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AN UNIDENTIFIED DEPRESSOR SUBSTANCE IN CERTAIN TISSUE EXTRACTS.

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Experiments with the rabbit's isolated intestine.

Following up the discovery by Dale and Dudley [1929] that acetylcholine is present in the fresh spleen of the ox and horse we tried to arrive at some idea of the distribution of this substance in different organs. Alcoholic extracts were made from various tissues obtained from horses by mincing the fresh tissue in five times its weight of alcohol. After removing the alcohol under reduced pressure, we tested the extracts on the longitudinal muscle of the rabbit's isolated intestine, which appeared to be very suitable for our purpose since it contracts in very low concentrations of acetylcholine and is very insensitive to histamine. Extracts of some tissues caused effects which probably were due to acetylcholine, but, in some cases, and particularly in extracts of intestine and brain, the contraction which occurred came on more slowly than that due to choline or acetylcholine, and was not much affected by doses of atropine which abolished the effect of these substances. This is illustrated in Fig. 1 which shows the action of a partially purified extract of horse's intestine. The action of this extract, which contained very little choline as shown by the effect of acetylation, was unaffected by atropine. Similar observations have been made by other investigators. Backmann [1921] showed that biodialysates, dialysates, and alcoholic extracts of uterus caused a contraction of the rabbit's isolated intestine after atropine, and Jendrassik [1929] observed the same effect with extracts of brain. It is clear that this effect is due to some substance other than a choline ester or histamine.

Extracts of some tissues produced a pure inhibition of the intestinal tone and rhythm, while in other cases inhibition occurred before the onset of the contraction (see Figs. 6 and 7). That this inhibition was due to a different substance was shown by its different stability in alkali. The stimulant substance was relatively stable to heating in acid solution, but

easily destroyed by brief boiling in alkali, which left the inhibitory substance intact. We shall give reasons for believing that the latter substance is probably adenylic acid or adenosine. It is with the stimulant substance, however, that we are chiefly concerned.

Effects on the blood-pressure of the rabbit.

When an extract from almost any organ was injected intravenously into a rabbit under ether it produced a fall of blood-pressure, which in many cases was not greatly affected by atropine. This phenomenon was first described by Osborne and Vincent [1900], and has since then been

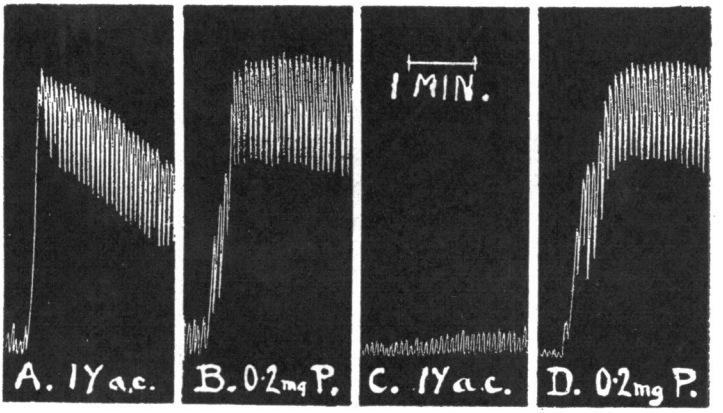

Fig. 1. Isolated rabbit's jejunum in 15 c.c. bath. A and B, in normal Tyrode solution. C and D, atropine added ($1/10^6$). A and C, 1 γ acetylcholine. B and D, 0·2 mg. of preparation *P*.

described by several investigators. It has been pointed out that this effect cannot be due either to histamine (which does not produce such an effect in the etherized rabbit), or to choline or choline esters (which produce no effect after atropine).

This depressor effect might be due to adenosine, which does cause a fall in blood-pressure in the rabbit after atropine. We have found, however, that most of the depressor effect produced by our extracts from small intestine and brain is due to some other substance, which, unlike adenosine, is unstable in alkali, and which can easily be separated from adenosine in several different ways. On the other hand, most of the extracts also contained an alkali-stable depressor substance, and we shall give reasons for believing that this was adenosine or some closely related substance.

