrenaline.

At a concentration of 1×10^{-15} g/ml., sodium fluoride potentiated isotonic responses in two preparations; in one experiment 1×10^{-12} g/ml. produced potentiation followed by depression (Fig. 4). In nine experiments higher concentrations were without effect, and in one experiment 1×10^{-9} g/ml. caused depression.

The influence of theophylline is shown in Fig. 5, and the effects of acetylcholine and zinc sulphate in Fig. 6. Concentrations of iminazole ranging from 5×10^{-6} to 2×10^{-5} g/ml. were without effect on the response to noradrenaline when contractions were recorded isotonically, but were clearly effective when they were recorded isometrically (Fig. 7).

In five experiments, in which isometric recording was used, prolonged exposure of the tissue to concentrations of 3′,5′-AMP ranging from 1×10^{-8} to 1×10^{-7} g/ml. not only potentiated the contractions to noradrenaline, but also increased the resting tension (Fig. 8). Repeated washing with Krebs solution for 3 hr did not reverse the effect. Control strips from

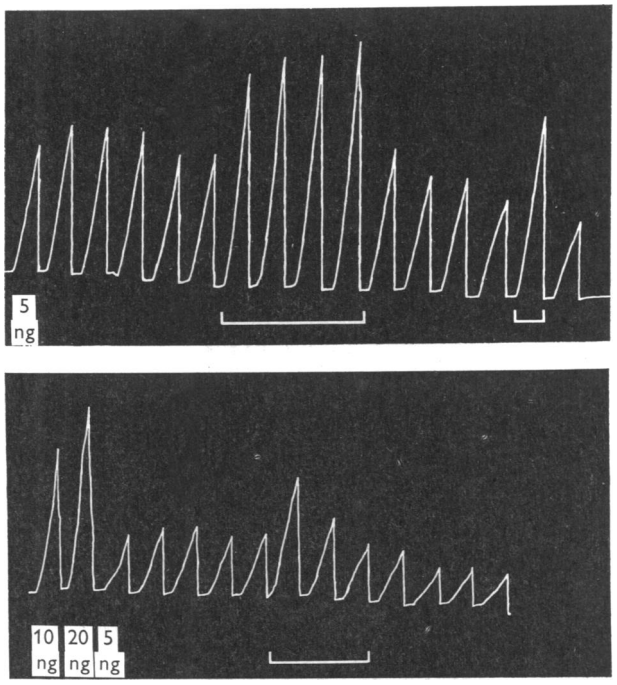

Fig. 4. Isolated aortic strips of the rat: isotonic contractions to noradrenaline. Upper trace shows the effect of 1×10^{-15} g/ml. of sodium fluoride (presence in the bathing fluid indicated by the bars) on the responses to 5 ng doses of noradrenaline given at 15 min intervals. Lower trace shows the effect of 1×10^{-12} g/ml. of sodium fluoride (presence as indicated by the bar) on the responses of a second preparation to 5 ng doses of noradrenaline, also given at 15 min intervals.

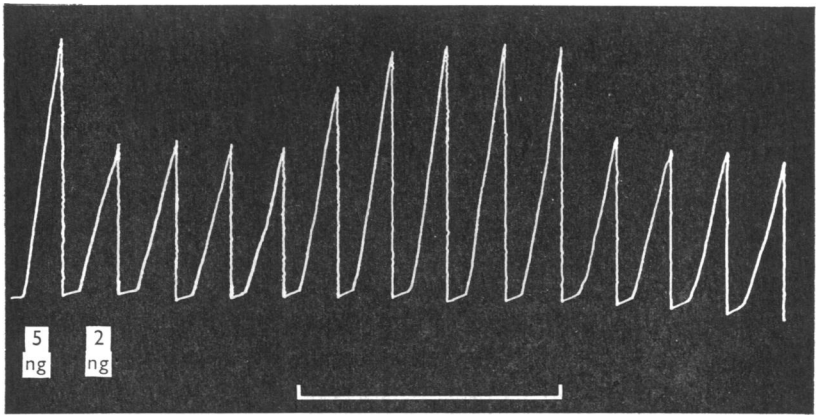

Fig. 5. Isolated aortic strip of the rat: effect of theophylline on the isotonic contractions elicited to 2 ng doses of noradrenaline given at 15 min intervals. Theophylline was present in the bathing fluid in a concentration of 10 ng/ml. for a period of 75 min as indicated by the bar. The response to 5 ng of noradrenaline during the control period is shown for comparison.

each aorta which were given doses of noradrenaline, but not exposed to 3′,5′-AMP showed neither a potentiation to noradrenaline nor an increase in the resting tension.

