THE EXCRETION OF SYMPATHOMIMETIC AMINES IN URINE

being

studies on urinary adrenaline and noradrenaline with reference to their extraction with Amberlite resin IRC 50, their estimations fluorimetrically and their excretion in certain normal and abnormal urine.

by

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INTRODUCTION

Holtz, Credner and Knoneberg (1947) demonstrated the presence in human urine, of what they termed "uro-sympathin," equivalent in amount to 100-150 μg/day of adrenaline or noradrenaline. Uro-sympathin was considered to be a mixture of adrenaline, noradrenaline and dopamine.[+] Holtz and two colleagues (1950), and Kroneberg and Schümann (1950) considered that adrenaline and noradrenaline were entirely or principally the effective pressor substances in the urine of human, and at least of some animals. Part of the uro-sympathin was found to be present in a form from which the free amines could be released by heating the amines at pH_2.

In 1951, von Euler, Hamberg and Hellner, using a chromatographic tachnique, found an extract of acid hydrolysed human urine to contain:

(a) noradrenaline;
(b) adrenaline;
(c) dopamine (0.1 to 0.2 mg/day, mainly in the free form).

In the same year, von Euler and Hellner (1951) reported/

[+] β (3:4 Dihydroxyphenyl)ethylamine (hydroxytyramine), often just "dopamine" for short.

reported the amounts of daily urinary sympathin excretion of 20 normal young adults to be 29 µg (SD 12.3) for noradrenaline and 11.5 µg (SD 6) for adrenaline, in terms of the hydrochlorides. The percentage of the methylated amine varied from some 9% to 45%.

Burn (1953) who estimated the total urinary sympathins in man in terms of noradrenaline, found that the daily sympathin excretion in urine increased with age; being low, with a mean of 18 µg/day, in children of 3 months to 10 years, rising to 80 µg SD 38.6 for a mean age of 50 years (SD 63 - 7 cases). The figures, however, showed considerable variations between individuals and between different days in the same individual. Some 50% of the amines were in a biologically inactive form.

A marked elevation of the uro-sympathin output in cases of pheochromocytoma was first noted by Engel and von Euler (1950), and the determination of the urinary sympathin level is now considered to be the most satisfactory dragnostic method for this condition (Goldenberg, 1951).

Two known major sources of urinary sympathins in the body were the adrenal medulla and the adrenergic nerves. From the results of separate determinations of noradrenaline and adrenaline in the/

the uro-sympathin, von Euler (1953) concluded in his review that these amines appearing in the urine were in normal persons, derived chiefly from the adrenergic nerve mediators, and not from the suprarenals; because their proportion in urine approximated to that found in adrenergic nerves, namely, 3 to 5 of noradrenaline to 1 of adrenaline. Consequently, an increase in the proportion of adrenaline in the uro-sympathin would indicate the stimulation of the adrenal medulla in which the adrenaline constitutes some 80% of the sympathins in adult humans (Shepherd and West, 1951). On the other hand, elevation of the uro-sympathin output with little or no change in the proportion of adrenaline and noradrenaline would indicate increased activity of the adrenergic nerves. On the basis of such an argument, von Euler and Hellner (1952) concluded that since in heavy muscular work, the urinary sympathin showed an increase, without a marked alteration in the noradrenaline-adrenaline proportion, the increase was to be related to the increased activity of the adrenergic nerves and not to a release of the adrenal medullary hormones.

It is evident from the previous work that the estimation of adrenaline and noradrenaline contents of the urine provides some measures for assessing their release in the body.

For/

For the measurement of the sympathin release in the body, the direct determination of these amines in the blood is the more logical procedure. But there is as yet, no agreement as to a proper technique for it. As a result, the blood sympathin figures obtained by different techniques differ much from one another. Moreover, the concentration of these sympathins in the blood is variable and it depends also on the emotional state of the subject at the moment of blood collection. Whereas a 24-hour sample of urine can equalize all these momentary variations. It was hoped that urinary sympathin estimation could circumvent all the difficulties mentioned; and by determining the total amount produced in 24 hours, an indication could be obtained as to the production of the sympathins in the body.

In the present work, the release of sympathin into the blood stream of the rat, as reflected by their output in the urine has been studied under various circumstances. In addition, some observations have also been made on the urinary sympathin concentrations of certain normal and pathological human urine.

This study necessitated the preliminary investigation of methods of collection, extraction and estimation, and the development of a suitable analytical technique.

METHODS.

Reagents.

(-)-noradrenaline-(+)-bitartrate ("Levophed," Bayer).

(-)-adrenaline (Burroughs Wellcome). This is a synthetic product and is free from noradrenaline.

Stock solutions (10^{-3}) with reference to the base, of each of these amines were prepared in 0.01N HCl at 5°C. Changes in their activities were not detectable even after 3 months of preparation; but as a routine, they were discarded when two months old.

(-)-ascorbic acid B.P. (Roche).

Acetone (A.R. quality).

Cation-exchange resin, Amberlite IRC 50 (H) (Rohn and Hans, supplied by British Drug Houses).

Ethanol "absolute" alcohol, refluxed with 5 g of NaOH (A.R.)/litre for 4 hours, and then doubly redistilled.

Heparin technical (Boots). 30 units/mg. Requisite amount, dissolved in 0.9% w/v sodium chloride just before use.

Hexamethonium bromide (May and Baker).

Mepyramine maleate, B.P. (Anthisan, May and Baker).

Other drugs. Unless the contrary is indicated, they were all of A.R. quality.

Water. Deionized water, obtained by passing tap water successively through beds of a cation exchanger Amberlite IR 120 (H), and an anion exchanger Amberlite IRA 400 (OH). The purity of the water was controlled by conductivity measurements.

Methods of estimation of the sympathin.

Fluorimetric

A modification of the fluorimetric method described by Lund (1949, 1950) has been used. The adrenaline or noradrenaline is oxidized at pH 6-7 to the corresponding "chrome" derivatives, adrenochrome or noradrenochrome, by shaking the solution with manganese dioxide. Under the influence of a strong alkali, the "chrome" undergoes intra-molecular rearrangement to yield adrenolutine and noradrenolutine respectively. These compounds show a greenish fluorescence in ultra violet light. The lutines are however, rapidly oxidized further by oxygen in the solution, with the loss of fluorescence intensity. In the presence of a reducing agent, such as ascorbic acid, the oxidation is avoided or at least considerably slowed down, thus permitting the measurement of the intensity of the fluorescence of the solution. It will be observed that the method represents a controlled production of the fluorescence which Oscar Loew (1918) first noted when an adrenaline solution was treated with a strong alkali, and then exposed to ultra violet light. Gaddum and Schild (1934) confirmed the great/

great sensitiveness of this reaction, a positive result being attainable with an adrenaline concentration of 10^{-8}. They found that the development of the fluorescence depended not only on a strongly alkaline reaction, but also on the presence of the dissolved oxygen. They observed that the intensity of fluorescence developed rapidly to its maximum, and also very rapidly decreased. The rapidity of development and fading made difficult the application of the reaction for quantitative measurements.

The detailed technique of the modified Lund's method is as follows:

In a 15 ml centrifuge tube, an aliquot of the test solution (< 1 ml) was diluted to 4 ml with 0.4% w/v $NaH_2PO_4 2H_2O$. One ml of 1.0% w/v $Na_2HPO_4 2H_2O$ was added, the solutions mixed and the pH checked by the use of Johnson and Johnson's narrow range indicator paper 5267. The pH should lie between 6.4 and 6.7. To the mixtures was then added approximately 50 mg manganese dioxide+, the tube stoppered and shaken for 30-35 seconds. After centrifuging/

+This substance was purified before use. About 250 g of MnO_2 were treated twice for 0.5 hour and overnight with 1 litre of approximately 2N acetic acid, with intermittent shaking. The acid was decanted off and the MnO_2 washed with distilled/

centrifuging at 3,500 r.p.m. for 30-35 seconds, the supernatent liquid was filtered through a double thickness of Whatman No. 41 filter paper (4.25 cm). One ml samples of the filtrate were transferred to each of two fluorimeter cuvettes. To one sample was added 0.02 ml of 0.5% w/v ascorbic acid (freshly prepared each day). After mixing by inversion twice, 0.2 ml of NaOH solution (20 g/100 ml water)[+] was added, and the solution mixed by inversion of the tube six times. Readings of the fluorescence intensity were taken at intervals of 30 seconds after the addition of the NaOH. To the other sample was added 0.2 ml of the NaOH solution, and the tube inverted/

distilled water by decantation until the supernatent liquid appeared ~~less~~ than pH4 to universal paper indicator. After filtration on a Buchner funnel, the MnO_2 was partially dried in an oven, and then heated in small lots in an evaporating porcelain basin over a Meker burner, with intermittent stirring until reduced to a fine powder, with a blue-black appearance instead of the former brownish-black colour. Usually, some 2-3 hours of heating were required. This intense heating was necessary since in its absence, the MnO_2 frequently gave rise to troublesome dark-brown colloidal solutions in the course of the amine determination. This was possibly due to the presence of some hydrated form of the oxide (Mellor, 1932).

[+]Stock solution, prepared and stored in a polythene bottle with a rubber stopper.

inverted six times. After 20 minutes, during which time complete destruction of any fluorescence due to adrenaline and noradrenaline would have taken place, 0.02 ml of the 0.5% ascorbic acid solution was added, the solution mixed by inversion twice, and the fluorescence intensity measured. This gave the "blank" value for the determination.

For the measurement of the fluorescence intensity, a Farrand Fluorimeter (Farrand Optical Co., Inc. New York) has been used, the primary filter being Wood's glass, transmitting the 3650 A^0 band of the mercury vapour lamp; and the secondary filter being Ilford No.625. This instrument has the advantage of allowing fluorescence measurements to be carried out on 1 ml of fluid.

This modification of the original Lund's method, introduced by Crawford in this department, lies essentially in the separate addition of the ascorbic acid and the NaOH solutions to the filtrate, instead of a mixture of ascorbic acid and NaOH, as suggested by Lund. In this way, the necessity for repeated preparations of the ascorbic-NaOH mixture in the course of a number of determinations, is avoided. A further modification lies in the amounts of ascorbic acid and of NaOH added. The amount of ascorbic acid is halved; while that of NaOH doubled the corresponding quantities used by Lund. As a result/

result, the reading for the intensity of fluorescence from a given amount of noradrenaline was increased by almost 100%, to become almost identical with that given by a like amount of adrenaline, as per Table 1 (p.64). Using the ascorbic acid-NaOH mixture advocated by Lund, noradrenaline under the condition of the present method of measurement, yields a fluorescence intensity of about half that of an equal amount of adrenaline. Besides, it has been Crawford's experience with using Lund's ascorbic acid-NaOH mixture, that the reading given by noradrenaline tended to vary quite considerably.

When the original Lund's ascorbic-NaOH mixture is used, the fluorescence intensity climbs to a maximum, and is not immediate as/ported by Lund, and remains constant for several minutes. An increase of NaOH concentration with a decrease of that of the ascorbic acid has the disadvantage that after the fluorescence has reached its maximum, it decreases rapidly. However, taking readings at 30 second intervals after the addition of the NaOH ensures that the maximum will not be missed. In the case of adrenaline, this maximum of fluorescence intensity is reached within 1-3 minutes, higher concentrations requiring the longer time; while with noradrenaline, 3-4 minutes are the usual time required. In spite

of/

of this difference, the maximum obtained for a mixture of adrenaline and noradrenaline were correct within the limits of experimental errors.

