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# REMOVAL OF INTERFERING NUCLEOTIDES FROM BRAIN EXTRACTS CONTAINING SUBSTANCE P. EFFECT OF DRUGS ON BRAIN CONCENTRATIONS OF SUBSTANCE P

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Several methods for removing interfering nucleotides, adenosine-5'-monophosphate and adenosine 5'-triphosphate from brain extracts have been studied. An enzymic method, using adenylic acid deaminase, has been found suitable. This deaminates adenosine monophosphate to 5'-inosinic acid, an inactive compound which does not influence the estimations of substance P. Owing to the adenosine triphosphatase content of the enzyme extract, adenosine triphosphate was also inactivated. For the estimation of adenosine monophosphate-deaminase activity, a simple colorimetric method is described which measures the ammonia liberated from adenosine monophosphate. Substance P in mouse brain extracts was estimated after treatment of the animals with various drugs, and after the enzymic removal of interfering nucleotides from the brain extracts. The drugs had no effect on the substance P content of mouse brain. The effect of drugs on the contractions of the guinea-pig ileum induced by substance P was also investigated, and the effect of drugs on the estimations of substance P in brain extracts is discussed.

Brain extracts, used for the estimation of substance P on guinea-pig ileum, contain adenosine mono- and triphosphate (Laszlo, 1963). These substances influence the contractions of the ileum and introduce a variable error into the assay. The wide scatter of the reported values for the concentration of substance P in brain is partly due to this error. The present work includes the removal of the interfering nucleotides, and an improved method of estimating substance P has been used to examine the effect of the administration to mice of centrally-acting drugs on the substance P content of their brains. Some of these results have been briefly reported (Laszlo, 1960).

## METHODS

### *Animal experiments*

The contents of substance P in the brains of male albino mice (body weight 22 to 30 g) were estimated.

*Administration of drugs.* Except where stated otherwise, control animals received 0.9% saline or the solvent used for the drug in the same volume and at the same time as the doses of drugs given to treated animals. The animals were subsequently killed by a blow on the head and the brain was removed immediately. Doses of drugs and details of the treatment of the animals are in Table 1.

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TABLE 1

TREATMENT OF ANIMALS WITH DRUGS

* Animals had no food or water during the time elapsed between treatment and killing. † Animals were kept without food for 2 hr before injections. I.p.=intraperitoneal; s.c.=subcutaneous injection

| Drug | Dose | Time of killing after treatment (hr) | Details of the treatment |
|---|---|---|---|
| Lysergic acid diethylamide | 17·7 mg/kg (total) | 0·5 and 1 | 3 i.p. injections at 15 min intervals |
| Reserpine | 5 mg/kg (total) | 24 | 2 i.p. injections at 24 hr interval; solvent contained ascorbic acid (25 mg/ml.) and sodium ascorbate (4 mg/ml.) |
| Morphine sulphate | 5 to 120 mg/kg (single doses) | 24 | Twice daily, 5 days/week, s.c. Doses were doubled after every 2 days up to 80 mg/kg, with the last dose 120 mg/kg |
| Chloroform and ether | | 0·75 | Dose causing surgical anaesthesia |
| Chlorpromazine | 10 mg/kg (total) | 5 | 2 s.c. injections at 24 hr interval* |
| Barbitone sodium | 100 mg/kg | 8 | S.c. injection |
| Insulin | 0·05 U/20 g mouse | After start of convulsions | *p*H of the solution was adjusted to 2·5† |
| Phenytoin sodium | 50 mg/kg | 4·5 | I.p. injection. *p*H was made alkaline with NaOH to give a clear solution |
| Leptazol | 160 mg/kg | After the hindleg tone had begun to relax | I.p. injection |
| Benzhexol hydrochloride | 1 mg/kg | 3 | S.c. injection* |
| Substance P | 220 U/ 20 g mouse | 1 | S.c. injection |

*Biological methods*

*Guinea-pig ileum.* Substance P was estimated on guinea-pig ileum superfused with Tyrode solution containing (g/l.) NaCl 8.00, KCl 0.20, $CaCl_2$ 0.20, $MgCl_2$ 0.01, $NaH_2PO_4$ 0.05 and $NaHCO_3$ 1.00; atropine sulphate ($10^{-7}$) and mepyramine maleate ($10^{-6}$) were added to inhibit the actions of acetylcholine and histamine, respectively. The piece of ileum was taken from about 4 cm from the ileocaecal junction. Animals of either sex were used, usually between 200 and 300 g in weight.