Figure 9 shows the potentiation to noradrenaline which occurred in the presence of from 1×10^{-7} to 2×10^{-5} g/ml. 3′,5′-AMP in five out of twenty-three experiments in which concentrations were recorded isotonically.

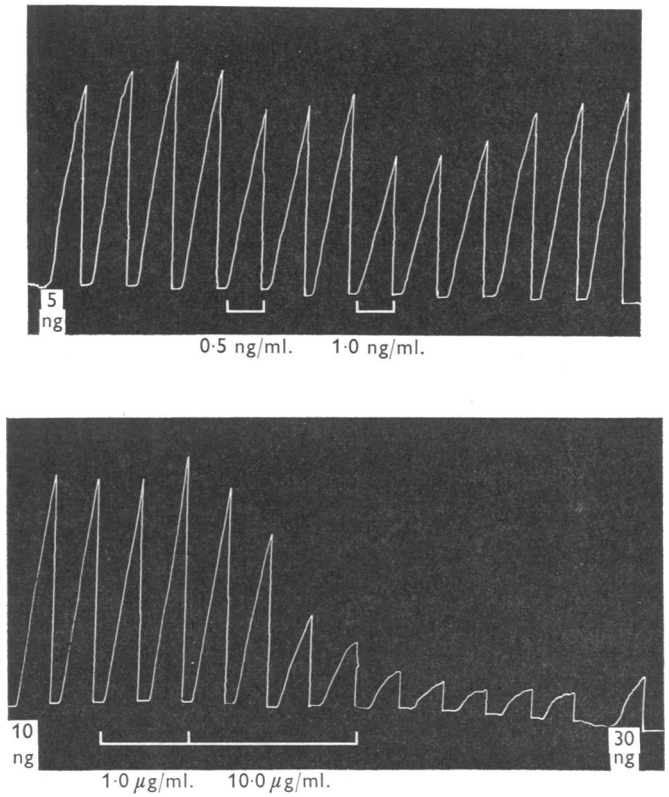

Fig. 6. Isolated aortic strips of the rat: isotonic contractions to noradrenaline. *Upper trace* shows the effect of acetylcholine on the contractions elicited to 5 ng doses of noradrenaline given at 15 min intervals. Acetylcholine was present in the bathing fluid in concentrations of 0·5 and 1·0 ng/ml. respectively for the periods indicated by the bars. *Lower trace* shows the effect of zinc sulphate on the contractions elicited to 10 ng doses of noradrenaline given at 15 min intervals. Zinc sulphate was present in the bathing fluid in concentrations of 1 and 10 μg/ml. respectively, as indicated by the bars.

Contractions of the isolated rabbit uterus

The influence of various drugs on isometric contractions of the rabbit uterus in response to noradrenaline are summarized in Table 3.

Drugs known to modify the metabolism of 3′,5′-AMP had inconsistent

effects on the response to noradrenaline of uteri from untreated rabbits. To determine whether or not these inconsistencies were due to domination of the uterus either by oestradiol or by progesterone, intact animals were pre-treated with these hormones to ensure dominance by either one or the other.