ADENYLIC ACID, FRÜHGIFT, AND COZYMASE.

The pharmacological activity of adenylic acid and adenosine was discovered by Drury and Szent-Györgyi [1929], who found that certain effects of various organ extracts on the heart were due to adenylic acid. They showed further that adenylic acid, or adenosine which had similar properties, caused a fall of blood-pressure in the dog even after atropine, and that the rabbit's isolated intestine was inhibited by concentrations as low as 0·002 p.c. Adenylic acid has been shown to be present in blood [Bass, 1914; Jackson, 1923], in skeletal muscle [Embden and Zimmermann, 1927], in heart and brain [Pohle, 1929 *a* and *b*], and also in kidney [Embden and Deuticke, 1930].

Quite recently Zipf [1930] has published an investigation on the chemical nature of the "Frühgift" of Freund [1920] which appears in the clotting of blood and causes a fall of blood-pressure, unaffected by atropine. He arrives at the conclusion, supported by pharmacological and chemical experiments, that the "Frühgift" is an adenylic acid. "Frühgift" or adenylic acid was shown by Zipf to be present also in liver, spleen, pancreas and lung. We also have found in extracts of all these tissues (except heart muscle which was not tested) substances which resemble adenylic acid pharmacologically in their action on the intestine, and which are not easily destroyed by alkali.

In their work on cozymase H. v. Euler [1930] and co-workers have found that the cozymase obtained from yeast and from several animal organs has the probable constitution of an adenylic acid, where the relation between adenine, pentose, and phosphoric acid groups is 1 : 1 : 1. They have also found that the activity of the purified preparations increases in parallel with the amount of phosphorus. After reprecipitation of its barium salt the activity of such a preparation remains unaltered. A sample of a highly purified solution of cozymase (with an A.Co. = 100,000) was tested by us. The preparation contained 6·5 mg. dry substance per c.c. and caused a fall of blood-pressure in atropinized rabbits and stimulation of a guinea-pig's isolated uterus. In both cases it was about equivalent in action to a solution of 4 mg. adenosine per c.c. The preparation of adenosine was obtained from British Drug Houses. It also inhibited the movements of the rabbit's isolated intestine (see Figs. 2 and 3). In these pharmacological respects its behaviour was like that of adenosine. Drury and Szent-Györgyi [1929] have shown that, though adenylic acid and adenosine have similar actions, adenosine is the more active by about 1½ times, probably owing to its lower molecular

weight. The cozymase preparation, therefore, appeared to have about the same activity as the preparation of adenylic acid used by Drury and Szent-Györgyi. This is of interest, since cozymase is known to be

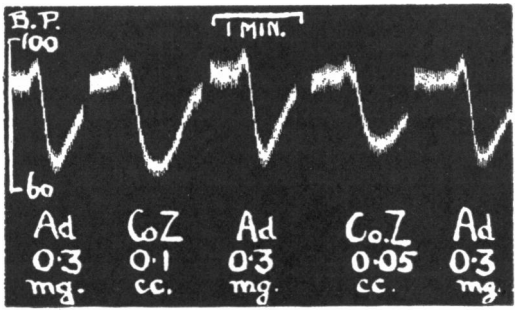

Fig. 2. Blood-pressure atropinized rabbit. Cozymase solution (CoZ) is equivalent to between 3 and 6 mg. adenosine (Ad) per c.c.