*Rabbit jejunum.* A length of rabbit jejunum was set up in an organ-bath at 37° C in modified Locke solution containing (g/l.) NaCl 9.00, KCl 0.42, $CaCl_2$ 0.24, $NaHCO_3$ 0.50 and glucose 1.00, together with atropine sulphate ($10^{-7}$).

*Preparation of standard extracts of substance P.* Standard preparations were made by the method of Amin, Crawford & Gaddum (1954) from cow, horse and pony intestines as described previously (Laszlo, 1963).

*Preparation of extracts of brain for the estimation of substance P*

As a result of the experiments described below, which aimed at the elimination of interfering nucleotides, the following method was developed for the estimation of substance P in brain.

Mouse brain without the cerebellum was homogenized in 20 ml. of acetone per g of brain, shaken for 1 hr and centrifuged. The supernatant fluid was discarded. The precipitate was shaken for 30 min with 20 ml. of 95% acetone per g of tissue, centrifuged, washed with 3 to 4 ml. of 95% acetone, centrifuged again, and the precipitate dried *in vacuo* at room temperature for a few hours. The dried acetone-precipitate was ground with 0.2 ml. of N-HCl, and then 1 ml. of water and 4 ml. of either potassium-free Tyrode solution or, in some experiments, 0.9% saline were added. The *p*H of the solution was adjusted to 5.5 with NaOH, using narrow range indicator paper (B.D.H.), and the solution was boiled for 2 min, cooled, and centrifuged for 10 min at 3,500 r.p.m. The precipitate was washed with 2 ml. of potassium-free Tyrode solution and centrifuged for 10 min at 3,500 r.p.m. The two supernatant fluids were mixed and kept overnight at 4° C. Next morning, 0.5 ml. of 50 mM-maleate buffer (*p*H 6.2) and a suitable volume (usually 0.2 to 0.4 ml.) of adenosine monophosphate-deaminase extract was added. The volume of deaminase extract depended on its activity, which had been estimated on the previous day. The *p*H was adjusted to 6.4 with 0.1 N-HCl and the mixture kept for 30 min at 35° C to allow dephosphorylation of adenosine triphosphate, and then for 1 hr at 25° C to allow deamination of adenosine monophosphate.

After incubation, the solution was neutralized with a few drops of 0.1 M-$Na_2CO_3$ solution, using indicator paper, and made up with Tyrode solution to 10 ml. Before the assay, suitable dilutions were made with Tyrode solution containing atropine sulphate ($10^{-7}$) and mepyramine maleate ($10^{-6}$).

*Adsorption chromatography of substance P.* Adsorption chromatography with aluminium oxide was tried for the purification of substance P. The aluminium oxide was treated as described by Pernow (1953). Its adsorption capacity, determined by the method of William (1946), was between grades II and III on his scale.

*Paper chromatography.* Paper chromatography was used for the separation of substance P from interfering nucleotides, after removal of salts and proteins by precipitation with alcohol at *p*H 8 and by electrolytic desalting. Proteins were removed by ultrafiltration through Visking tubing (Laszlo, 1963).

*Identification of substances on paper chromatograms*

Substances on the chromatograms were identified (a) by the action of the eluates on guinea-pig ileum and rabbit jejunum ; (b) by absorption in ultra-violet light at 254 mμ wavelength ; and (c) by phosphate reaction on the paper after irradiation with ultra-violet light (Wood, 1958).

*Enzymic methods*

*5'-Nucleotide phosphatase.* This enzyme was extracted from potatoes by grinding them with two volumes of distilled water and filtering through cloth (Kornberg & Pricer, 1950). The enzyme activity was measured by the estimation of the phosphate liberated when adenosine monophosphate was incubated with the mixture for 1 hr in 0.1 M-sodium acetate buffer (*p*H 5.0) or in 0.1 M-glycine buffer (*p*H 9.5) at 40° C.

*Adenosine deaminase.* Adenosine deaminase was extracted from rat heart with water (Ostern & Mann, 1933). A water extract of Taka-diastase was also used as an enzyme source (Mitchell & McElroy, 1946). The activity of the enzyme was estimated by measurement of the ammonia liberated on incubation of the extract with adenosine for 1 hr in 0.1 M-phosphate buffer (*p*H 7) at 40° C.