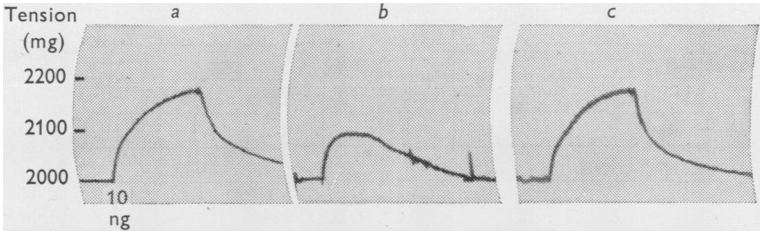

Fig. 7. Isolated aortic strip of the rat: isometric responses to 10 ng doses of noradrenaline given at 15 min intervals: (*a*) the response to noradrenaline during the initial control period, (*b*) the response to the same dose of noradrenaline 10 min after the addition of 10 μg/ml. of iminazole to the bathing fluid, and (*c*) the response to noradrenaline 60 min after removal of the iminazole.

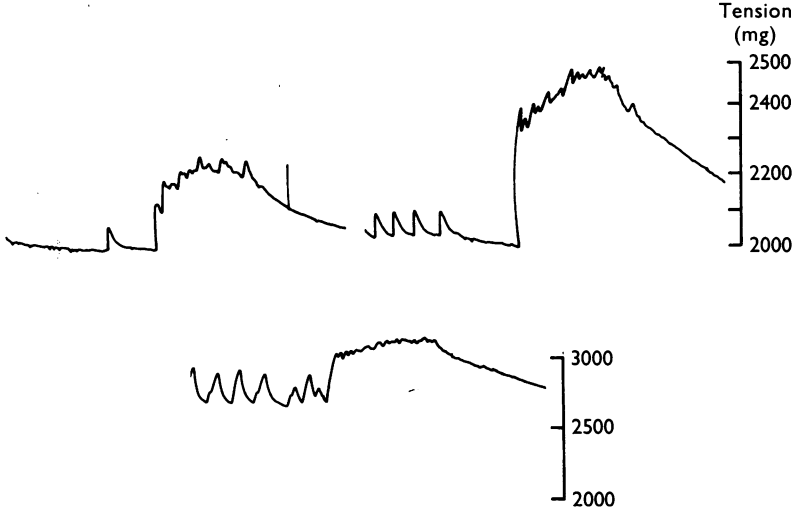

Fig. 8. Isolated aortic strip of the rat. Isometric contractions, 10 ml. bath. Upper traces: both contractions were in response to 2 ng of noradrenaline, the first during the control period and the second 165 min after progressively increasing doses of 3′,5′-AMP added to the bath at 15 min intervals following the wash after every noradrenaline response. The concentrations of 3′,5′-AMP increased progressively from 10 to 100 ng/ml. Lower trace was obtained in response to 2 ng of noradrenaline 80 min later during which time the concentration of 3′,5′-AMP in the bath was maintained at 100 ng/ml. Note that both the resting tension and the maximal tension developed in response to noradrenaline are increased, but that the change in tension in response to noradrenaline is reduced.

The lower doses of oestradiol and progesterone used in these experiments have been shown by McPhail (1934) to be adequate to produce the histological changes in the uterus usually associated with them and they were sufficient to reduce the inconsistencies in these experiments. High doses of oestradiol increased the sensitivity of the uterus to noradrenaline so much, that the responses were often 'all or none'. By contrast, after high doses of progesterone suitable contractions to noradrenaline could not be obtained.