Crawford has observed that a linear relationship exists between the fluorescence intensities and the concentrations of adrenaline or noradrenaline solutions from 0.01 to 0.2 µg/ml. From experiments with pure solutions of adrenaline and of noradrenaline, he has determined that the standard deviation of a single estimate in the range of concentrations already mentioned to be 10% for adrenaline (27 determinations) and 9% for noradrenaline (27 determinations). The lower limit of the concentration range was that, which, in pure solutions, yield a reading about double that of the "blank". The limit of detection, i.e. the lowest concentration of adrenaline or noradrenaline, giving a reading definitely greater than the blank value, was estimated to be about one quarter of this.

The close similarity of the fluorescent intensities yielded by equal amounts of adrenaline and noradrenaline under the present experimental conditions had the advantage that the total sympathin in a mixture of these two amines could be determined in terms of either adrenaline or noradrenaline, the result being the sum of the concentrations of the

two/

two components, irrespective of their proportions in the mixture.

For routine assays, the instrument was calibrated, using standard adrenaline or noradrenaline solutions. Stock solutions, 10^{-3} in terms of the base,[+] were prepared in 0.01N HCl, and stored at 5°C. Such solutions remained stable for at least two months. Standard solutions for fluorimetry, (1 µg/ml) were prepared fresh, each day, from the stock solutions by dilution in 0.4%(w/v) NaH_2PO_4 $2H_2O$. Usually, the instrument was calibrated, using duplicate samples of 0.4 µg adrenaline or noradrenaline which would give a concentration of 0.08 µg/ml in the sample, to which the ascorbic acid and NaOH solutions were added.

As a stable fluorescent solution for the presetting, and time to time adjustment of the sensitivity of the fluorimeter, a solution of quinidine sulphate (15 µg %) in 0.01 N H_2SO_4 proved satisfactory.

In the analysis of urine extracts by this technique, it was necessary to ensure that the extracts did not contain any substance which could interfere/

[+](-) adrenaline base, (Burroughs Wellcome), synthetic; and (-) noradrenaline (+)-bitartrate (Bayer) were used for the preparation of these solutions.

interfere with the development of the fluorescent intensity of the amines, by exerting either a quenching or masking action or a potentiation. This was tested by the addition to a second aliquot of the test extract, of a known amount of the standard adrenaline or noradrenaline solution, usually containing 0.2 µg of the amine. The fluorescence of this muxture was developed and the intensity determined. If the additional fluorescence was less than 90% or greater than 110% of the expected value, this test was repeated. Should the two observations with the added amine agree, the fluorescence of a duplicate sample of the test solution was determined. If the duplicates agreed, then the estimated adrenaline or noradrenaline of the extract was corrected to allow for the observed masking or potentiation.

It should be remarked at this stage, that only very infrequently, such corrections were found necessary.

Dopamine produces a fluorescent substance on similar treatment with MnO_2. The intensity of the fluorescence is about 0.6% of that of adrenaline or noradrenaline. A mixture of noradrenaline and dopamine in 20-fold the noradrenaline concentration results in a 15% over estimate of the noradrenaline concentration if it is assumed that only noradrenaline is present.

Fluorimetric/

present.

Fluorimetric examination of the extract from the relevant portion of paper chromatogram of rat's urine failed to show the presence of dopamine (p.164) Fluorimetric determination of the urinary sympathin would not therefore be in error from this source.

Biological Assays

In the present work, biological assays were used mainly to serve as controls for the sympathin estimations, obtained by the fluorimetric technique.

Rat's blood pressure preparation.

A rat (200-250 g) of either sex was anaesthetized with urethane (175 mg or 0.7 ml of a 25 g/100 ml aqueous solution per 100 g body weight, subcutaneously), and tied in a supine position to a board. Through a midline incision in the neck, the trachea was cannulated and one carotid artery dissected ready for cannulation. The vagus nerve of the same side was cut; while a loose ligature was put around the opposite vagus which was cut after the cannulation of the carotid artery. A venous cannula was tied into a femoral vein. Its distal end was joined by a short piece of rubber tubing to a 1 ml burette fitted, just above the tap, with a side arm which was connected/

connected by means of a piece of rubber tubing with a spring clip to a reservoir containing normal saline (0.9%). The burette can thus be filled without detaching it. Heparin (200 unit/100 g rat) in 0.5 ml of normal saline is injected through the venous cannula and washed in with saline.

The arterial cannula is then inserted and tied. It is connected to a Condon's manometer (1953), normal saline being used as the liquid bridge.

The remaining vagus and the femoral nerves are cut.

Mepyramine maleate, B.P. 80 µg per rat, is given subcutaneously over the abdomen, and the rat is left for 20-30 minutes before an assay is made.

The rat's blood pressure preparation (Crawford and Outschoorn, 1951) provides a suitable biological method for the assay of noradrenaline and adrenaline. With noradrenaline, a dose of 1 ng usually gives a response notably different from that given by the control saline injection, when the rat has been pre-treated with hexamethoniam (10 mg/kg body weight, intravenously). A difference of 1-2 ng in dosage is clearly distinguishable. With adrenaline the threshold dose in the hexamethonium treated preparation is in the region of 30 ng.

In/

In the absence of hexamethonium, the threshold doses for noradrenaline and adrenaline are about 20 and 50 ng respectively. With doses near the threshold level, the inequality of the responses to adrenaline and noradrenaline appear to be at a minimum. Consequently, assays of extracts for sympathin (adrenaline and noradrenaline mixture) were carried out on rats, untreated with hexamethonium, using doses which yielded responses just in excess of the threshold. Noradrenaline being the major constituent in the sympathin in normal urine, was used as standard. In the case of estimation of adrenaline and noradrenaline in eluates from paper chromatogram, hexamethonium-treated rats were used, and assays were done against standard adrenaline and noradrenaline respectively.

A dose was given every three minutes, the dose being injected from a 1 ml tuberculin syringe through the rubber connection to the venous cannula, and washed in with sufficient saline from the burette to give a total volume of 0.3 ml fluid administered at one time. Doses of standard and test were alternated, the response to a dose of test solution being bracketed by doses of standard giving a just visibly lower and a just visibly higher response. If sufficient amount of extract was available the response

to/

to this was, in addition, matched with a dose of standard.

The result of the assay was calculated from the matching dose of standard, or from the mean of the two bracketing doses of the standard, the range of the estimate reported in the tables of results being calculated from the upper and lower bracketing doses of the standard.

It seems likely that the major interfering substance in the assay of the urinary extract on the rat's blood pressure preparation would be histamine which would tend to mask the pressor effects, as a result of its depressor effect. The effect of this substance was blocked by the prior injection of mepyramine. The small dosage levels adopted in the present work would, it is felt, tend to minimize the amount of other interfering substances, likely to be introduced with each dose of urinary extract, and so reduce the extent of the error in the assay.

Rat's isolated uterus preparation.

Assays using the rat's isolated uterus were carried out in principle, as described by Gaddum and Lembeck (1949). The test depends on the inhibitory effect of adrenaline and noradrenaline on the uterine contraction, induced by a constant dose of carbachol, at fixed intervals. With the uterus suspended/

suspended in a 2 ml isolated organ bath, (29° to 31°C), a dose of adrenaline as small as 0.5 ng was detectable, and the effect of a difference of 1 ng between doses was found to be easily distinguishable. The equiactive dose ratio, noradrenaline/adrenaline is 75-300 (Gaddum, Peart and Vogt,1949).

5-hydroxytryptamine caused the contraction of isolated rat's uterus, the oestrous uterus being considerably more sensitive than the non-oestrous one (Amin, Crawford and Gaddum, 1954). For adrenaline assays, routine use of a non-oestrous uterus would help to reduce errors should any 5-hydroxytryptamine be also present in the test solution. It is however, probably wise to pre-treat the uterine preparation before the assay, with lysergic acid diethylamide (LSD), 1 μg/ml of bath concentration for 10 minutes. This would abolish the 5-hydroxytryptamine effect entirely. The slight anti-adrenaline effect of LSD would be exerted on both the test and standard solutions, and so would be of no consequence, apart from making the uterine preparation somewhat less sensitive.

The rat uterus preparation was invaluable for the assay of a test solution of rather low concentration of adrenaline. In the present work, the concentration of adrenaline in the test solution was/

was sufficiently high to permit assay on rat's blood pressure, and rat's uterus preparation was used only when specially indicated.

Extraction of sympathins from urine

For an accurate assay of the sympathin content of urine, it is first necessary to obtain the sympathins in a sufficiently concentrated solution, and reasonably free from interfering substances.

A method of extraction devised by von Euler (1948) and subsequently adopted for use on urine (Euler and Luft, 1949; Euler and Hellner, 1951), has been used quite extensively in the past. It depends on the adsorption of the sympathins on aluminium hydroxide formed in the urine by addition of aluminium sulphate solution, and adjustment of the pH to 7.5 with NaOH. The precipitated aluminium hydroxide, containing the adsorbed sympathins is filtered or centrifuged off, and dissolved in dilute sulphuric acid. The pH is then adjusted to 3.5, and the salts removed by precipitation with a mixture of acetone and ethanol (50:50 mixture by volume). This almost salt-free supernatent liquid is evaporated to just dryness at reduced pressure. The dry extract/

extract can be taken up in a suitable volume of saline. Such extracts appear to be sufficiently pure pharmacologically to permit their assay on biological preparations. von Euler claimed a recovery per cent of 70-75, which has been verified in this work. Extracts prepared by this method were invariably pigmented, and proved unsuitable for the fluorimetric assay for sympathins, as a considerable masking of the fluorescence of added adrenaline or noradrenaline was noted, especially when the urine had been subjected to acid hydrolysis (see p. 46).

An extract suitable for use fluorimetrically is obtained if the urinary amines are adsorbed on a column of weak cation ion-exchange resin, Amberlite IRC 50, buffered at a pH of 7 to 7.5. Elution with sulphuric acid, followed by pH adjustment and salt removal as in von Euler's method, gives a clear, very faintly coloured extract, suitable for assay both fluorimetrically and biologically. This technique gives a recovery of about 80%. The investigation into the possibility of using the resin IRC 50 for the purpose of extraction of urinary sympathin was started by Crawford, and with his guidance, the method was standardized in the present work.

The credit of the first use of ionic exchange for the isolation of adrenaline went to Whitehorn (1923) who used permutit for the purpose. Bergström, von/

von Euler and Hamberg (1949, 1950) investigated the use of cation exchangers for the isolation of catechol bases from the crude extracts of the adrenals. In 1951, Bergström and Hansoon employed IRC 50 resin for the adsorption of adrenaline. They found that the quantitative recovery of adrenaline from the resin was almost complete at 2 mg level, using pure drug in water solution, mixed with citrated human plasma, and estimating directly on the effluents, without evaporating them to dryness.

Both the techniques have been used in the present work, although von Euler's method was employed only on relatively few occasions, mainly with the purpose of obtaining a few basic figures for comparison with the results obtained by IRC 50 resin procedure.