*Adenylic acid deaminase.* Various methods of preparing this enzyme were tried (Conway & Cooke, 1939 ; Kaplan, Colowick & Ciotti, 1952 ; Schmidt, 1955 ; Lee, 1957), but for routine purposes a preparation from rabbit skeletal muscle, made by the method of Nikiforuk & Colowick (1956), was used. Minced rabbit muscle was washed four times with four volumes of 0.9% saline for 20 min, homogenized and extracted with one volume of a 2% solution of $NaHCO_3$ for 1 hr. The extract was squeezed through cloth and stored at 0° C. Before use a suitable volume of the extract was neutralized with N-HCl or, in a few experiments, with N-acetic acid. The suspension was centrifuged, and the supernatant fluid contained the enzyme.

The adenosine triphosphatase activity of extracts of the adenosine monophosphate-deaminase was investigated at different temperatures (between 22 and 37° C) by incubating the mixtures containing 34 $\mu$g to 2 mg of adenosine triphosphate in 2 ml. of maleate buffer (*p*H 6.4) for 20 to 60 min at 22 and 37° C. Breakdown of adenosine triphosphate was measured using the fire-fly method of Strehler & Totter (1957), and by the estimation of liberated inorganic phosphate.

Buffer solutions mentioned above were made according to the tables published by Dawson & Elliott (1959).

### *Chemical estimations*

*Inorganic phosphate.* This was estimated by the method of King (1947).

*Adenosine monophosphate-deaminase.* The activity of this enzyme was measured in a few experiments by a spectrophotometric method (Kalckar, 1947). Adenosine monophosphate was incubated in the cell of the spectrophotometer at 25° C. in 0.06 M-succinate buffer (*p*H 5.9) and the decrease in absorption at 265 m$\mu$ was followed.

*Ammonia.* Routinely the activity of adenosine monophosphate-deaminase and, in a few experiments, of the adenosine deaminase was estimated from the amount of ammonia liberated by a modification of Russel's (1944) method. Since it was found that the enzyme extract did not interfere with the estimation of ammonia, ammonia in the incubation mixture was measured directly with a photoelectric colorimeter, without using Conway's diffusion technique. Solutions containing 200 $\mu$g of adenosine monophosphate and from 0.1 to 0.4 ml. of enzyme extract were incubated, in glass stoppered tubes, in 50 mM-maleate buffer at *p*H 6.3 (total volume, 1.5 ml.) at 25 and 35° C for 50 min. Ammonium sulphate solutions, containing 5.0 and 2.5 $\mu$g of ammonia, were used as standards; maleate buffer containing adenosine monophosphate or enzyme were blank solutions. Dilutions were made before the estimation of the ammonia. The reagents for the ammonia estimations, described by Russel (1944), were added. The difference between the measured ammonia and the ammonia content of the enzyme extract represents the amount of ammonia liberated by the enzyme.

### *Materials*

A standard preparation of substance P was kindly given by Professor J. H. Gaddum; a preparation containing 11 U/mg, used for the animal experiments, was given by Sanabo, Vienna. Other chemicals and drugs were: acetylcholine (Roche), histamine acid phosphate (B.D.H.), 5-hydroxytryptamine creatinine sulphate (Abbott), adenosine monophosphate-$Na_2H_2O$ (Boehringer & Light), adenosine diphosphate-Na and adenosine triphosphate-$Na_2.3H_2O$ (Sigma), inosinic acid (Nutritional Biochemicals), adenosine and adenine (B.D.H.), reserpine and solvent (Ciba), chlorpromazine (May & Baker), and lysergic acid diethylamide (Sandoz). Aluminium oxide (Savory & Moore) was used for adsorption chromatography.

## RESULTS

In the first part of this work several methods for the elimination of nucleotides from the brain extract were tried.

(1) *Adsorption chromatography.* Substance P extract was adsorbed on aluminium oxide (Pernow, 1953), but the recovery was poor (27%).

(2) *Paper chromatography.* A number of the solvents used in the identification of nucleotides (Laszlo, 1962) were tried. A mixture of pyridine and water (65:35) gave a fairly satisfactory separation of nucleotides from substance P but the recovery of substance P was also very poor (about 25%).

(3) *Extraction of 5'-adenylic acid with acetone.* Separation of adenosine monophosphate and substance P was attempted on the basis of their differential solubility

in 95% acetone in which, between *p*H 7.0 and 7.2, adenosine monophosphate was soluble. Extraction in this *p*H range reduced the nucleotide content of brain extract but much substance P was lost.