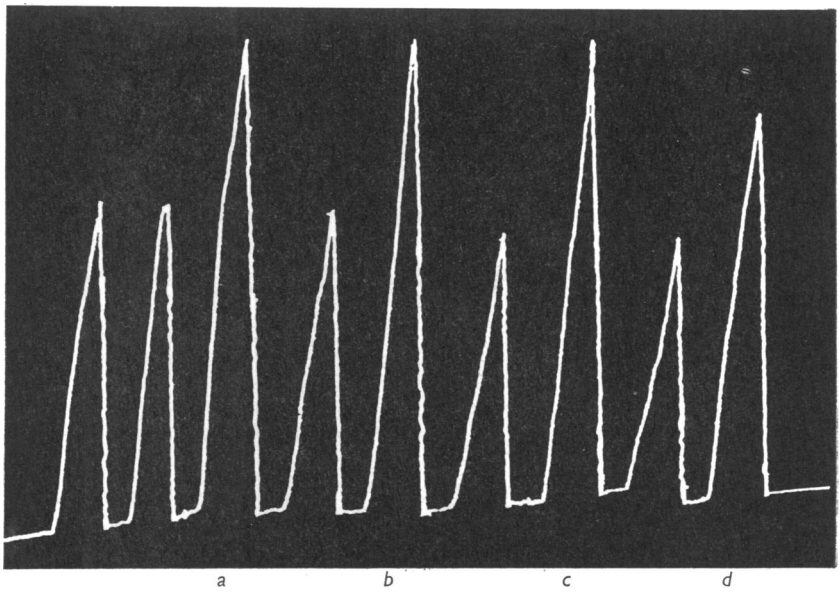

Fig. 9. Isolated aortic strip of the rat. Isotonic contractions, 5 ml. bath. All contractions are in response to 2 ng doses of noradrenaline given at 15 min intervals. Potentiated responses *a* and *b* were obtained 10 min after adding 10 μg of 3′,5′-AMP to the bath, *c* was obtained 10 min after adding 20 μg, and *d* 10 min after adding 1 μg of 3,5-AMP. The contractions between the potentiated responses were obtained in the absence of 3,5-AMP.

The effects of vasopressin are shown in Fig. 10. Three uteri isolated from rabbits given high doses of oestradiol (not included in Table 3) gave 'all or none' responses to noradrenaline. Vasopressin did not affect the magnitude of these responses, but it halved the delay of 49 sec between adding noradrenaline to the bath and the response to it. The responses of two uteri from progesterone-treated animals were unaffected by vasopressin initially, but were potentiated by it after exposure to theophylline.

Figure 11 shows the effect of sodium fluoride on the responses of the uterus to noradrenaline.

In one experiment on a uterus from an untreated rabbit (not included

TABLE 3. The effect of drugs on isometric contractions of the isolated rabbit uterus in response to noradrenaline

Drug	Concentration μ-u./ml.	Pre-treatment of rabbit	No. of expts.	Effect on response	Time to max. effect (min.)	Time for recovery (min)	Comments
Vasopressin	100 to 1 m-u./ml.	None	4	Increase in 4	10–15	5	—
		Oestradiol	3	Increase in 3	10–15	5	For effects of high doses of oestradiol see text
	10 to 500 μ-u./ml.	Progesterone	5	None	—	—	See text
NaF	1×10^{-15} to 1×15^{-12} g/ml.	None	2	Increase in 1 No effect in 1	5	10	—
		Oestradiol	3	Increase in 3	5	10–20	—
		Progesterone	3	None	—	—	Contact with NaF for up to 1 hr without effect
Theophylline	1×10^{-12} to 1×10^{-6} g/ml.	None	6	None No effect in 2	—	—	See text
	5×10^{-10} to 5×10^{-7} g/ml.	Oestradiol	5	No effect on magnitude in 4 Small increase in 1	—	—	See text
		Progesterone	5	Increase in 4 No effect in 1	10	5	See text
Iminazole	5×10^{-7} g/ml.	None	5	Decrease in 2 No effect in 3	5–40	10	See text
		Oestradiol	4	None	—	—	—
		Progesterone	3	Decrease in 3	40	10	See text
Zinc sulphate	2×10^{-5} to 5×10^{-4} g/ml.	None	2	Decrease in 2	20–30	5–10	Doses in excess of 1×10^{-4} g/ml. caused precipitation in the Krebs solution
		Oestradiol	2	Decrease in 2	20–30	5–10	—
		Progesterone	2	Decrease in 2	20–30	5–10	—
Acetylcholine	1×10^{-7} to 5×10^{-4} g/ml.	None	2	None	—	—	—
3',5'-AMP		Oestradiol	2	None	—	—	—
		Progesterone	2	None	—	—	—

in Table 3), theophylline potentiated the responses to noradrenaline after it had been exposed to 10 μ-u./ml. of vasopressin for 15 min. In one of the five uteri from rabbits treated with oestradiol, the responses to noradrenaline were 'all or none'. Theophylline did not affect the magnitude of the response in this preparation, but it did reduce the delay between the addition of noradrenaline to the bath and the response from 39 to 7 sec.