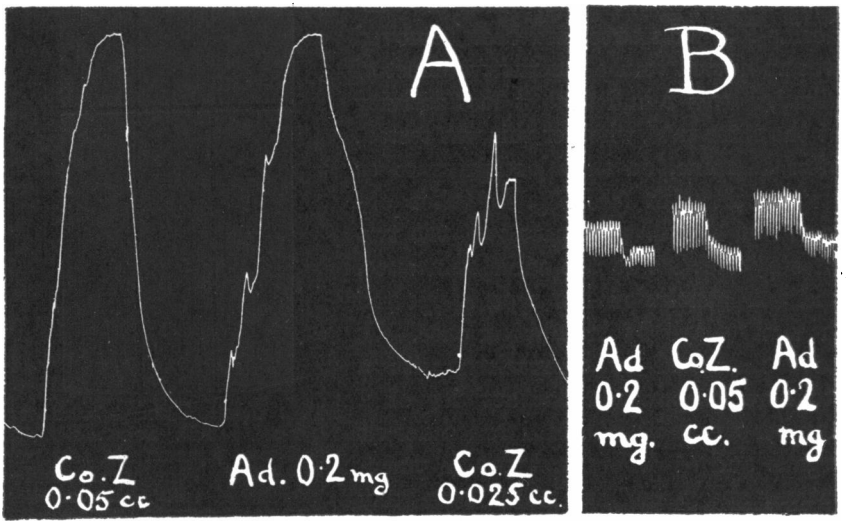

Fig. 3. A. Guinea-pig's isolated uterus. B. Rabbit's isolated jejunum in atropine ($1/10^6$) in 15 c.c. bath. Cozymase solution (CoZ) is equivalent to 4 mg. adenosine (Ad) per c.c.

present in most organs, and therefore might play a rôle as a depressor substance in organ extracts. It must be pointed out, however, that cozymase is apparently not identical with any of the adenylic acids that have previously been isolated from yeast and muscle.

Though adenylic acid is insoluble in absolute alcohol it is soluble in mixtures of water and alcohol and would, therefore, be likely to appear in our extracts. It is thus reasonable to assume that the alkali-stable substance in our extracts, which causes inhibition of the intestine and a fall of blood-pressure, is adenosine or adenylic acid. This assumption was confirmed by the observation that this substance in the extracts was insoluble in absolute alcohol, and that, though it was but little affected by boiling for half-an-hour in normal alkali, it was destroyed by a few minutes' boiling with normal acid. Its behaviour in the presence of the precipitants used in the study of the other substance pointed to the same conclusion.

Purification of the unidentified substance.

We now come to what has been our main interest—the stimulant effect of the extracts on the atropinized intestine and their depressor effect (after atropine) which was not due to adenosine.

When an extract from which the adenosine-like substance had been removed was subjected to the destructive effect of alkali, it was found that it lost activity at the same rate, whether this was tested, after atropine, on the blood-pressure or on the intestine (see Fig. 4). This observation led us to suspect that the two effects were due to the same substance, and this conclusion has been supported in many ways by later experience. We have measured by comparison with a standard preparation the activity of a number of extracts obtained from different organs and at different stages of purification, and there has never been any significant discrepancy in the results obtained by the two tests.

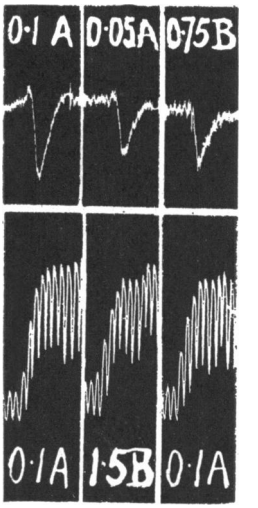

Fig. 4. *Upper.* Blood-pressure atropinized rabbit. *Lower.* Rabbit's isolated jejunum in atropine ($1/10^6$). A. Solution of purified powder *P*. B. Same solution boiled with 0·67 *N* NaOH for 2 min. and neutralized. In both cases A is 15 times as active as B.

The extracts have all been tested in the first place on the blood-pressure of rabbits, after the subcutaneous injection of 5 mg. of atropine sulphate. Confirmatory experiments on the intestine have been carried out only at the more important stages of the purification.