Extraction method of von Euler

To an aliquot (10 ml rat urine; 50-100 ml human urine) of the 24 hour urine sample was added aluminium sulphate (Analar 20% w/v) solution in the proportion of 4 ml of the solution to 100 ml of the urine. The pH was then adjusted to 7.5 (glass electrode) by careful addition of 0.5N NaOH, dropwise and with constant stirring. With the human urine/

urine samples, 2N NaOH might be used to bring the pH to about 7, the final adjustment to 7.5 being made with 0.5 N NaOH. This served to keep the volume down to reasonable levels, and also to speed up the neutralization process. After standing for 2-3 minutes, the pH was checked and readjusted to 7.5 if necessary. The suspension of precipitated aluminium hydroxide was centrifuged at 3,500 r.p.m. for 20 minutes. The clear supernatent liquid was decanted off, and the sediment washed twice by stirring up with 0.9% NaCl, which was removed by centrifuging and subsequent decantation. In washing the sediment, normal saline invariably gave a clear supernatent liquid while distilled water tended to give a slightly cloudy one. The dirty-white or slightly-brownish sediment was then dissolved in a minimal amount of 2N H_2SO_4. The pH of this solution, usually 1 to 1.5, was adjusted to 3.5 with 2N NaOH. Some precipitation was noted when the pH reached about 2.5.

Removal of salts:

To the solution at pH 3.5 were added 4 volumes of a 50:50 mixture (by volume) of acetone (A.R.) and ethanol (refluxed with NaOH and doubly redistilled). Precipitation was allowed to continue at 5°C for 2 to 3 hours, or preferably overnight.

Removal/

Removal of the precipitated salts was effected by either filtration or centrifugation. For filtration, a sintered glass funnel (Pyrex SF 3N 3) of 200 ml capacity was used. Transfer of the last traces of the sympathins by washing of the precipitate was carried out with small volumes of the acetone-ethanol mixture already referred to.

For the removal of the precipitated salts by centrifugation, the suspension of salts was transferred to a hard glass centrifuge tube of suitable size, the transfer being completed by washing twice with 2 ml portions of the acetone-ethanol mixture. The suspension was centrifuged at 3,500 r.p.m. for 20 minutes. The clear supernatent was decanted off, and the sediment stirred up with 5 ml of acetone-ethanol mixture and re-centrifuged, the supernatent being added to the original.

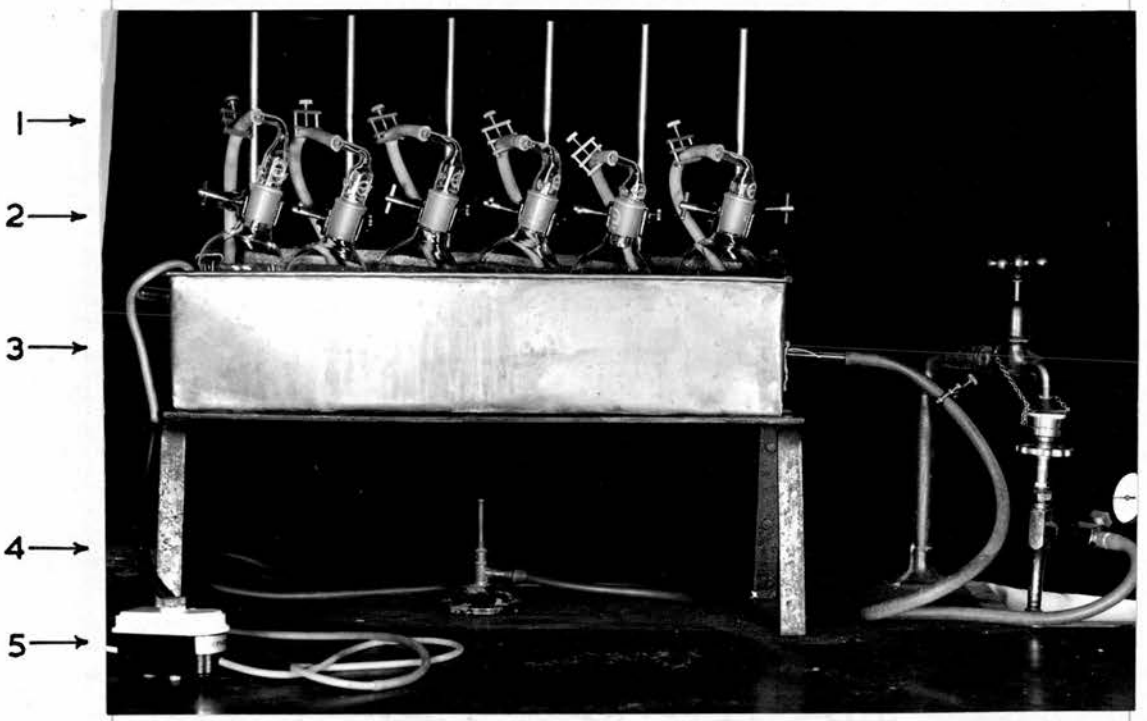
Both methods of salt removal proved equally efficient; but from the practical point of view, centrifugation required less time. Against this, however, there is the ever present fear of centrifuge-tube breaking, with the resultant loss of the sample.

Removal of the solvent:

After the removal of the salts, the extract was transferred to a 150 ml R.B.flask fitted with a standard joint and evaporated just to dryness, under reduced/

Figure I

Apparatus for mass evaporation.



1. Screw clips, for the regulation of suction pressure.
2. Pyrex evaporating flasks, containing material for evaporating.
3. Brass water-bath.
4. Microburner.
5. Aerator for agitating the water in the bath.
6. Water suction pump, with pressure gauge. (Thermometer, not shown).

reduced pressure (water pump) and at 33-35° (the temperature of the water-bath). During the process of evaporation, care was exercised to see that the level of the extracts in the flasks did not fall below that of the water in the bath, as it was found that there was a decrease of sympathin activity if this point was not observed. This fact was also reported by Serlin and Goldenberg (1953), as being due to etherization of noradrenaline in acid ethanol.

When a number of samples was to be evaporated, much time and labour could be saved by the use of the manifold evaporator (shown in figure I) in which up to six samples could be evaporated at one and the same time. One drawback to the use of this evaporation system was the danger of bubbling and the subsequent loss of extracts by being sucked away. While it was definitely safer to drive off all the acetone and most of the ethanol, at the beginning, by manual shaking over the water-bath, the end-point being indicated by the cessation of bubbling, this could be avoided by a careful regulation of the suction force at the initial stages, provided that the flasks were not more than three-fourths full.

Assays:

The residue from the evaporation was dissolved in normal saline, and the pH of the solution was adjusted/

adjusted to about 6 with solid sodium bicarbonate (A.R.) just prior to the actual assay, on the rat's blood pressure preparation.

The extraction of urinary sympathins by adsorption on cation-ionic exchange resin, Amberlite IRC 50.

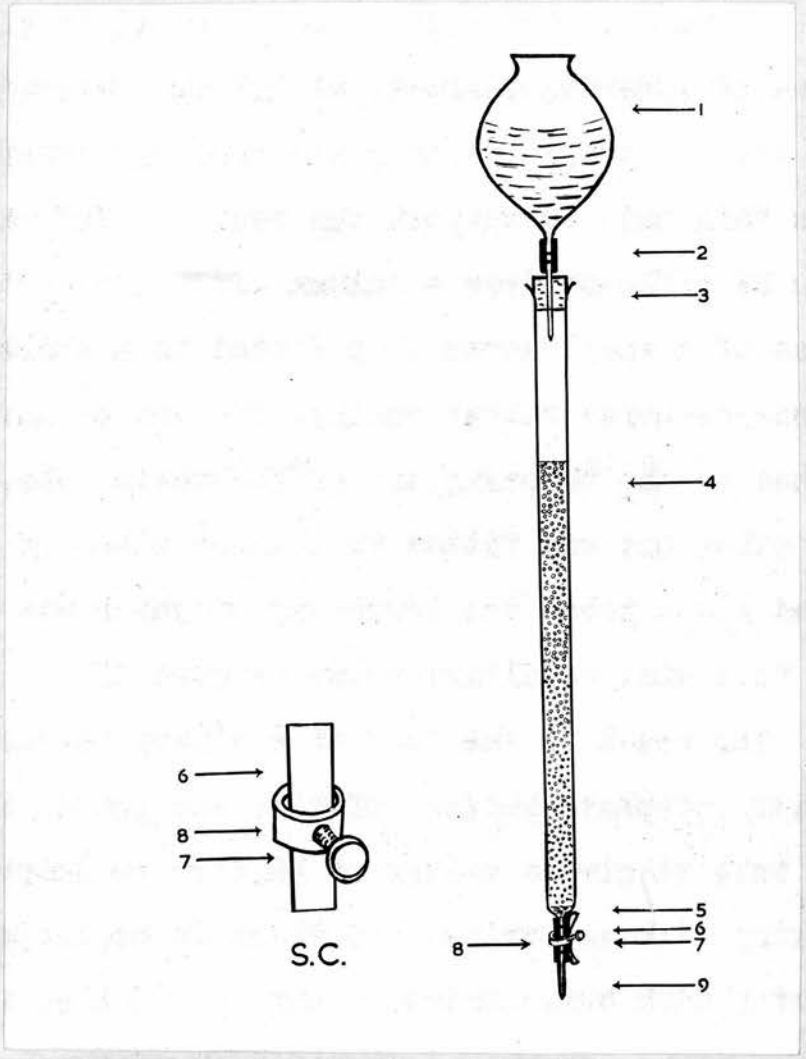
Preparation of the resin columns:

The resin as supplied was in bead form (16-50 mesh) and in the H^+ state. It was first converted to the sodium form by treatment with 2N NaOH, after washing with de-ionized water.[+] One volume of 2N NaOH was added to one volume of the wet resin, and the mixture stirred intermittently for 15 minutes. The supernatent liquid was decanted off, and 2 volumes of 2N NaOH added to the resin. The mixture was stirred, and left overnight. The supernatent liquid which should show a definitely alkaline reaction, was decanted off, and the resin washed with copious amounts of de-ionized water until the pH of the washings was about 7 (as tested with Universal indicator paper). The resin was then/

[+]De-ionized water was prepared by passing tap water successively through a column of the cation exchange resin, Amberlite IR 120 (H) and a column of anion exchange resin IR 400 (OH). The purity of the effluent was assessed arbitrarily by conductivity measurements in a cell inserted in the course of the outlet tube from the last resin column.

Figure II

IRC 50 Resin Column



1. Mercury levelling bulb, 250 ml capacity.
2. Rubber connection.
3. Rubber stopper, perforated.
4. IRC 50 resin in glass tube, 1.1 c.m. internal bore (much more closely packed than drawn).
5. Glass wool plug.
6. Metal strip to press the rubber tubing.
7. Screw to press the metal strip.
8. Metal ring.
9. Glass tubing with capillary exit.

S.C. - screw clip.

then stored under 0.2M sodium phosphate buffer pH 6.5 for at least two days before use.