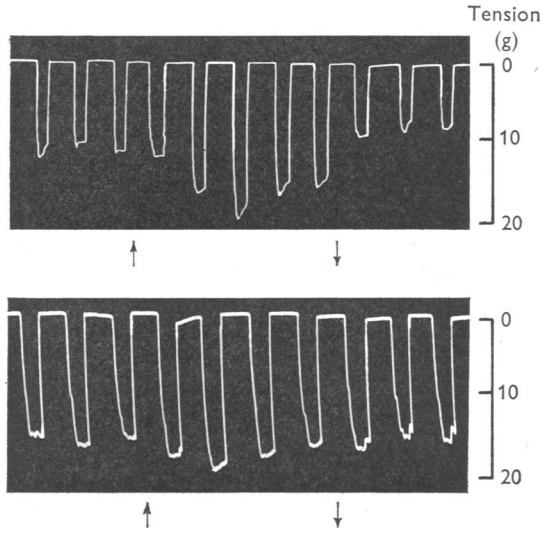

Fig. 10. Isolated uterus of the rabbit: isometric responses to noradrenaline. Upper trace shows the effect of 500 μ-u./ml. of vasopressin (present in the bathing fluid for the period between the arrows) on the responses of the uterus of a rabbit treated with oestradiol to 2 μg doses of noradrenaline given at 5 min intervals. Lower trace shows the effect of the same concentration of vasopressin (present between the arrows) on the responses of the uterus of a rabbit treated with progesterone to 5 μg doses of noradrenaline given at 5 min intervals.

In two of the experiments on uteri from progesterone-treated animals in which theophylline potentiated the response to noradrenaline, exposure of the tissue to 10 μ-u./ml. of vasopressin for 15–20 min markedly enhanced the potentiation produced by theophylline when the latter drug was introduced a second time 20–40 min after the vasopressin was washed out.

Iminazole completely blocked the response to noradrenaline in one of the two preparations from untreated animals in which it caused inhibition. The inhibition of noradrenaline responses produced by iminazole in one of the preparations from progesterone-treated animals was not reversed 45 min after removing the iminazole from the bath, but at this time sodium fluoride (1×10^{-12} g/ml.) doubled the response to noradrenaline within 10 min.

Acetylcholine contracts the uterus when applied in sufficient doses. Concentrations up to 1×10^{-5} g/ml. did not contract the uterus, but increased the contractions to noradrenaline, while they were present in the bathing fluid. When the acetylcholine was removed from the bath, the contractions to noradrenaline were reduced by as much as 80% in 5 min and returned to control values after 30 min. This dual effect was seen in each of five uteri whether they were from rabbits treated with hormone or not. Concentrations of acetylcholine below 1×10^{-7} g/ml. produced only the potentiation.

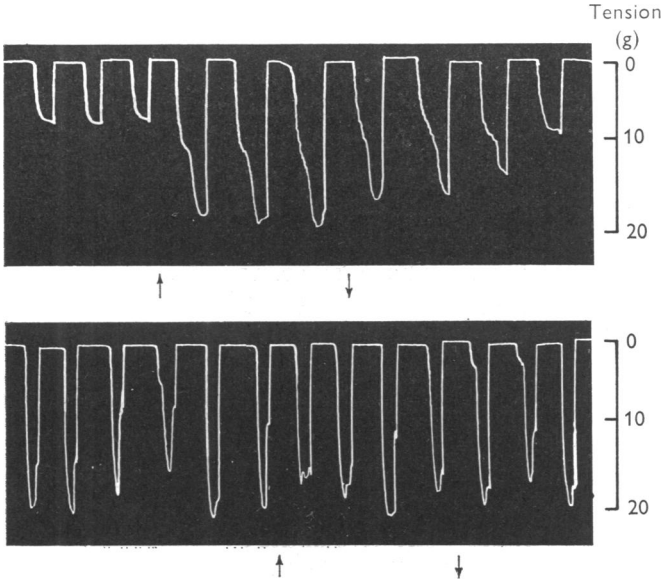

Fig. 11. Isolated uterus of the rabbit: isometric responses to noradrenaline. Upper trace shows the effect of 1 pg/ml. of sodium fluoride (present in the bathing fluid for the period between the arrows) on the responses of the uterus of a rabbit treated with oestradiol, to 2 μg doses of noradrenaline given at 5 min intervals. Lower trace shows the effect of the same concentration of sodium fluoride (present as shown by the arrows) on the responses of the uterus of a rabbit treated with progesterone to 5 μg doses of noradrenaline given at 5 min intervals.