(4) *Precipitation of substance P.* Dahlstedt, Euler, Lishajko & Östlund (1959) estimated substance P after precipitating it with ammonium sulphate. However, no precipitate was obtained with the small amounts of substance P present in mouse brain.

(5) *Enzymic methods.* These were successful in removing adenosine mono- and triphosphate.

### *Removal of adenosine monophosphate*

The effects of the degradation products of adenosine monophosphate on the contractions of the guinea-pig ileum induced by substance P were first studied. These products were much less active in reducing these contractions than was adenosine monophosphate itself (Table 2). Two enzymic methods of destroying adenosine monophosphate were tried.

TABLE 2

EFFECT OF ADENOSINE MONOPHOSPHATE AND RELATED COMPOUNDS ON THE SUBSTANCE P ASSAY ON GUINEA-PIG ILEUM

Results were obtained by superfusion. Values indicate the threshold concentration (μg/ml.) in which the given substance diminished the submaximal contractions induced by substance P

| Compound | Concentration (μg/ml.) | Effect |
|---|---|---|
| Adenylic acid | 5 | Inhibition |
| Adenosine | 20 | Inhibition |
| Adenine | 125 | Inhibition |
| Inosinic acid | 500 | No effect |

*(a) 5'-Nucleotide phosphatase and adenosine deaminase.* These enzymes, which produce adenine from adenosine monophosphate, were rejected since the only buffer found to be suitable for both was citrate which, in concentrations as low as 10 mM, rendered the guinea-pig ileum insensitive.

*(b) 5'-Adenosine monophosphate-deaminase.* Inosinic acid, the end product of this enzyme reaction (Fig. 1), in concentrations up to 500 μg/ml., does not influence contractions of the guinea-pig ileum induced by substance P (Table 2). Rabbit skeletal muscle was used as the source of enzyme, since this tissue contains it in high

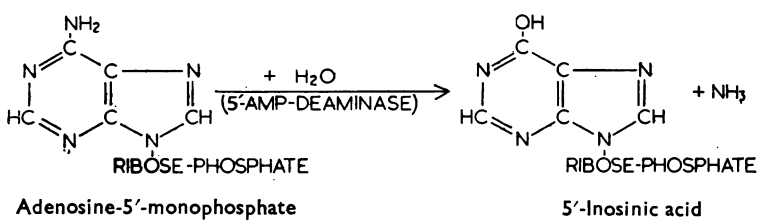


Fig. 1. Enzymic deamination of adenosine monophosphate.

concentration (Conway & Cooke, 1939) and contains little polypeptidase (Gullbring, 1943), which inactivates substance P. The activities of extracts containing adenosine-monophosphate-deaminase, made by different methods, were compared by spectrophotometry. The extracts showed about the same activity, except for Taka-diastase extract which contained very little enzyme. Deamination was nearly complete in maleate buffer which, in concentrations up to 30 mM, did not influence the subsequent estimation of substance P. Conway & Cooke (1939) also found the greatest deamination in this buffer. The results with different buffers are summarized in Table 3.

TABLE 3

EFFECT OF BUFFERS ON THE DEAMINATION OF ADENOSINE MONOPHOSPHATE AND ON THE ESTIMATION OF SUBSTANCE P

The concentration of buffers during the incubation of adenosine monophosphate was 40 to 50 mM (2nd column), whereas in the bioassay it was 5 to 10 mM (3rd column). Tris=2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride

| Buffer | Degree of deamination (%) | Contraction of guinea-pig ileum induced by substance P |
|---|---|---|
| Citrate | — | Abolished |
| Tris | — | Increased |
| Succinate | 45 | Irregular |
| Phthalate | 46 | Increased |
| Barbitone | 27 | No effect |
| $NaHCO_3$-$CO_2$ | 0 | — |
| Maleate | 97 | No effect |

Since published values for the optimum *p*H of this enzyme are conflicting (Nikiforuk & Colowick, 1956), this problem was reinvestigated. The optimum *p*H lay between 6.1 and 6.5, and hence this range was used in the further experiments. Before the assay the incubation mixture was neutralized with sodium carbonate (0.1 M). Fig. 2 shows the effect of different concentrations of maleate buffer on the final *p*H and on the enzymic deamination of adenosine monophosphate. Over 60% of deamination was obtained even with a very low concentration (2.5 mM) of maleate, although in this case it was necessary to adjust the *p*H of the solution before incubation.