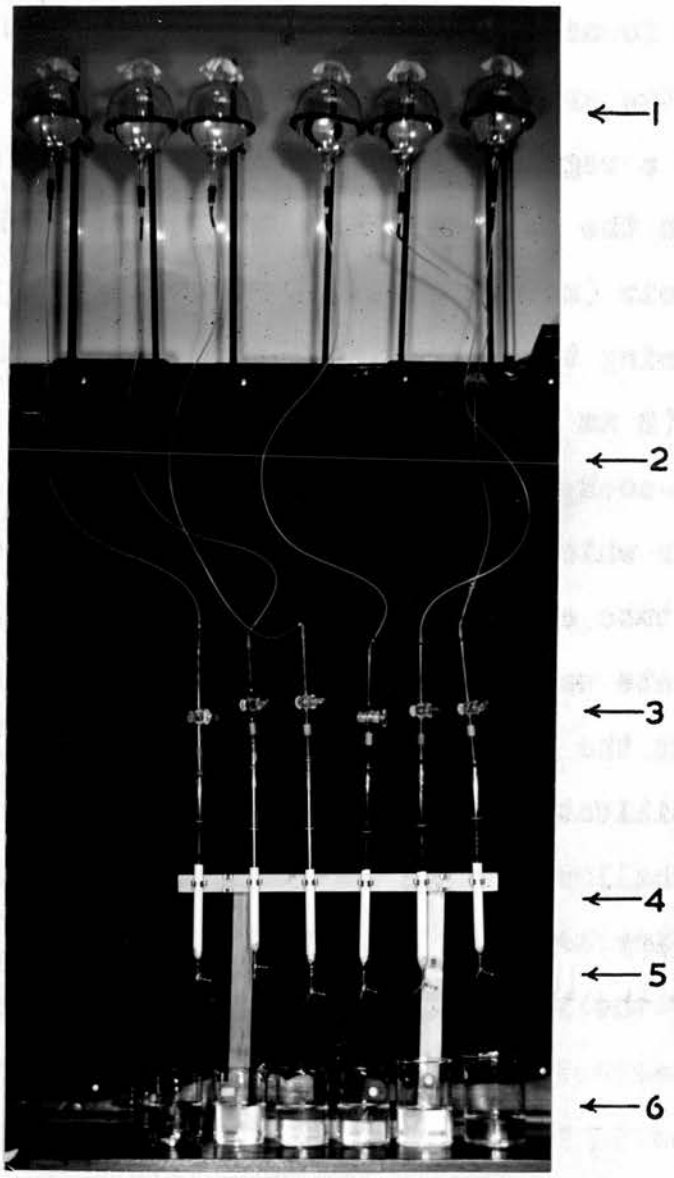
Columns of the resin were prepared in glass tubes of internal diameter of 1.1 cm, tapered at one end. A small wad of glass wool was inserted into this end, to support the resin. The rate of flow of effluent from a column was controlled by means of a small screw clip fitted to a short piece of narrow-bored rubber tubing, one end of which was fitted to the tapering end of the resin tube, while the other end was fitted to a short piece of narrow-bored glass tube, the lower end of which was drawn out to a wide capillary point (Figure II).

The resin in the form of a slurry in 0.2M sodium phosphate buffer, pH 6.5, was poured into the tube to give a column of 10.5 cm in height when working with rat urine, and 21 cm in height when working with human urine. Any air bubbles entrapped in the resin column were removed by stirring with a fine glass rod. Due to closer packing of the resin beads on the continuous passage of the buffering fluid, the heights of the resin columns became approximately 10 cm and 20 cm respectively. A 10 cm column required the equivalent of approximately 2 g of dry resin in the H^+ form.

The resin in the columns was then buffered to the/

Figure III

Apparatus for buffering the resin columns.

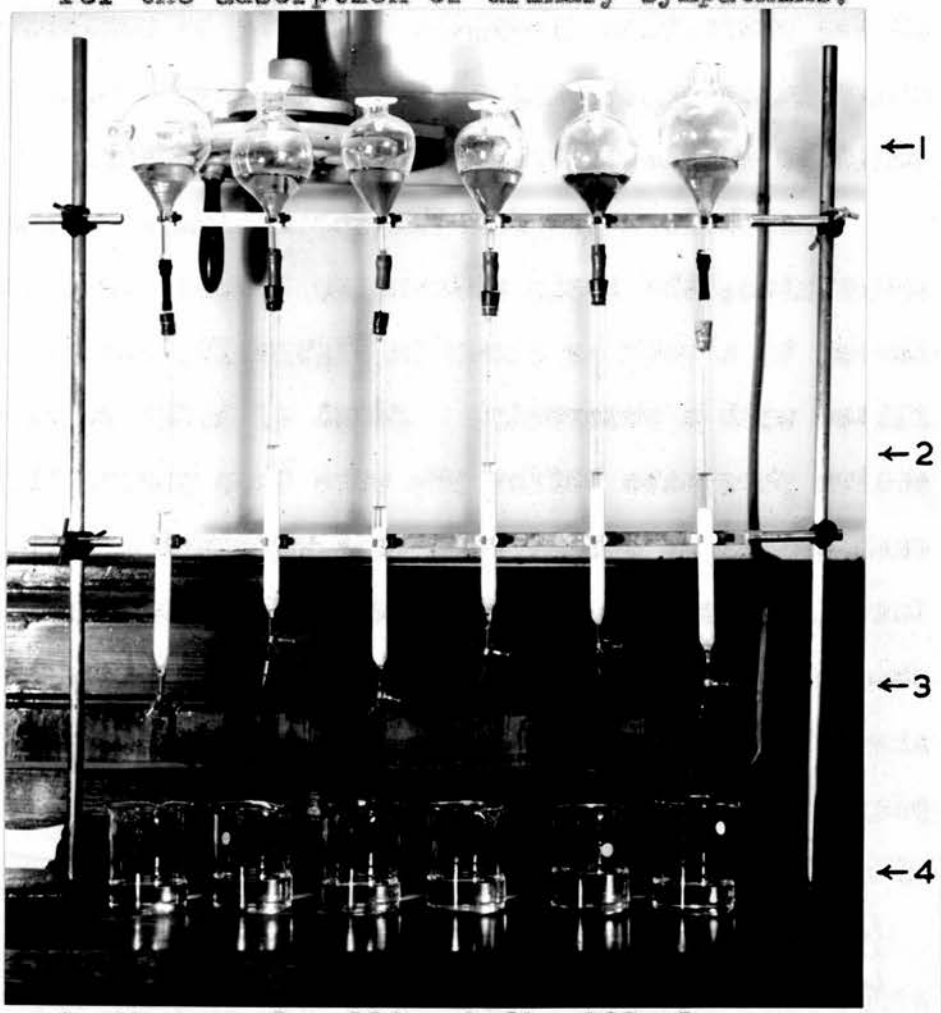


1. Reservoir, 400 ml capacity.
2. Polythene tube (narrow bore).
3. Stopcock, grooved as described in text.
4. Resin column.
5. Screw clips.
6. Beakers for waste materials.

the pH of approximately 7, by the passage firstly, of 300 ml of 0.2M sodium phosphate buffer 6.5 through each column, at a flow rate of 1.5 ml ± 0.5 ml/min. It was found convenient to carry out this process, using the apparatus shown in Figure III. It permitted a regular flow rate of the buffer solution through the columns. Each unit consisted of a reservoir (mercury levelling bulb of 400 ml capacity) containing the buffer solution, connected by narrow-bored (2 mm internal diameter) polythene tubing to a stop-cock, the other end of which carried a rubber stopper which fitted tightly into the top of the glass tube containing the resin. Control of the flow rate was maintained by the stop-cock, the screw clip at the lower end of the column being wide open. To facilitate the adjustment of the flow rate, two fine shallow grooves were filed in the stop-cock, extending some two-thirds of the way round from each end of the hole. These channels were such that they were deepest towards the hole, becoming more and more shallow in the opposite direction. By setting the reservoir at some height (100 cm) from the top of the columns, the drop in the fluid level in the reservoirs produced only negligible alterations in the flow rate through the columns. Once set up and the flow adjusted, no further attention was needed. When the buffer in the reservoir was exhausted, the

Figure IV

IRC 50 resin columns, mounted on the rack, for the adsorption of urinary sympathins.



1. Mercury levelling bulb, 200 ml capacity.
2. Resin column.
3. Flow rate regulator.
4. Beaker, for waste materials.

flow would automatically stop, while almost the original height of the buffer solution would remain over the top of the resin column. Thus, this stage of the extraction procedure could be conveniently started the last thing at night when the buffering would be completed by the following morning.

Just before use for the adsorption of urinary sympathins, the resin columns so treated were transferred to a rack as shown in Figure IV, and each fitted with a reservoir. 50 ml of 0.02M solution of sodium phosphate buffer pH 7 were then passed through each column at a flow rate of 2 ml/minute. The last 10 ml of the effluent were collected and the pH checked (glass electrode). Usually, this was about 7.5. 25 ml of de-ionized water were next passed through at the same flow rate. The last 5 ml of the effluent had a pH of 7.5-8.

The columns were now ready for the adsorption of urinary sympathin.

Adsorption of sympathins from urine, on the IRC 50 resin column.

To the urine sample (10 ml in the case of rat urine; 50-100 ml in the case of human urine) was/

was added ascorbic acid (1 mg/ml),[1] and the pH of the sample adjusted to about 6.5 (glass electrode) by the dropwise addition of NaOH (using 0.5N for rat urine, but 2N for the larger volumes with human urine samples) with constant stirring. The solution was then diluted[2] threefold (in terms of the original urine volume) by the addition of de-ionized water, and the pH finally adjusted to 7 with 0.5N NaOH.

The urine sample was then passed through a column of IRC 50 of suitable size, previously buffered as described above. In the case of 10 cm column used for rat urine samples, the flow rate was maintained as near 0.5 ml/minute as possible, and never allowed to exceed 0.75 ml/minute. With the 20 cm columns used for human urine samples, a flow rate of 1-1.5 ml/minute was maintained, care being taken that the upper value was not exceeded.

When all the urine sample had been passed in, each column was washed by passing through it, 25 ml of 0.02M sodium phosphate buffer pH7, followed by 25 ml of de-ionized water.

[1] and [2] The addition of ascorbic acid and the threefold dilution of the urine sample were considered by Crawford (unpublished yet) to be necessary for the maximum recovery of the sympathins. The ascorbic acid would tend to prevent oxidation of the sympathins in the protracted period of maintenance at pH7 during their adsorption on the resin. The dilution of the urine sample reduced the salt concentration to a level such that they did not interfere with the adsorption. The need for either or both these steps has not been investigated in the present work.

Elution of the adsorbed sympathins.

Elution of the adsorbed sympathins was carried out by the passage of, firstly, 2N H_2SO_4 through the resin columns at a flow rate of 0.3-0.5 ml/minute, the eluate being collected in 50 ml measuring cylinders, containing a drop of 2N H_2SO_4 to acidify the neutral solution initially issuing from the columns, and thus stabilizing the sympathins it contained. Preliminary experiments showed that 6 ml of the 2N acid (for a 10 cm column) and 13 ml of the same acid (for a 20 cm column) were required to complete the acidification of each resin column. As a routine, therefore, an appropriate volume of the 2N acid was allowed to pass through each column. When almost all this amount of the acid had passed through, the effluent was tested with a thin strip of Universal paper indicator to ensure that the effluent was indeed acidic. If not, usually the passage of an additional millilitre of the 2N acid would suffice. At this stage, 10 ml of 0.01 N H_2SO_4 were pipetted onto the top of the resin column, and the flow stopped for an hour. The flow was then restarted, and continued with 0.01 N H_2SO_4 until a total of 20 ml of the eluate (in the case of 10 cm column) and of 40 ml of the eluate (in the case of a 20 cm column) were collected./

collected. Omission of this stationary stage in the elution resulted in a decreased recovery of the sympathins, possibly due to the fact that sufficient time was not given then for the acid to diffuse into, and for the desorbed sympathins to diffuse out of the interior of the resin beads.