DISCUSSION

To establish a hypothesis by means of circumstantial evidence, it is desirable that there should be a variety of approaches all indicating the same conclusion, and that there should be no equivocation in the results. At first sight, the second of these conditions does not seem to be fulfilled, there being considerable equivocation in the results obtained in the experiments on the rat aortic strip and on the rabbit uterus. However, closer examination of the results on the aortic strip shows that, except for

the results with 3′,5′-AMP, they were unequivocal when the contractions were measured isometrically by a system having little or no inertia. Equivocal results in experiments on the rabbit uterus were eliminated when the observations were made on uteri from animals treated with oestradiol or progesterone.

3′,5′-AMP was without effect on the bulky tissue of the rabbit uterus and produced the expected effect in only thirty-eight out of hundred experiments on the more delicate aortic strip. The incidence of negative effects with 3′,5′-AMP was reduced when the contractions were recorded isometrically, but by contrast with the experiments using other compounds, equivocation remained. The reason for this remaining variation is most likely to be related to difficulty in penetrating the cell membrane. Indeed it has been supposed that 3′,5′-AMP does not penetrate the membrane at all, but Butcher, Ho, Meng & Sutherland (1965) have shown it to be capable of stimulating lipolysis in the epididymal fat pads of the rat. A dibutyryl derivative of the nucleotide was shown to be equally effective at lower concentrations and this was thought to be due to its being more resistant to hydrolysis by phosphodiesterase and perhaps to increased entry into cells. These results, taken together with the experiments described here, suggest that 3′,5′-AMP can penetrate the cell membrane, albeit with difficulty. Slow penetration coupled with varying activity of phosphodiesterase within the cell would explain the negative results in the experiments on the aortic strip.

In experiments on whole animals, only iminazole produced equivocal results, though sodium fluoride was without effect in the spinal cat. Since these agents were uniformly effective under the right conditions on isolated tissues, it seems likely that failures in the whole animal were caused by variation in their metabolism. Some increase in pulse pressure was observed when sodium fluoride or theophylline was infused into pithed rats. Rall & West (1963) have shown that theophylline increases the contractile force of isolated rabbit auricles and considerably potentiates their response to noradrenaline. They judged these results to be consistent with the hypothesis that 3′,5′-AMP is a mediator of the inotropic action of sympathomimetic amines. It seems probable, therefore, that the increase in pulse pressure observed in the present experiments was due to an accumulation of 3′,5′-AMP in the heart muscle.

The evidence that the compounds used in these experiments influence the enzymes responsible for the metabolism of 3′,5′-AMP has all been obtained in broken cell systems, and there is no evidence that they produce similar effects *in vivo*. Indeed the estimation of 3′,5′-AMP in living tissue is difficult. Butcher *et al.* (1965) have described a method and applied it to the production of lipolysis in the epididymal fat pads of the rat. In

these experiments, caffeine (1·0 mM) produced an insignificant increase in the tissue 3′,5′-AMP but a significant increase in lipolysis. However, the same concentration greatly increased the effectiveness of adrenaline in increasing both the tissue content of 3′,5′-AMP and lipolysis. This argues that the xanthines will only produce significant effects *in vivo* in the presence of an agent which stimulates cyclase. This could account for the effectiveness of theophylline in the present experiments and would suggest that a pressor agent that did not activate cyclase would not be affected by it. Preliminary experiments on the effects of angiotensin in the pithed rat show that they are potentiated by infusions of vasopressin and sodium fluoride, which activate cyclase, but not by theophylline, which inhibits phosphodiesterase. It is not known whether angiotensin affects cyclase or phosphodiesterase. These preliminary experiments suggest that it does not affect either and lend support to the proposal that the effects reported in the present experiments are the result of changes in the tissue content of 3′,5′-AMP, especially as the nucleotide itself can potentiate the contractions of the rat aortic strip in response to noradrenaline.