It has been found possible to measure the depressor activity of a

solution with an error of certainly not more than 30 p.c. by matching it with a standard preparation, given in alternate doses. The effect on the active principle of various chemical and physical procedures has thus been studied quantitatively.

Our most active preparations have been made from the small intestine of the horse. The mucous membrane contained only little activity of this kind and was scraped off. The plain muscle was minced and suspended in a solution of alcohol so that the final concentration of alcohol was 72–80 p.c. 1 c.c. of normal sulphuric acid was added to every 100 c.c. of alcohol. The addition of sulphuric acid increased the yield, and made filtration easier. After standing for about an hour at room temperature with stirring, the suspension was filtered, and the filtrate was placed in the cold room overnight. The next morning the

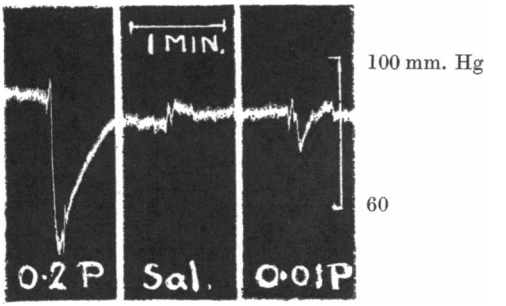

Fig. 5. Blood-pressure atropinized rabbit. Effect of 0·2 mg. and 0·01 mg. of purified powder *P*. Sal. control injection of saline.

alcohol was removed under reduced pressure. Fats were removed with ether, in which the active principle was practically insoluble. The watery extract obtained in this way was highly depressant on the blood-pressure and stimulating in action on the rabbit's isolated intestine. The solution was found to be very stable when the solution was made just acid to congo-red. If such a solution was concentrated, made alkaline with sodium carbonate and, as far as possible, dried by rubbing up with anhydrous sodium sulphate, as much as 90 p.c. of the activity could be extracted with absolute alcohol. When the solution was not made alkaline before drying, absolute alcohol extracted only a small amount of the activity. The addition of sulphuric acid to the solution in absolute alcohol produced a precipitate which, in a dose of 0·01 mg., caused a just perceptible fall of blood-pressure in a sensitive rabbit (see Fig. 5).

This precipitate contains practically no adenosine-like substance, as judged by its action on the rabbit's isolated intestine, and its lack of effect on the blood-pressure after treatment with alkali. However, it probably contained a small proportion of both histamine and choline. The evidence for this is discussed below. It also contained about 65 p.c. of ash, and it was thus clear that it consisted of a precipitate of inorganic sulphates which had carried down the active substances with them. We have used a powder prepared in this way as a standard in a number of experiments. This standard preparation, which we call *P*, dissolved easily in water to form a practically clear solution.

In trying to purify the substance further we found that it was carried down by phosphotungstic acid and by mercuric chloride, but we were unable to recover more than a few p.c. of the activity from the precipitates, even with careful decomposition. We were more successful with Reinecke acid [$(NH_3)_2Cr(SCN)_4$. H (see Kapfhammer and Eck, 1927)] and recovered about 60 p.c. of the activity by decomposing the precipitated compound with silver sulphate. Practically none of the adenosine-like depressor substance was precipitated by the Reinecke acid.

We suspected that the active principle was carried down in these cases adsorbed on other substances, and at Dr Dudley's suggestion we used another method of purification. A quantity of hydrochloric acid was added to an active solution, which was then poured into a solution containing a quantity of sodium benzoate equivalent to the hydrochloric acid. This caused a crystalline precipitate of benzoic acid, which was collected, washed, and then dried in a desiccator. When completely dry the precipitate was shaken up with ether, which dissolved the benzoic acid and left a very active residue. It is possible in this way to remove most of the histamine, which is not so readily adsorbed as the active substance we have been studying.

Some physico-chemical properties of the depressor substance.