The pH of the eluate (1.5-2) was adjusted to 3 (glass electrode) with dropwise addition of 2N NaOH, and much stirring, and finally to 3.5 with 0.5 N NaOH.

Using the apparatus illustrated and described above, it is possible to cope with six urine samples simultaneously.

Assay of the sympathins in the eluate.

Where it was desired to carry out both biological and fluorimetric assays, and especially when further paper chromatographic separation of adrenaline from noradrenaline was also contemplated, the salts in the eluate were removed by treatment with 4 volumes of a 50:50 acetone-ethanol mixture (by volume), and the desalted extracts evaporated as already described (p.22).

For the estimation of total sympathins, the dry residue was taken up in 3 ml of 0.9% saline. A maximum of 0.5 ml was used as the aliquot volume for each/

each fluorimetric determination.

On occasions, separate determination of adrenaline and noradrenaline were required. This was carried out after the separation of the amines by paper chromatography, the mixture of the amines being extracted from the dried residue resulting from the evaporated salt-free eluates from the resin columns, by means of HCl-acidified ethanol, and this acid-ethanol extract of the amines applied to the paper (see p.36).

If only a total sympathin determination was required by the fluorimetric method, only partial removal of the salts from the eluate was necessary, prior to the evaporation by using only 2 volumes of the 50:50 acetone-ethanol mixture. Decreasing the volume in this way decreased the time required for evaporation, and the presence of the remaining extra salt content was not detrimental to the fluorimetric assay. Indeed, if the sympathin (adrenaline + noradrenaline) concentration of the eluate exceeded 0.5 µg/ml, direct assay by the fluorimetric method was possible.

Separate/

Separate determination of adrenaline and noradrenaline in the urine.

For the determination of adrenaline and noradrenaline, Bergström, von Euler and Hamberg (1950) used parallel assays on the cat's blood pressure and the hen's rectal caecum preparations; and from the results and a knowledge of the activity ratios of the two amines on the two preparations, they calculated the amounts of adrenaline and noradrenaline present. Doubts as to the accuracy of such a method, especially when one of the two amines was present in great excess, were expressed by Gaddum and Lembeck (1949) in their study of a similarly conceived method, depending on parallel assays, using the isolated rat's uterus and isolated rat's colon.

Colorimetric methods for the separate determination of adrenaline and noradrenaline in mixtures, without preliminary separation of the amines, have been described by von Euler and Hamberg (1950) and Schuler and Heinrich (1948, 1949 a and b). These methods, however, lack sensitivity, some 20-40 μg of the amines being required for a determination.

Goldenberg, Faber, Alston and Chargaff (1949) described a method in which the amines were separated by paper chromatography, the amount of each amine/

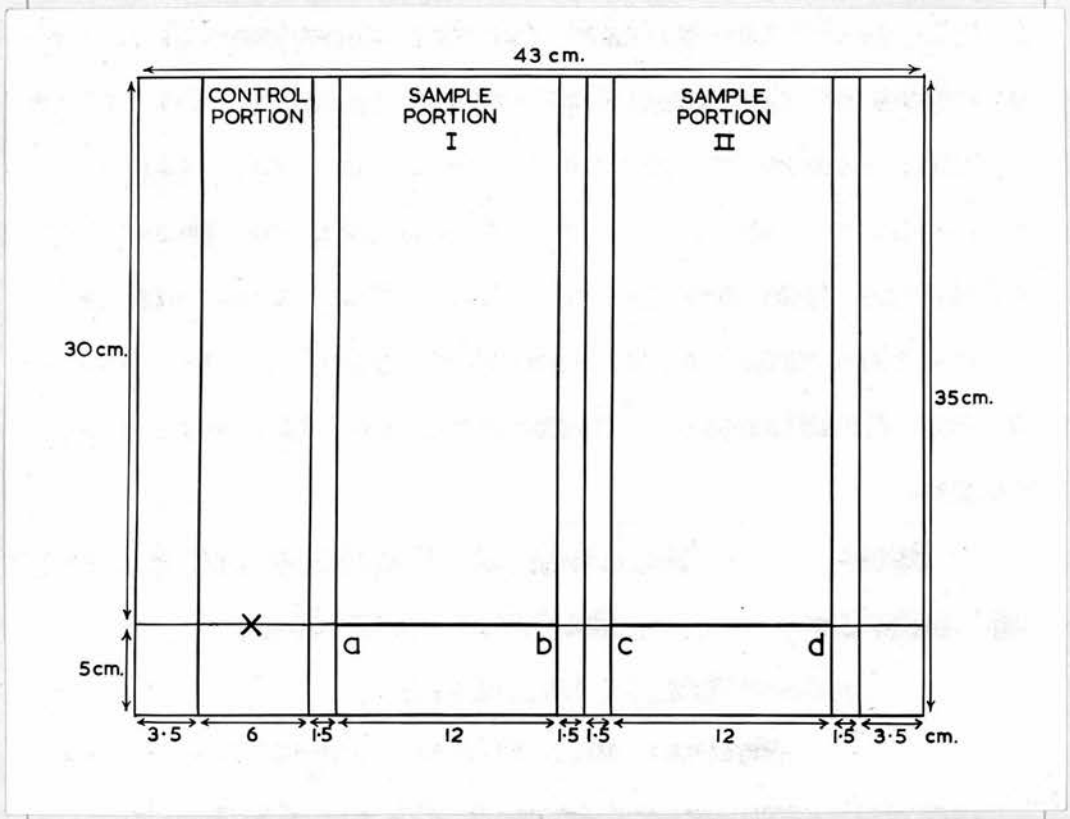
amine being then determined by planimetry of the Prussian blue spots formed on the chromatogram at the adrenaline and noradrenaline positions, as a result of spraying the paper with potassium ferricyanide, followed by ferric sulphate solution. The limit of sensitivity of this method was not less than 2 µg for each amine.

As there were only some 2 µg of total sympathin in the 24 hour sample of a rat's urine, methods for the separate determinations of the adrenaline and noradrenaline constituents that required larger quantities were at once excluded from consideration.

In the present series of experiments, extensive use was made of the paper chromatographic technique described by Crawford and Outschoorn (1951), as modified later by Crawford (unpublished yet: see Vogt 1952a). In this method, the amines were first separated by ascending paper chromatography in a suitable solvent and the separated amines eluted from the appropriate paper strips, and determined in the eluates. Using the rat's blood pressure preparation for the final assay, Crawford and Outschoorn claimed recoveries of 75-125% from pure solutions, and from plasma with quantities of adrenaline and noradrenaline, ranging from 0.25 µg to 20 µg, with either amine in twentyfold excess. With slight modifications/

Figure V

Mapping out a chromatograph paper, before cutting.



x a b c d - starting line

x - spot for applying standard control solution.

a b c d - along which test solutions were applied.

modifications, the technique has been used successfully by Vogt (1954) in her determination of the distribution of adrenaline and noradrenaline in the central nervous system. As originally carried out, the separation technique of Crawford and Outschoorn (1951) could not be used for the fluorimetric determination of the separated amines owing to the interference caused by the presence of ascorbic acid in the eluate, and of some material from the paper resulting from the use of SO_2. The technique has since been modified by Crawford (page 7) to permit of the fluorimetric determination of the separated amines.

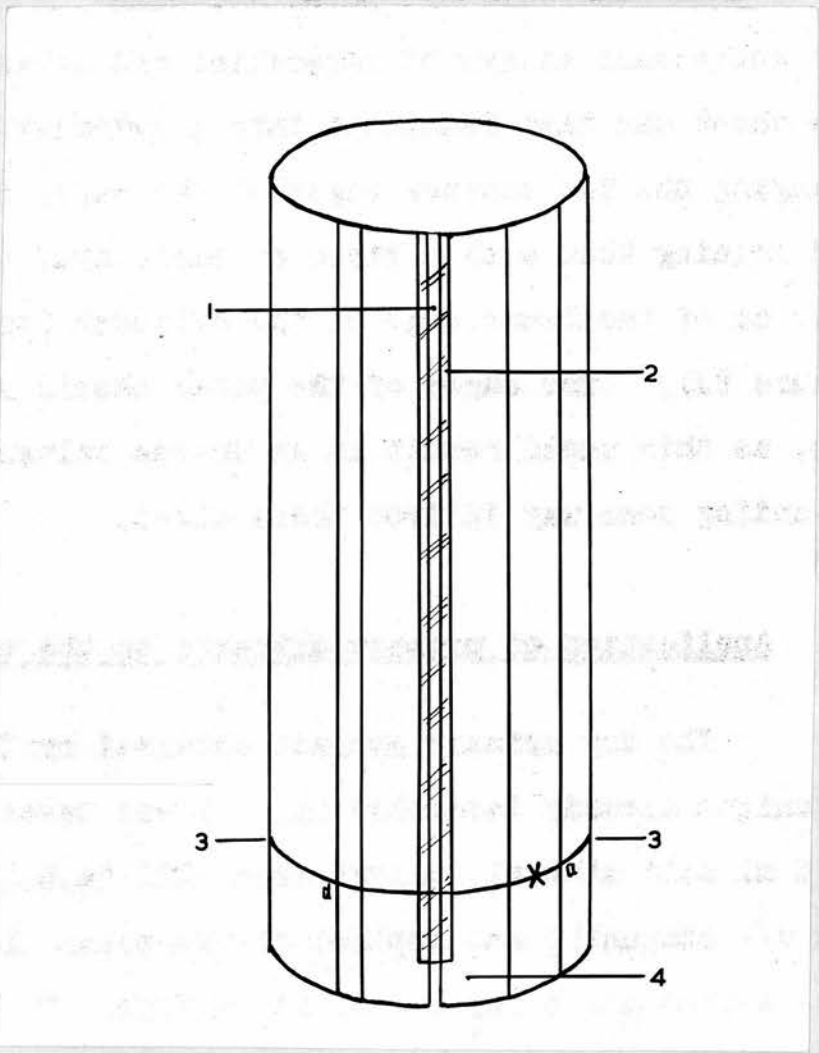
Paper chromatographic separation of adrenaline and noradrenaline in the urine extracts.

Preparation of the paper.

Whatman No.1 filter sheets "for chromatography" were washed with 0.01N HCl (A.R.) by descending chromatography for 12 hours. After drying at room temperature, each sheet was ruled off in pencil, as illustrated in Figure V, the longer side of the 43 cm x 35 cm rectangle being in the direction of the acid flow during the washing, and the right edge (as in the illustration) being towards the side of the original sheet that was dipped in the solvent trough. By so doing, it was hoped that at the end of/

Figure VI

Chromatographic paper cylinder.



1. The join, 1 mm apart (exaggerated in the drawing).
2. Sellotape.
3. Starting line.
4. Lower end of sellotape, leaving a space of 2 c.m.

x - spot for standard control solution.

a, d - respective outer ends of sample portions I and II.

of the washing period, any slowly moving contaminants on the paper would be located towards the left (as in illustration) of the sample portions where their presence would not interfere with the samples and subsequent assays of adrenaline and noradrenaline. The sheet was then fashioned into a cylinder by bringing the two shorter edges of the paper together and joining them with a piece of sellotape, to within 2 cm of the lower edge of the cylinder (see Figure VI). The edges of the paper should not overlap, as this would result in an uneven solvent flow extending some way in from these edges.

Application of urinary extracts to the paper.