The work was supported by a grant from the Medical Research Council to P. A. Nasmyth for scientific assistance and by Public Health Service research grant HE-05792-03 to H. J. Bartelstone from the National Heart Institute.

REFERENCES

BARTELSTONE, H. J. (1960). Role of the veins in venous return. *Circulation Res.* **8**, 1059–1076.

BARTELSTONE, H. J. & NASMYTH, P. A. (1963). Possible implication of adenosine 3′,5′-phosphate in vascular responses produced by vasopressin and catecholamines. *Fedn Proc.* **22**, 541.

BARTELSTONE, H. J. & NASMYTH, P. A. (1965). Vasopressin potentiation of catecholamine actions in dog, rat, cat and rat aortic strip. *Am. J. Physiol.* **208**, 754–762.

BARTELSTONE, H. J., NASMYTH, P. A. & TELFORD, J. M. (1964). A possible role of adenosine 3′,5′-phosphate (3′,5′-AMP) in controlling the vascular response to noradrenaline. *J. Physiol.* **171**, 48–49*P*.

BROWN, E., CLARKE, D. L., ROUX, V. & SHERMAN, G. H. (1963). The stimulation of adenosine 3′,5′-monophosphate production by antidiuretic factors. *J. biol. Chem.* **238**, 852–853.

BUTCHER, R. W., HO, R. J., MENG, H. C. & SUTHERLAND, E. W. (1965). The measurement of adenosine 3′,5′-monophosphate in tissues and the role of the cyclic nucleotide in the lipolytic response of fat to epinephrine. *J. biol. Chem.* **240**, 4515–4523.

BUTCHER, R. W. & SUTHERLAND, E. W. (1962). Adenosine 3′,5′-phosphate in biological materials. I. Purification and properties of cyclic 3′,5′-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3′,5′-phosphate in human urine. *J. biol. Chem.* **237**, 1244–1250.

CSAPO, A. (1954). Dependence of isometric tension and isotonic shortening of uterine muscle on temperature and on strength of stimulation. *Am. J. Physiol.* **177**, 348–354.

MCPHAIL, M. K. (1934). The assay of progestin. *J. Physiol.* **83**, 145–156.

MURAD, F., CHI, Y-M., RALL, T. W. & SUTHERLAND, E. W. (1962). Adenyl cyclase. III. The effect of catecholamines and choline esters on the formation of adenosine 3′,5′-phosphate by preparations from cardiac muscle and liver. *J. biol. Chem.* **237**, 1233–1238.

NASMYTH, P. A. & BARTELSTONE, H. J. (1962). Interaction of vasopressin and norepinephrine on the circulation. *Pharmacologist* **4**, no. 2, 150.

ORLOFF, J., HANDLER, J. S. & PRESTON, A. S. (1961). Vasopressin-like effects of adenosine 3′,5′-phosphate (cyclic 3′,5′-AMP) and theophylline in the toad bladder. *Biochem. biophys. Res. Commun.* **5**, 63–65.

RALL, T. W. & SUTHERLAND, E. W. (1962). Adenyl cyclase. II. The enzymatically catalysed formation of adenosine 3′,5′-phosphate and inorganic pyrophosphate from adenosine triphosphate. *J. biol. Chem.* **237**, 1228–1232.

RALL, T. W. & WEST, T. C. (1963). The potentiation of cardiac inotropic responses to norepinephrine by theophylline. *J. Pharmac. exp. Ther.* **139**, 269–274.

SHIPLEY, R. F. & TILDEN, J. H. (1947). A pithed rat preparation suitable for assaying pressor substances. *Proc. Soc. exp. Biol. Med.* **64**, 453.

SUTHERLAND, E. W., RALL, T. W. & MENON, T. (1962). Adenyl cyclase. I. Distribution, preparation and properties. *J. biol. Chem.* **237**, 1220–1227.