Conway & Cooke (1939) showed that tissue extracts contain substances which inhibit the activity of this enzyme. It was therefore necessary to demonstrate deamination of adenosine monophosphate in brain extracts. This was done by testing for adenosine monophosphate using rabbit jejunum in an organ-bath. The results of an experiment are shown in Fig. 3. After incubation of brain extract with adenosine monophosphate-deaminase extract, the inhibitory effect was abolished because inosinic acid, the end product of the reaction, has no effect on the rabbit jejunum. Similarly, when adenosine monophosphate was incubated with the enzyme extract its inhibitory effect was abolished. When brain extract and adenosine monophosphate were incubated with boiled extract of enzyme, their inhibitory effect was not affected. Enzyme extract and buffer solution alone had no effect on rabbit jejunum. The preparation, from which Fig. 3 was recorded, was rather insensitive to substance P, the action of which was therefore not seen.

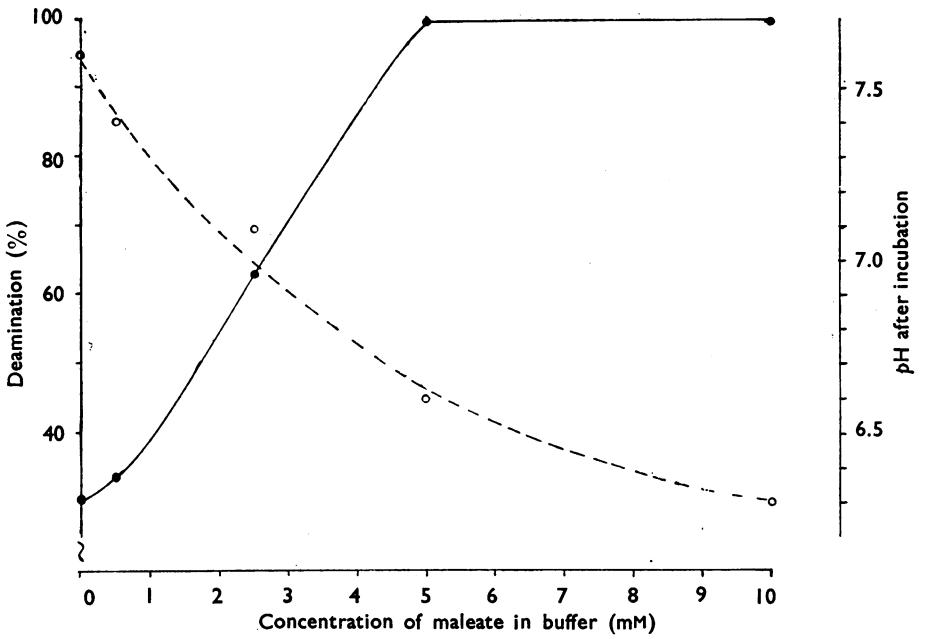


Fig. 2. Relationship between the concentration of maleate buffer (abscissa, mM) and the enzymic deamination of adenosine monophosphate (ordinate, % of initial content). ●——● Deamination of adenosine monophosphate (left-hand scale); ○ - - - ○ *p*H after incubation (right-hand scale).

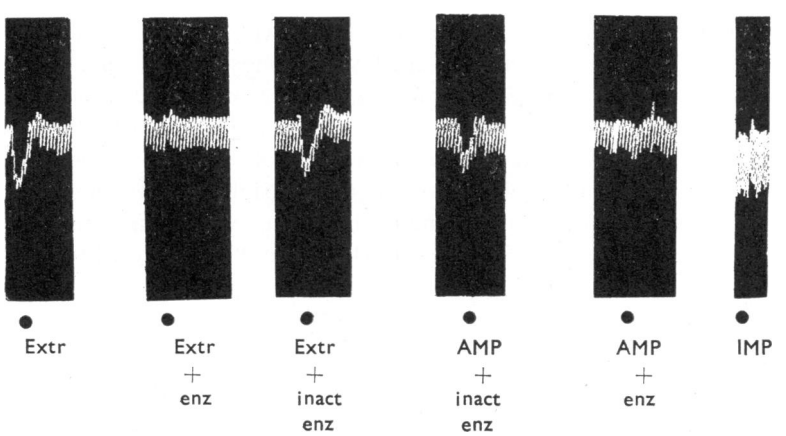


Fig. 3. Effect on rabbit jejunum of brain extract and adenosine monophosphate after incubation with adenosine monophosphate-deaminase. Extr=brain extract; Extr+enz=brain extract+adenosine monophosphate-deaminase; inact enz=enzyme extract boiled before incubation; AMP=adenosine monophosphate; IMP=5'-inosinic acid.