The dry urinary extract obtained by 'IRC 50' technique already described (p.25) was taken up in 0.75 ml acid ethanol (0.1 ml conc. HCl (A.R.)/100ml 75% v/v ethanol), and applied to the paper along the line marked a - b (or c - d) as in Figure V, by means of a 0.2 ml pipette drawn out to a fine capillary point with a bevelled tip. In applying the solution to the paper, care was taken that the width of the moisture band did not exceed 1 cm at any point when the solvent spread on the paper. After the solvent had evaporated (at room temperature), a further application of the solution was made,/

made, and the process repeated until all the solution of the extract had been transferred on to the paper. The residue left was further taken up in 0.5 ml acid ethanol, and this too was similarly applied to the paper. Finally, to ensure the completeness of the transfer of the amines to the paper, the residue was further extracted with 0.25 ml of the acid ethanol, and the solution similarly applied to the paper.

Alternatively, and this was more usually the procedure adopted in this work, the dry extract of a sample was dissolved in a suitable small volume of de-ionized water, and a known portion of the solution taken, evaporated under reduced pressure at 33-35°C (external temperature), taken up in acid ethanol, and applied to the paper. The remainder of the solution was used for the assay to determine the total sympathin content.

Markers for adrenaline and noradrenaline positions on the developed chromatogram.

5 µl of a standard solution (containing 5 mg each of (-) adrenaline, and (-) noradrenaline bases per millilitre in 0.01 N HCl) was applied at the position marked x (Figure V) and allowed to dry at room temperature.

Development/

Development of the chromatogram.

The chromatograms were developed using phenol containing 0.1N HCl (A.R.) in an atmosphere of N_2, by ascending technique.

Phenol (500 g) was purified by distillation from zinc dust (20 g) at atmospheric pressure in an all-glass apparatus, a quantity of glass wool being present in the distillation flask to avoid bumping. The distillate, usually about 460-470 g was collected in a tared 1 litre flask, and to it was added 15 ml of 0.1N HCl (A.R.) for every 100 g distillate, gentle heating being applied to effect solution if the distillate had already solidified. This mixture yielded a one phase system and constituted the developing solvent for the chromatcgrams. The mixture was poured into a glass tank (45 cm x 32 cm x 15 cm) to give a layer of fluid about 1.5 cm in depth.

The paper cylinder carrying the dried on sample was inserted so that the lower end rested on the floor of the tank. Two such cylinders could be accommodated in the tank at one time, so that it was possible to chromatograph four urinary samples simultaneously in one tank. Care was taken to see that the cylinders did not touch either each other, or the walls of the tank. The tank was closed by a

piece/

piece of plate glass and air tight seal between the top of the tank and the glass plate was effected by a liberal use of soft paraffin. The air in the tank was then displaced by passing nitrogen gas in a gentle stream into the tank via the inlet tube, which almost reached the phenol HCl at the bottom of the tank, and held in position by a one holed rubber stopper insert in a hole in one corner of the plate glass. In a hole in the diagonally opposite corner of the glass plate, a short outlet tube was fitted. Nitrogen gas was passed in for 15 minutes after which the inlet and the outlet tubes were clamped off. The chromatograms were allowed to develop at room temperature[+] for 20-24 hours, during which time the solvent had ascended the paper to 23-25 cm above the line of application of the samples.

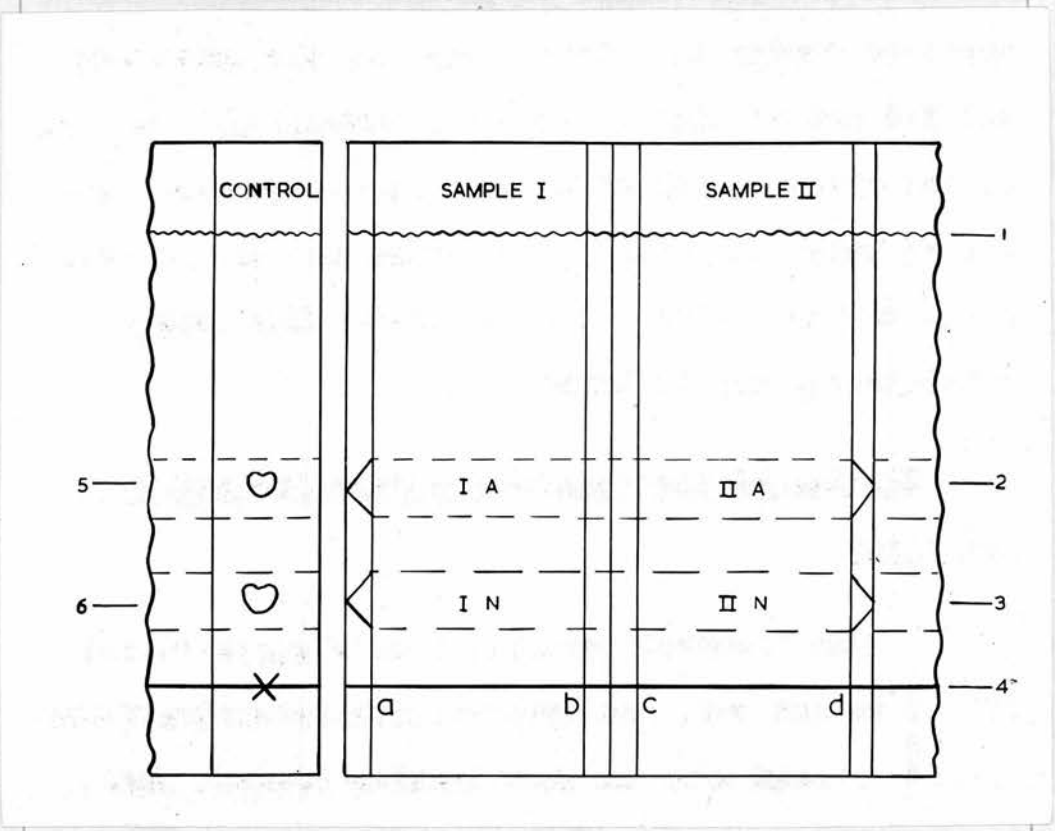
Removal of phenol from the developed chromatograms.

The paper cylinders having been removed from the tank, the part of the cylinder, carrying the sellotape was cut away. The paper sheet, held by the/

[+]In cold weather, it was customary to place the tank in a fume cupboard, and to maintain the temperature at 20°-25°C by means of a Bunsen burner burning in the vicinity.

Figure VII

Preparation of strips from the paper chromatogram for elution.



1. Solvent front.
2. Strip, containing adrenaline (IA, IIA, adrenaline from sample I, and sample II respectively).
3. Strip, containing noradrenaline (IN, IIN, noradrenaline from samples I and II respectively).
4. Starting line. a b, that for sample I, c d, that for sample II, x, spot for applying control solution.
5. Adrenaline spot, developed after spraying $k_2Fe(CN)_6$ solution.
6. Noradrenaline spot, after the same procedure.

the dry upper end, was pulled backward and forward three or four times through a layer of benzene (A.R.) contained in a large photographic developing dish. In this way, the paper was washed free of almost all the phenol. The traces of phenol remaining, disappeared during the later phases of the experiment and did not require any special attention. For two papers about 600 ml of benzene were required. The papers were then hung up to dry in the air at room temperature. After 30-45 minutes, they were sufficiently dry to handle.

Elution of the separated adrenaline and noradrenaline.

The "control portion" (see Figures VI and VII) were cut out, and sprayed with potassium ferricyanide (0.44% w/v) in 0.2M Na_2HPO_4 (James, 1948). Two reddish coloured spots appeared on the paper; the centre of one (noradrenaline) was situated 3-5 cm from the starting-line, and that of the other (adrenaline) at the position 10-11 cm from the starting-line when the height of the solvent front was 23-25 cm, and the ambient temperature 23-25°C. The mean R_F values calculated from 22 experiments were 0.21 (SD 0.04)+ for noradrenaline and 0.53 (SD 0.04)+

+SD - standard deviation of a single observation.

for adrenaline.

The position of the control amine spots indicated the appropriate strips to be cut from the sample portions. To allow for possible minor variations in the position of the amines in the sample portions, from that indicated by the control marked strip, the upper limit of the transverse strips were always taken 1 cm higher than the upper limit of the marker spot, while the lower limit was taken 1 cm lower than the lower limit of the marker spot (see Figure VII). Each transverse strip was then suitably marked in pencil for future indentification. Each paper strip was cut out to a point at one end, care being taken not to remove any paper on which adrenaline or noradrenaline was likely to be present.

The non-tapered end of each strip was put in between two microscopic slides and secured with a small piece of rubber band. This end was dipped, held firmly by another glass slide, placed at a right angle to the first two slides, in the eluting fluid, contained in a small celluloid trough. The eluting fluid was 7.5 ml of 0.4% w/v Na $H_2PO_4 2H_2O$. If the eluate was to be assayed biologically also, 0.01N HCl (A.R) in aqueous solution was used instead. The tapered end projected into but did not touch, the mouth of the collecting tube. Elution was generally allowed to proceed overnight at room temperature, in

a/

a closed airtight chamber, with a beaker of water placed inside it, to saturate the atmosphere with water vapour.

The next morning, about 4 ml of eluate would be found collected in each tube. Each was transferred to a 150 ml evaporating flask and evaporated to just dryness over a water-bath (33° to 35°C) under reduced pressure. It was during this evaporation that the last traces of phenol were removed.

If it was desired to carry out the estimation by fluorimetry only, this step of evaporation could be omitted, and the eluates used directly. If however, biological assays were to be done, this evaporation step was necessary to remove the phenol.

Each residue was then taken up in a suitable volume of 0.9% w/v sodium chloride just before use. For biological assays, this saline extract would be found too acidic, and would need neutralizing. For this, a small portion only was taken out and the pH adjusted to about 6 (just yellowish-green to Universal paper indicator, by the addition of small pinches of solid sodium bicarbonate, A.R.

Acid/

Acid hydrolysis of urine to determine the proportion of the free and conjugated sympathins.

Current theories of the methods of inactivation of adrenaline and noradrenaline in vivo are:

(1) Oxidation by amine oxidase (Burn, 1951, 1952).
(2) Formation of adrenochrome and noradrenochrome (Bacq, 1949).
(3) Conjugation, forming sulphate esters or glucuronides (Richter, 1940; Beyer and Shapiro, 1945; Dodgson et al. 1947; Clark et al. 1950) which are excreted in the urine.
(4) Urinary excretion of the free amines.
(5) Taking up by the tissue cells (Bacq, 1949).

Urinary sympathin therefore consists of a mixture of free and combined forms, and for the determination of the latter, liberation of the free amines is required.

G.P. Burn (1953) stated that boiling human urine for 20 minutes at pH 1-2 hydrolysed any conjugated noradrenaline and rendered it free. von Euler, Hamberg and Hellner (1951) reported that boiling human urine under similar conditions increased the yield of biologically active catechol amines by 30 to 100%.

On the assumption that the above holds true, and that all the combined forms are hydrolysed into the free forms under these conditions, the proportion of the free and combined forms of the amines present in/

in the urine could be determined. Two equal volumes of urine could be taken; one sample hydrolysed under the conditions stated above, and the other not. Assay of the sympathin in the unhydrolysed sample would give the amount of the free amines; while the difference between the assay results of the hydrolysed and of the unhydrolysed samples would be the amount of the combined amines.

However, in the course of present study, it was noticed that the problem was not so simple as that. For, hydrolysis of a sample of urine at pH 0.5 for 20 minutes gave a greater yield of free catechol amines than that done at pH round about 2.