The substance is dialysable, and filters under pressure through a cellophane membrane. It has already been mentioned that the substance can be adsorbed on benzoic acid, and it seems on the whole to be rather easily carried down by precipitants. This circumstance suggests that the substance is of a complicated nature, but it is not carried down in appreciable amounts by colloidal iron.

Solubility.

The solubility of the substance in some organic solvents is given in the table below.

TABLE I. Solubility of depressor substance.

A. In neutral or acid state:	
In absolute alcohol	Slightly soluble
In ether	Insoluble
In acetone (dry)	Insoluble
B. In alkaline state:	
In absolute alcohol	Soluble
In ether	Insoluble
In chloroform	Slightly soluble
In amyl alcohol	Slightly soluble

Stability.

Table II shows some of the results obtained by various treatments of the standard preparation *P*.

TABLE II.

Temperature	Medium	Time	Activity destroyed p.c.
0° C.	Water just acid to congo-red	96 days	50
Room temp.	0·04*N* H_2SO_4	1 hr.	None
,,	0·2*N* H_2SO_4	1 hr.	10
Boiling	Water just acid to congo-red	½ hr.	None
,,	0·6*N* HCl	1½ min.	None
,,	0·6*N* HCl	2 min.	25
,,	*N* HCl	2 min.	66
Room temp.	Sat. Na_2CO_3	1 hr.	33
,,	*N* NaOH	1 hr.	50
Boiling	0·67*N* NaOH	½ min.	75
,,	0·67*N* NaOH	2 min.	93
,,	*N* NaOH	2 min.	100
0° C.	Absolute alcohol	34 days	50

When crude extracts were boiled in normal sodium hydroxide the unidentified depressor substance was rapidly destroyed, and the activity which we attribute to adenylic acid was practically unaffected. Acids destroyed both forms of activity at much the same rate, though the preparation *P* was slightly more stable in acids than pure adenosine.

An experiment was carried out to test whether the active principle was destroyed by blood. The injection of small quantities of defibrinated goat's blood was found to have no effect on the blood-pressure of an atropinized rabbit. One c.c. of goat's blood was added to 3 c.c. of a solution of the preparation *P*, and kept at room temperature for 45 minutes. When the mixture was tested directly it was found that this treatment produced no detectable loss of activity.

PHYSIOLOGICAL PROPERTIES OF THE ACTIVE PRINCIPLE.

Our purest preparations have always contained at least one physiologically active substance which resists treatment with alkali. A solution of the powder *P*, which contained no adenosine, was found to produce a contracture of the virgin guinea-pig's isolated uterus, and the action was not affected by boiling the solution for two minutes with an equal volume of 2*N* NaOH. The solution was compared with a solution of histamine and the conclusion was reached that this effect was due to the presence in *P* of between 0·5 and 1 p.c. of histamine. Similar results were obtained in experiments on the rabbit's isolated uterus, and also in experiments on the blood-pressures of a cat and a goat. In all these cases the activity could be quantitatively accounted for by assuming that the preparation contained between 0·5 and 1 p.c. of histamine. There was no evidence that the unknown active principle had any effect in these experiments.

In other experiments preparation *P* was treated with alkali, and then acetylated and tested on the rabbit's isolated intestine in comparison with acetylcholine. It was thus shown that the preparation probably contained about 1 p.c. of choline. This quantity of choline would probably not affect the action of *P*, but the presence of such a proportion of histamine has prevented us hitherto from studying satisfactorily any other effects which the active substance may produce, in addition to its effects on the blood-pressure and on the intestine of the rabbit.

The effect on the blood-pressure of the atropinized rabbit is due to peripheral vaso-dilatation, being produced equally well by direct injection into the aorta and with a shorter latent period than by intravenous injection. Table III shows the times between injection and beginning of fall in blood-pressure, using the two methods of injection in two experiments.

TABLE III. Showing the time between injection and first visible fall in blood-pressure.

	Arterial injection	Venous injection
Expt. 1	2·2; 2·5 sec.	4·9; 6·2 sec.
Expt. 2	1·1; 1·4 sec.	3·1 sec.