Experiments with guinea-pig ileum showed that substance P was not destroyed by incubation for 1 hr at 25 or 37° C with adenosine monophosphate-deaminase. It was also found that an extract of enzyme previously inactivated by boiling did not influence contractions of the guinea-pig ileum induced by substance P. 0.1 to 0.3 ml. of enzyme extract was always sufficient to deaminate the adenosine monophosphate in one mouse brain, so potassium chloride was not added to the incubation mixture (Lee, 1957).

*Effect of phosphate on the deamination of adenosine monophosphate.* Phosphate (30 mM) inhibits the activity of adenosine monophosphate-deaminase (Nikiforuk & Colowick, 1956). For this reason the content of inorganic phosphate in brain extract was estimated and the effect of various concentrations on the deamination of adenosine monophosphate was investigated. The inorganic phosphate content of the extract during the incubation was 0.32 mM, but inorganic phosphate did not diminish the deamination in a concentration up to ten-times higher (3.2 mM).

*Removal of adenosine triphosphate*

The effect of incubating adenosine triphosphate and brain extracts with adenosine monophosphate-deaminase at 25 and 35° C was also investigated. Adenosine triphosphate disappeared after incubation with this deaminase in 5 mM-maleate buffer at *p*H 6.2 and 35° C. After the incubation free inorganic phosphate was found in the solution. Since the amount of adenosine triphosphate, from which phosphate was removed, was up to 600 μg, the activity of the enzyme was judged sufficient to dephosphorylate the amount of adenosine triphosphate in the brain extract, the upper limit of which was 140 μg per mouse brain (Laszlo, 1963). The liberation of inorganic phosphate occurred within a wide *p*H range of 5.2 to 9.0, with an optimum at 6.2.

The disappearance of adenosine triphosphate from the brain extract and the liberation of inorganic phosphate after incubation with adenosine monophosphate-deaminase is very likely due to myosin-adenosine triphosphatase, since the *p*H for the extraction of adenosine monophosphate-deaminase from rabbit muscle is suitable for the extraction of myosin-adenosine triphosphatase (Engelhardt, 1946 ; Kielley & Meyerhof, 1950). Hermann & Josepovits (1949a) observed that adenosine monophosphate-deaminase extracts often show adenosine triphosphatase activity. Another possibility, the deamination of adenosine triphosphate by adenosine monophosphate-deaminase extract (Lohman & Schuster, 1934), was not encountered in these experiments.

*Effect of temperature on the inactivation of nucleotides*

The optimal temperature for the activity of adenosine triphosphatase is 37° C (Engelhardt & Ljubimova, 1939), while adenosine monophosphate-deaminase is active at lower temperatures, namely 25° C (Kalckar, 1947 ; Nikiforuk & Colowick, 1956) or 30° C (Lee, 1957). The inactivation of adenosine triphosphate was found to be unreliable below 35° C, therefore this temperature was chosen. Incubation at this temperature did not impair the activity of adenosine monophosphate-deaminase, and therefore incubation was continued at 25° C with the same enzyme extract to inactivate adenosine monophosphate.

These investigations led to the procedure of estimating substance P in brain described in "Methods."

*Effect of drugs on the substance P content of mouse brain*

The method described above was used to determine the effect of drugs on the substance P content of mouse brain. The estimations of substance P were made after incubation of the brain extracts with adenosine monophosphate-deaminase at 25° C. In the experiments in which benzhexol and substance P were injected into mice the extracts were also incubated at 37° C. Doses of drugs and details of administration are given in Table 1.