In the course of hydrolysis, there was a certain amount of racemization of the free amines already present in the urine. If the hydrolysis was done at a pH of 2 and for only 20 minutes, this racemization was minimal (von Euler and Hellner, 1951). This was found to be correct.

The question of racemization assumed importance only when assay was to be done biologically, as on rat's blood pressure preparation, where the dextro-form is much less active than the levo-form. In assays by the fluorimetric method, racemization would be of no consequence, as the assay results would be the same from the same amount of amine, regardless/

regardless of its optical activity.

For the present study, hydrolysis was carried out around pH2, in spite of our knowledge that more of the combined forms of the amines could be freed at a lower pH. This was for two main reasons. Firstly, hydrolysis at the lower pH would give more racemization, and therefore the fluorimetric assay results would always be greater than the biological results for a given sample. Hence, verification of the results would not be possible when desired. The second reason was that all the data in the available literature were results based on hydrolysis at pH2, and so for the sake of ease of comparison, the conventional method of hydrolysis at pH2 had been followed.

Evidences obtained in the present study, considered together with all those available in the literature, indicated that it was only safe to say that:

(a) adrenaline and noradrenaline were excreted in the urine partly in the free form and partly in a biologically inactive combined form; and that
(b) hydrolysis at pH round about 2 for 20 minutes could liberate free amines from the combined forms only partially and with a minimum of racemization.

Hence, the assay figures for hydrolysed samples of urine in the present work, represented only that part of the combined amines that could be freed at the pH indicated, plus the free amines already present.

Method of acid hydrolysis

The urine sample (10 ml for rat urine; 50-100 ml for human urine) was adjusted to pH 1.8 (glass electrode) by the addition of 2N H_2SO_4, and heated in a vigorously boiling water-bath for 20 minutes. In the course of the heating, the pH of the urine sample tended to rise. It was brought back to around 2 by the addition of 2N H_2SO_4 at the 5th, 10th and 15th minutes of heating, and the flask was shaken to mix the contents. The addition of 0.1 ml of the acid each time for the three occasions mentioned was found to be sufficient for the purpose, with rat's urine, while 0.15 ml (for 50 ml sample) and 0.3 ml (for 100 ml sample) were sufficient in the case of human urine. The pH could be tested at intervals during the hydrolysis, with small strips of paper indicator, but it was found not necessary. The pH at the end of the hydrolysis conducted as described, usually came out near to 2.

After cooling rapidly in cold water, the hydrolysed sample was extracted and assayed in parallel with an untreated sample of the same urine of an equal volume.

Determination/

Determination of the accuracy of the analytical techniques by the assessment of the percentage recoveries of added sympathin.

Two methods have been employed to assess the accuracy of the techniques of determining urinary sympathins, which had been used in the present work. These may be conveniently referred to as the "direct method" and the "indirect method." Both the methods depend on determining the percentage recovery of known amounts of (-) adrenaline or (-) noradrenaline added to the urine samples.

Indirect method.

Two equal portions of the same urine sample were taken; and to one of them was added a known amount of adrenaline and/or noradrenaline. Both portions were then subjected to the same extraction and assay procedures. The difference in the assay results of the two portions represented the amount of the added amine recovered.

Direct method.

The principle involved is to destroy the sympathins originally present in the urine, and to this "de-aminated" urine, a known amount of adrenaline or noradrenaline is then added and then subjected to the extraction and assay procedures. The assay result represents the amount of the added amine/

amine recovered.

The pH of a suitable volume of a urine sample was adjusted to 10 (glass electrode) by the addition of 2N NaOH. The solution was brought to boiling and then simmered gently for 20 minutes over a small Bunsen flame. The pH of the solution was maintained at about 10 (narrow range indicator paper, Johnson and Johnson 8.4-10) by the addition of the 2N NaOH at intervals. In this way, the sympathin originally present in the urine was destroyed. After cooling, the pH was adjusted to 4 if the recovery from an unhydrolysed urine sample was to be assessed; or to 1.8 if that of a hydrolysed one was to be investigated. After the addition of a known amount of adrenaline and/or noradrenaline, the sample was subjected to the same extraction and assay processes as used for the experimental urine samples. The amount of the amine found in this sample gave a direct measure of the recovery of the added amine.

In both the methods, the amount of the added amines was in the region of that expected to be present in the urine under investigation and not unduly large.

In the initial stages of the present study, the "indirect method" was employed; but later, when it/

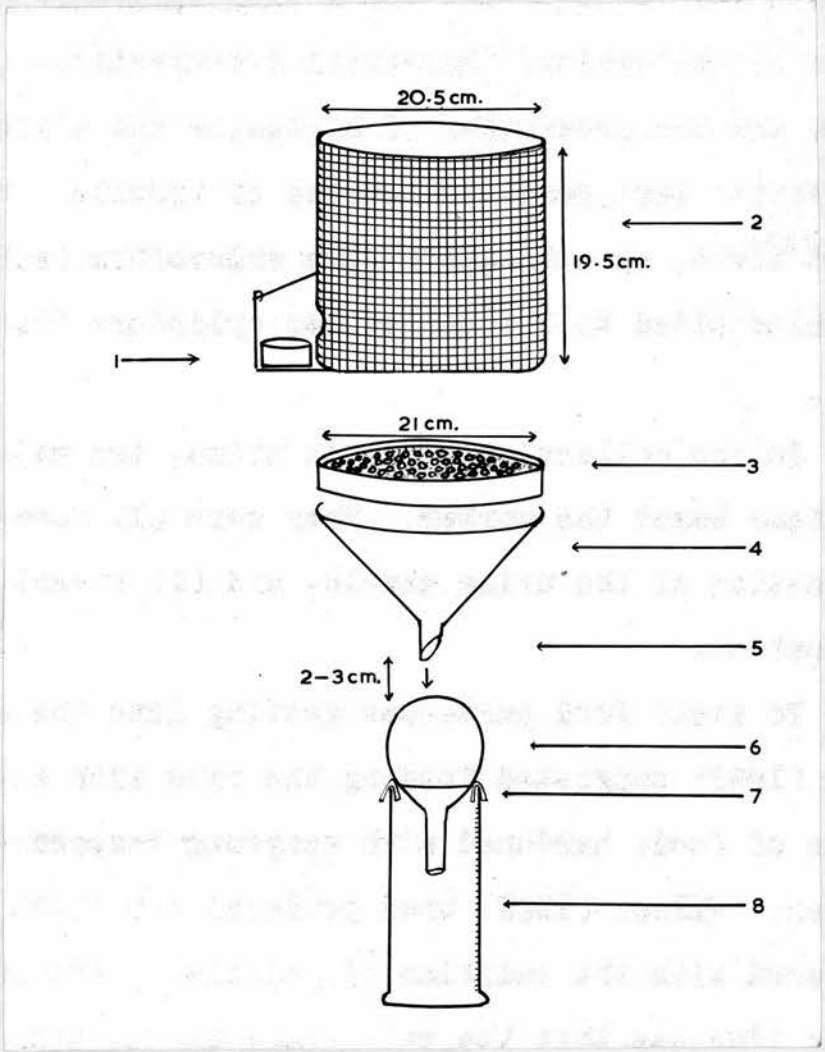
it was discovered that the "direct method" yielded comparable results, the latter procedure was used, as it required the extraction and the assay of only one sample, as against two for the "indirect method." It thus represented a considerable saving in labour and materials, particularly in view of the fact that the recovery percentage was determined with each batch of analysis, in order that the experimental results might be corrected by a recovery factor obtained under conditions as nearly identical as possible. This was considered necessary because the recovery percentage varied slightly from experiment to experiment.

Collection of rat's urine.

Both adrenaline and noradrenaline are easily oxidized by molecular oxygen at neutral or alkaline reaction in pure solution; but they are fairly stable in body fluids or extracts (Mann, 1953). In heparinized blood at 5°C, they are stable for several days. von Euler and Hellner (1951) reported that in normal urine, there is a slight inactivation after 48 hours at room temperature. In acidic medium and in the cold, no change was noted. At pH 3-4, brought about by the addition of an acid, no change was detectable even at room temperature for the/

Figure VIII

Urine collecting apparatus.



1. Water cup.
2. Body of metabolic cage.
3. Cage floor (waxed).
4. Large glass funnel.
5. Cut and bevelled stem.
6. Bulb.
7. "U" shaped glass support.
8. Collecting cylinder.

Body of cage was made of galvanized wire gauze sheet, mesh 5 to the inch.

Cage floor was made of perforated zinc sheet, and waxed with paraffin wax; perforations were 0.9 c.m. in diameter.

the same length of time.

As a routine measure therefore, Analar sulphuric acid was added to the collecting cylinders at the start of collection. Bacterial decomposition of the urine and the production of histamine and allied substances were possible sources of trouble. To avoid these, an antiseptic like chloroform (A.R.) was also added to the collection cylinders beforehand.

In the collection of rat's urine, two major problems beset the worker. They were (1) food contamination of the urine sample, and (2) faecal contamination.

To avoid food particles getting into the urine, Dyer (1933) suggested feeding the rats with solid balls of food, hardened with compound tragacanth powder. Wilson (1952) used powdered rat food, hardened with the addition of gelatine. The underlying idea was that the rats would nibble off a small piece at a time, out of the hardened mass and eat it. But the rats did not behave like that: they did not eat all that they had chipped off, and food particles found their way into the urine glass below.

Next, a gruel-like preparation was used, being placed in the side cup of the metabolism cage (see Figure VIII). However, particles of food

invariably/

invariably got stuck to the paws and the snouts, and these eventually found their way into the urine. Powdered rat cake was tried, and for a similar reason, the practice was discarded.

It was finally decided to feed the rats outside the cages, for a fixed period of 30 minutes, with ordinary rat cake nuts, at fixed times twice a day. This was found satisfactory. By taking the precaution of coaxing the rats to urinate before taking them out of the cages for feeding, the risk of the loss of urine was much reduced. Though this method of feeding entailed the need of attending them twice a day, it eliminated food contamination of the urine effectively, and also the trouble of preparing the hardened rat food.