These experiments were made in heparinized rabbits. The injections were made through narrow glass tubes which were passed down the left sub-clavian artery into the arch of the aorta, and down the right jugular vein into the superior vena cava. Our preparation did not, however, produce vaso-dilatation in a rabbit's isolated ear perfused with Tyrode's solution

[Pissemski, 1914]. It caused vaso-constriction quantitatively equivalent to that caused by the amount of histamine which we had reason to believe that the preparation contained.

We have also investigated the effect of the depressor principle on the denervated gastrocnemius of a cat in two experiments. These showed that 4γ of acetylcholine caused a fall of blood-pressure and strong contraction of the muscle, and that 0·4 mg. of our standard powder *P* produced a fall of blood-pressure (probably due, as indicated above, to the histamine in the preparation) but no contraction of the denervated muscle.

The distribution of activity in different organs.

We have prepared acid-alcoholic extracts at room temperature of a number of organs obtained from horses and, after removing the alcohol, found that most of them produced a temporary fall of blood-pressure in the atropinized rabbit under ether. The activity of each preparation was measured by comparing it with our standard preparation *P*. The results are shown in Table IV.

Table IV. Depressor activity of alcoholic extracts from horses on atropinized rabbits under ether.

Organ	Activity (mg. *P* per gm. of tissue)
Small intestine	0·7–2
Brain	0·7 1·9
Stomach	0·7
Bladder	0·3
Blood	0·1
Uterus	0·05
Striated muscle	*ca.* 0·05
Lung	*ca.* 0·02
Liver	*ca.* 0·02
Kidney	< 0·03
Pancreas	< 0·03
Spleen	None

Most of these extracts appeared to contain adenylic acid, but it is improbable that our extracts contained all the activity of this kind originally present in the tissue, and we have made no attempt to trace the distribution of this substance in any detail. In the case of the first four extracts shown in Table IV we can state with confidence that the major part of the depressor effect was not due to adenylic acid. We have no reason to doubt that it was due in all four cases to the same unknown depressor substance. In the other extracts the total amount of activity was so small that we were unable to decide in what proportion these two

kinds of depressor substance occurred. In any case the table shows that most of the tissues contained very little of the substance we are studying.

Extracts of the muscular coat of the small intestine gave the biggest effects, and these appeared to contain comparatively little adenylic acid.

Brain appeared to contain almost as much of the active principle as the small intestine. The first observation of this kind of depressor action was made with extracts of the central nervous system by Osborne and Vincent [1900], but data were not then available for distinguishing between adenylic acid, in which brain is comparatively rich, and the other alkali-unstable depressor substance. It might be pointed out here, too, that Jendrassik made from brain his extracts which caused a contraction of the rabbit's isolated intestine after atropine.

Osborne and Vincent [1900] found that the grey matter of the brain was particularly rich in depressor substances, and recently Leimdörfer [1930] has found that extracts of the region of the thalamus produce a larger fall of blood-pressure in the cat than extracts of the hemispheres. These phenomena might be due to an uneven distribution of various different depressor substances, and we have therefore made similar experiments on the atropinized rabbit.

In Fig. 6 an experiment is reproduced which shows the effect of extracts from the basal ganglia and from the hemispheres on the rabbit's blood-pressure and intestine. The experiment on the intestine shows that both tissues contain some of the inhibitory adenosine-like substance, but the additional depressor activity of the extract of basal ganglia was accompanied by an additional stimulant effect on intestinal muscle, and it appears probable that this was due to the other substance we have been studying. Fig. 7 shows the effect of boiling an extract of whole brain (obtained from a rabbit) with normal sodium hydroxide for 3 minutes. The substance stimulating the intestine is removed by this treatment, but the inhibitory substance is left.

The depressor effects of extracts used by other investigators.