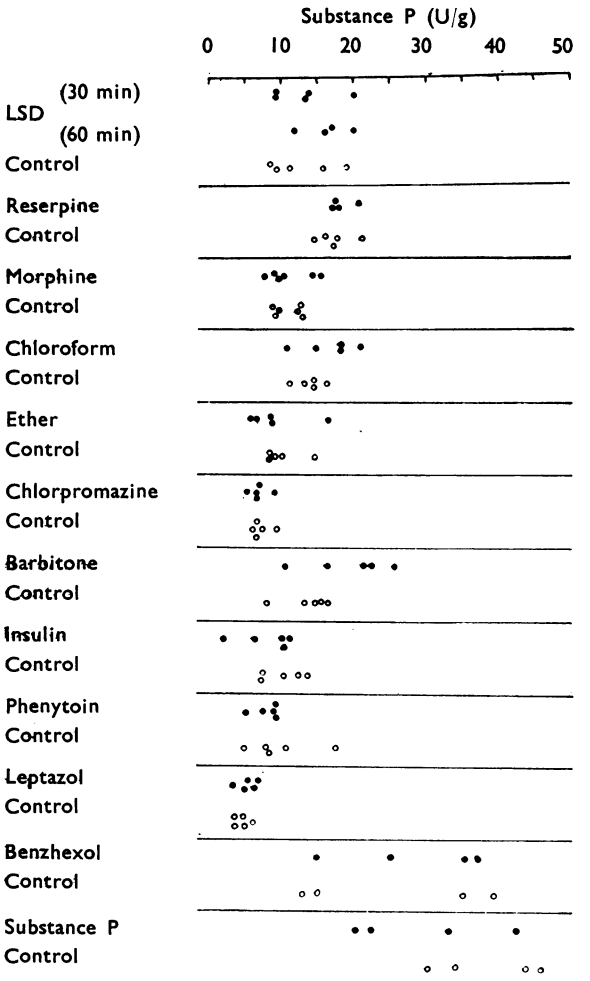


Fig. 4. Substance P content of mouse brain after treatment with various drugs. Estimations were performed with the modified method described in the text. Times in parentheses are the periods between the last injections and killing the animals. LSD=lysergic acid diethylamide. ○, substance P content in the control animals; ●, in the treated animals.

Comparison of the substance P contents in brains of the treated and control animals (Fig. 4) shows that none of the drugs caused any striking change.

*Effect of drugs on the contractions of guinea-pig ileum induced by substance P*

These experiments were done by superfusion, except where otherwise stated, with solutions which contained substance P and the drug to be investigated. The results are given in Table 4.

TABLE 4

EFFECT OF DRUGS ON THE CONTRACTIONS OF GUINEA-PIG ILEUM INDUCED BY SUBSTANCE P

| Drug | Concentration (mg/ml.) | Effect |
|---|---|---|
| Lysergic acid diethylamide | 0·10 | Inhibition |
| Barbitone | up to 2·00 | No effect |
| Leptazol | up to 0·50 | No effect |
| Benzhexol | 0·05 | Inhibition |

Besides these experiments, the effect of lysergic acid diethylamide was investigated in experiments which used an organ-bath. The drug was added to the bath 90 sec before substance P, and both drugs were washed out 40 to 50 sec after adding substance P (Krivoy, 1957). In four such experiments lysergic acid diethylamide had no action on the contractions induced by substance P. Thus, the potentiating effect of lysergic acid diethylamide on such contractions (Krivoy, 1957) was not confirmed.

## DISCUSSION

The removal of nucleotides interfering with the assay of substance P in brain was achieved by incubation with adenosine monophosphate-deaminase which produces inosinic acid. This compound has no effect on contractions of guinea-pig ileum induced by substance P. The elimination of adenosine triphosphate from the extract was obtained with the same enzyme extract, due to its adenosine triphosphatase activity. The conditions under which the incubation of the brain extract takes place are favourable for the action of adenosine triphosphatase. $Mg^{++}$ (10 mM) inhibits myosin-adenosine triphosphatase (Engelhardt, 1946), but the $Mg^{++}$ concentration in the Tyrode solution in which the brain extract was incubated was only 0.10 mM. Myosin is soluble only in a higher salt concentration (0.5 M-KCl or LiCl), while the molarity of the Tyrode solution is 0.154 M. According to Engelhardt (1946) at this molarity only 11% of the total protein of myosin is dissolved. However, this amount of myosin seems to be sufficient for the dephosphorylation of the adenosine triphosphate to diphosphate in the brain extract. Adenosine triphosphate, after incubation with the enzyme extract, was dephosphorylated to adenosine diphosphate, which did not affect the substance P estimation in the concentration in which it was present in the extract. It is thought likely that the enzyme extract removes adenosine diphosphate as well, because Hermann & Josepovits (1949b) showed that myosin removed all adenosine di- as well as triphosphate and that the end product was inosinic acid.

*Effect of drugs on the concentration of substance P in brain*

The question whether inadequate dosage accounts for the absence of effects has to be considered for each drug.