No attempt will be made to review the very large number of accounts which have been published of the depressor effect of tissue extracts. Bibliographies will be found in the papers by Vincent and Curtis [1926], Dale and Dudley [1929] and Gley and Kisthinios [1929].

There are probably at least five different types of substance which may occur in tissue extracts and which may produce vaso-dilatation:

(1) Histamine.

(2) Choline and choline esters.

(3) Substances allied to adenosine.

(4) The unidentified substance with which this paper is chiefly concerned.

(5) Kraut and Frey [see Frey, Kraut and Schultz, 1930] have described a depressor substance, "Kallikrein," which is present in urine and pancreas and which has been shown to be neither histamine nor choline. It is clearly not allied to adenosine since it increases the heart

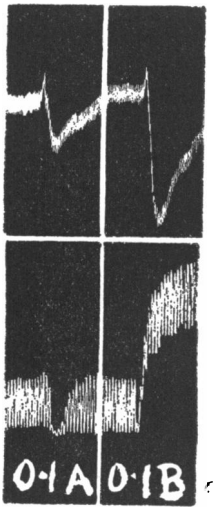

Fig. 6.

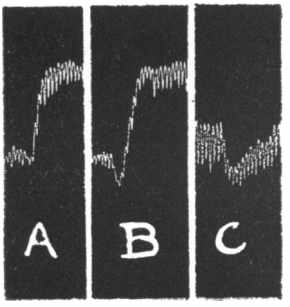

Fig. 7.

Fig. 6. *Upper.* Blood-pressure atropinized rabbit. *Lower.* Rabbit's isolated jejunum in atropine ($1/10^6$). A. Extract of cerebral hemisphere. B. Comparable extract of basal nuclei (from horse).

Fig. 7. Rabbit's isolated jejunum in atropine ($1/10^6$) in 15 c.c. bath. A. 0·2 mg. purified powder *P*. B. 0·15 c.c. extract of whole rabbit's brain. C. Equivalent dose to B after treatment with alkali.

rate. It is undialysable and easily inactivated by blood. It is therefore almost certainly distinct from any of the other substances mentioned above. Our extracts are presumably free of this substance, which is insoluble in alcohol as strong as 80 p.c.

Depressor effects of tissue extracts on atropinized rabbits have been observed by Osborne and Vincent [1900], Vincent and Sheen [1903], Vincent and Cramer [1904], Roger [1922], Vincent and Curtis [1926], Best, Dale, Dudley and Thorpe [1927], Gley and Kisthinios [1929], Major and Weber [1929, 1930] and Fontaine and Jung [1930].

Most of these authors have pointed out that such effects cannot be due either to histamine or to choline. They might, however, be due to any of the other types of substance (3), (4), or (5) and in most cases there is not sufficient evidence to enable us to say which of these substances the extracts contained. When, as in certain cases, the extract produced bradycardia it is probable that some substance allied to adenosine was present. Kallikrein, on the other hand, accelerates the heart. Adenosine is stable in alkalis, whilst the substances (4) and (5) are not. Adenosine inhibits the rabbit's intestine, which is stimulated by substance (4). It is, however, clear that it is not at present possible to devise simple and reliable pharmacological tests which will measure the relative concentrations of all these substances in a tissue extract.

SUMMARY.

The nature of the vaso-dilator substances in tissue extracts is discussed.

Extracts in cold acid alcohol have been made from various tissues obtained from horses and tested on the blood-pressure of atropinized rabbits.

In some cases the depressor effect is probably due to substances allied to adenosine. Highly purified cozymase preparations have the same effect. These substances also inhibit the rabbit's isolated intestine and stimulate the guinea-pig's isolated uterus.

Evidence is given for the presence in these tissue extracts, particularly in those from intestinal plain muscle and brain, of another substance, which lowers the arterial blood-pressure of the atropinized rabbit by peripheral vaso-dilatation, and also stimulates the tone and rhythm of the rabbit's isolated intestine after atropine.

Some of the chemical and physical properties of this substance have been studied.

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