*Lysergic acid diethylamide.* In these experiments 17.7 mg/kg was injected intraperitoneally into mice. Zetler & Ohnesorge (1957) injected 10 mg/kg into mice, and found no change in the substance P content of mouse brain. The concentration of lysergic acid diethylamide in mouse brain after twice the dose used in this experiment was 6.8 μg/g 10 min after the injection (Lanz, Cerletti & Rothlin, 1955). Since the drug, in concentrations up to 50 μg/ml., does not influence substance P estimations (Table 4), the presence of lysergic acid diethylamide could not interfere with the estimations of substance P in brain extracts.

*Reserpine.* After the same dose as that given in the present experiments, Lessin & Parkes (1957) observed that the temperature of mice decreased.

*Morphine.* Doses similar to those used here were given to rats by Cochin & Axelrod (1959) in chronic experiments; tolerance and changes in liver enzyme activity resulted. Morphine, in a concentration as low as 200 ng/ml., depresses the contractions of guinea-pig ileum induced by substance P (Lewis, 1960) and, taking into account the concentration in brain after treatment (Woods, 1954), it is possible that estimations of substance P were influenced by this drug, and that a rise in content may have been missed.

*Ether and chloroform.* These substances were given in sufficient doses to anaesthetize the animals, which were then killed; they obviously cannot influence the assay of substance P owing to their evaporation from the brain extract during the extraction procedure.

*Chlorpromazine.* The dose given has a depressant effect and causes hypothermia in rat and mouse (Courvoisier, Fournel, Ducrot, Kolsky & Koetschet, 1953).

*Barbitone.* Comparison of the concentration found by Vogt (1935) in dog brain (0.24 mg/g) with the relatively high concentration (up to 2 mg/ml.) in which it was tested on the assay of substance P (Table 4) excludes any effect of this drug on the estimations of substance P.

*Insulin.* The dose of insulin caused convulsions of the animals.

*Phenytoin.* The injected dose is similar to that which was effective against hyponatraemic electroshock seizure in mice (Swinyard, Brown & Goodman, 1952). Since this drug is soluble only at alkaline *p*H, it cannot be present in the brain extract, therefore it does not influence the estimations.

*Leptazol.* The dose caused convulsions, but the estimations were not influenced by this drug (Table 4).

*Benzhexol.* Comparison of the injected dose with the concentration tested on guinea-pig ileum (Table 4) shows that the estimations of substance P could not have been influenced by this drug.

*Substance P.* The dose used is one which protects against convulsions due to strychnine or picrotoxin (Zetler, 1959). Since the injection of substance P did not influence its content in the brain, it is possible that it was inactivated either during

the absorption from the site of the injection, or after reaching the brain. The other possibility, that substance P did not pass the brain-blood barrier, can be excluded by the observation of Stern & Milin (1959), who found increased substance P in brain after its intravenous injection, and by the similar results of Serafimov & Stern (1958) with intraperitoneal injection. On the other hand there are reports that substance P is inactivated before reaching the brain if it is given intravenously (Lechner & Lembeck, 1958).

Conflicting results of various authors on the changes of substance P concentration in brain after treatment with the drugs used in these experiments can be seen in Table 5. One explanation for the disagreement is the interference produced by nucleotides (Laszlo, 1963). The effects of drugs might be due to changes in the nucleotides of brain, or the effects of drugs on substance P might be obscured by the presence of nucleotides in the extracts (Laszlo, 1962).

TABLE 5

EFFECT OF TREATMENT ON BRAIN CONCENTRATION OF SUBSTANCE P

0=No effect; +=increased substance P; −=diminished substance P

| Treatment | Species | Effect | Reference |
|---|---|---|---|
| Chloroform | Mouse | − | Zetler & Ohnesorge (1957) |
| Chlorpromazine | Rat | 0 | Stern & Kocic-Mitrovic (1960) |
| Ether | Dog | 0 | Paasonen & Vogt (1956) |
| Insulin | Dog | 0 | |
| Lysergic acid diethylamide | Mouse | 0 | Zetler & Ohnesorge (1957) |
| Morphine | Mouse | + | |
| Reserpine | Rabbit | + | Stern & Kocic-Mitrovic (1959) |
| | Rat | + | |
| | Rat | + | Stern & Kocic-Mitrovic (1960) |
| | Dog | 0 | Paasonen & Vogt (1956) |
| Substance P | Rat | + | Serafimov & Stern (1958) |
| | Hare | + | Stern & Milin (1959) |

While this work was in progress, Cleugh, Gaddum, Holton & Leach (1961) described a method for the estimation of substance P on fowl rectal caecum, eliminating the effect of nucleotides by specific tachyphylaxis.

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