Faecal contamination proved more difficult to overcome. Perforations of various sizes in the cage floor were tried, and those of 0.9 cm diameter were found to be about the best. Below the collecting funnel on which the cage rested, was situated a glass bulb riding over the mouth of the collecting jar (Figure VIII). This bulb served to deflect the falling pieces of faeces, while allowing the urine to trickle down its side into the collecting jar below. Faecal separation from the urine was by no means complete; but it was about the best one could hope/

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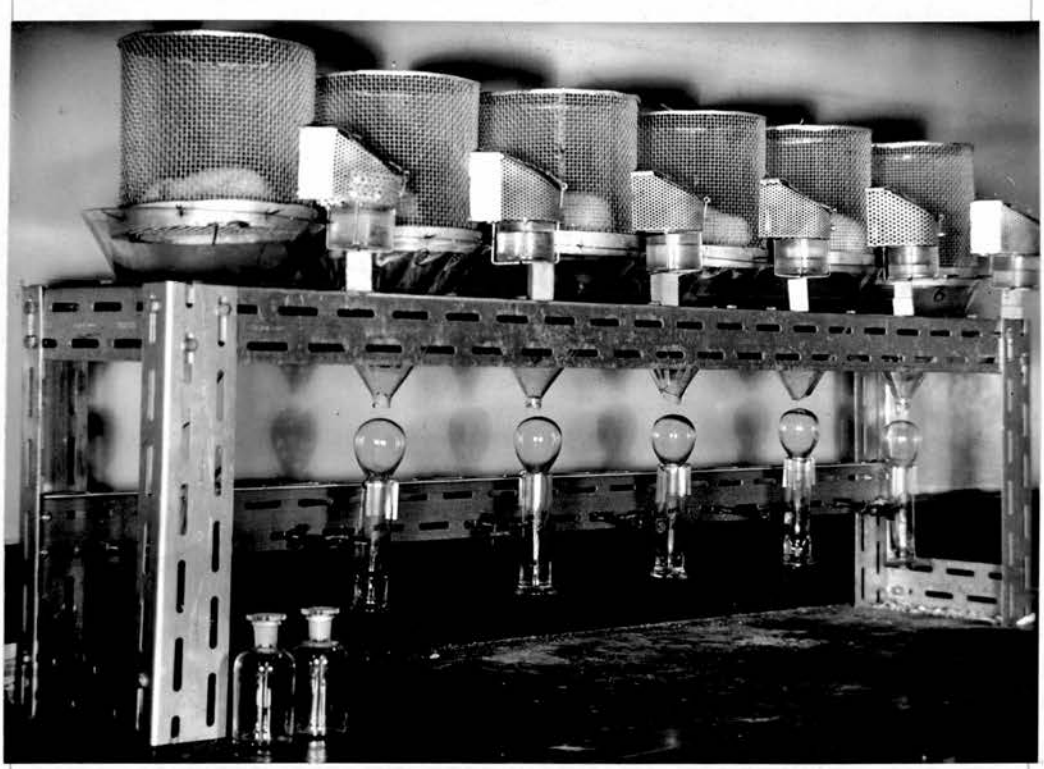
Figure IX

Complete set-up for the urine collection from rats.



1. Metabolic cages.
2. Glass funnel.
3. Inverted kjeldahl flask.
4. Collecting cylinder.
5. Coloured bottles: Analar $CHCl_3$; and
   2N Analar H_2SO_4.

hope for, and the urine samples so obtained were reasonably free from faeces.

Detailed description of rat's urine collection and results.

The rats were confined in metabolic cages, one in each cage, and kept in a corner of a spacious room, well ventilated, but free from draught. In winter, an electric heater was put on to supply heat from a varying distance. The degree of warmth was recorded by a minimum and maximum thermometer, kept hanging near the cages. The temperature ranged from 18 to 21°C.

The design of the metabolism cages and of the urine collecting apparatus is shown in Figure VIII. It consisted of a metabolic cage of the type, material and dimensions as indicated in the figure, resting on a large glass funnel which in turn rested on a metallic supporting structure (see Figure IX). The tip of the cut and bevelled funnel stem, at 2 to 3 cm distance, pointed to the centre of the ellipsoidal bulb of an inverted Kjeldahl flask the neck of which had been cut short. The bulb of the flask in its turn, rested on 3 inverted "U"-shaped glass rods which gripped the sides of the glass collecting cylinder and acted like a tripod.

Drinking/

Drinking water was provided in a cup attached to the side of the cage.

Feeding was done in a duplicate series of similar cages. Each rat was offered about 15 g (an excess) of rat cake nuts,+ twice a day, at 9.15 a.m. and 8.30 p.m. daily, with drinking water ad lib in a separate cup, attached to the side of the cage. The rats were allowed to eat for 30 minutes on each occasion after which they were replaced in the original cages. Usually, for the first few days after being placed in the individual metabolism cages, they did not eat well, and were restless. After four or five days, however, a rat would eat about 20 g of rat cake nuts per day. With this regime of feeding, the rats were found to maintain or increase slightly their original body weight when checked over ten-day periods.

9.15 a.m. was the dead line; 9.15 a.m. of one day to the same time the next day was taken as one collecting/

+Rat cake nuts were supplied by the North-Eastern Agricultural Co-op. Soc. Ltd. Aberdeen. The approximate composition, according to Lane-Petter and Dyer (1952) was:

Protein	24.8%
Fat	4.7%
Starch	49.3%
Fibre	4.9%
Ash	9.5%
Vitamins, added q.s.	

collecting day. Every day at this hour, thermometer readings were noted, and while the rats were feeding, changing of the cage floors, funnels, bulbs and collecting cylinders, was done. The volume of urine in each cylinder was read off, reaction taken with a small piece of Universal indicator paper, and the specific gravity of the pooled urine was taken with a small hydrometer. Whenever there was a chance of encountering freshly passed urine, its reaction was also tested with the paper indicator.

Just before removing the rats from the cages for feeding purposes, each rat was coaxed to urinate by gently holding up its tail by its tip, and stroking gently the back of the rat with fingers. Alternatively, a gentle squeeze around the abdomen would also be effective. It was found that female rats were much easier to be so persuaded than the males. With male rats, the second method was more effective. This procedure was taken to obviate the possible loss of urine if passed during the feeding. Should a rat pass urine in the feeding cage, the urine so collected was added to the next day's sample. At 8.30 p.m. the rats were similarly fed. Any urine passed during this feed was to be added to the same day's sample. So far, there was no occasion to do this in all the series that were to be assayed.

On/

On those days when urine was required for assay, 0.5 ml of 2N sulphuric acid (A.R.) and two drops of chloroform (A.R.) were placed and shaken to mix, in each collecting cylinder before the collection started. The cage floors too, were waxed with hard paraffin, B.P. On other days, these procedures were omitted.

Collected thus, the urine was free from food contamination. Faecal contamination too was reduced to the minimum. Ordinarily, the faecal lumps were dry, and they dropped through the perforations in the cage floors readily enough into the funnel and then fell on to the bulb and bounced off. Wet diarrheic stools had a tendency to stick to the cage floor, the funnel, and the bulb surface, but healthy rats on the diet given did not pass wet stools.

Occasionally, displacement of the funnel caused the centre of the funnel stem (instead of the bevelled tip) to become perpendicular to the surface over the centre of the bulb where one or two lumps of faeces might sit. Ordinarily, with the bevelled tip of the funnel stem pointing to the centre of the top of the bulb surface and at 2 to 3 cm distance faecal lumps just bounced off.

A longer funnel stem, especially when its bevelling was slight, coupled with the chance of faecal lumps falling down sideways, was more likely to/

to get blocked with faeces. Even in this case, urine trickled through, down the sides of the funnel stem and touched the faeces only at certain points for a very short duration of time. There was then a definite risk of contamination with the faeces, but it was temporary and slight. It would probably be better to have the stem end of a funnel cut off, such that the circumferance of the outlet so made was just smaller than the diameter of the bulb of the Kjeldahl flask. With such funnels, the question of faecal block would not arise. However, it was technically difficult to cut a glass funnel that way, although a funnel made of polythene might be readily adjusted in this way.

The size of the perforations in the cage floor was 0.9 cm in diameter. A bigger size would certainly facilitate the passage of faecal lumps, but would also allow the feet of the rats to fall through. Smaller perforations were found to hold back faeces, and with the rats trampling about, the cage floors became covered with faecal matter, and the likelihood of faecal contamination of the urine sample was greatly increased.

The cage floors were waxed with hard paraffin, B.P. on those days when the urine was wanted for assay. On other days, this was omitted. Waxing of the /

the funnel and the bulb had been attempted also, with the idea of minimizing the loss of urine due to its adhesion and drying on the surface of the funnel and the bulb. But this was given up, as it was found that urine drops would bounce off from the waxed surface and be lost. Instead, a little de-ionized water was used to wash down the dried up urine patches.

In connection with the collection of rat's urine, the following observations were made:

(a) Specific gravity. The specific gravity of pooled urine of 6 rats had been determined daily for 100 days. It ranged from 1040 to 1060, with the usual figures round about 1055.

(b) Colour. Usually, it was straw-yellow. On those days when sulphuric acid and chloroform were added, it was dark-brown.

(c) Reaction. Tested with Universal paper indicator, freshly voided rat's urine had a pH of 6, while a 24-hour old sample was usually 7 or 7^{+}. Sometimes, it was 8, and very occasionally even 9. 24-hour samples collected into 1 ml of 2N H_2SO_4 and 2 drops of chloroform usually had a pH value of about 2. When collected into 0.5 ml of the acid, the pH of the 24-hour sample was usually 3 to 3.5.

(d) Volume. This varied somewhat with different batches of rats. One particular batch gave a mean of 14.6 ml per rat per day, (SD 2.4 for 70 samples).

Collection/

Collection of human urine samples.

With human adults, the urine collection was easy. Healthy men or ambulant patients could be instructed to pass their urine directly into a wide-mouthed coloured bottle which had 10 ml of 2N sulphuric acid (A.R.) and 1 ml of chloroform (A.R.) at the bottom, and after urinating, to shake the bottle to mix the content. With bed-ridden cases, a clean urinal (free from any antiseptics) was used as an intermediate collecting vessel, and the urine could then be poured into the coloured bottle and the bottle shaken to mix.

With male infants, a small rubber funnel-like receptacle with a drainage tube, somewhat like the apparatus for suprapubic drainage of operated urinary bladder cases, was fixed on to the genital area. The tip of the drainage tube was dipped into a bottle, containing 5 ml of 2N H_2SO_4 (A.R.) and 0.5 ml of chloroform (A.R.) Frequent checking and readjustment were needed to prevent leakage of urine from the funnel-like receptacle. No female infants were included in the present study, as the collection of their urine was very difficult.

Method/

Method of administration of drugs to the rats.

The effect of several drugs on the urinary sympathin output of rats was studied. These drugs were: adrenaline. noradrenaline, morphine, Marsilid (1-isonicotinyl-2-isopropyl hydrazide), insulin, thyroxine and ether.

Except in the case of ether, the subcutaneous route was used for the administration of the drugs, as it ensured more complete absorption than the oral route; and the action of the drugs so given was comparatively much less acute, and lasted longer than when given by the intravenous route. The injections were made into the skin fold, pinched up between the thumb and the forefinger, above the neck. On withdrawing the needle, the skin was lightly massaged to prevent oozing and to spread the injected substance into the subcutaneous tissues.

To prevent the rats struggling during injection and thus ensure that the entire dose was in fact injected, each rat was, except for the head and a portion of the neck, rolled up in a towel, and then tied loosely with a cord. This did not appear to disturb the animals, and helped considerably in the performance of the injection, single-handed, as was the case with all the injection experiments in the present/

present study. Alternatively, especially when the rats were "trained," they could be injected without the wrapping. But one could never be sure when a rat would start resenting an injection and struggle, and so causing the needle to slip and thereby spilling the drug. As a routine therefore, where only one injection was to be given, the rats were wrapped up. When repeated injections were to be given, they were injected without wrapping. They were then coaxed to stay on the cage floor over the funnel, with the collecting cylinder below, and were then injected. It was done like that, as the rats frequently urinate during the injection, in spite of the fact that they had been coaxed to urinate before injecting. This was found to occur more frequently with male rats than with the female.

Unless otherwise stated, all the injections were done in the forenoon, round about 11 a.m. That is to say, about one to two hours after feeding. The body weight of the rats were taken the previous day, before feeding.

Control injection of 0.9% sodium chloride, or whatever solvent it was intended to dissolve the test substance in, was made the previous day or week at about the same hour, and the urine for the 24-hours following, collected. Control injections of saline were/

were necessary, as the urinary sympathin excretion thereafter showed a statistically significant increase over that of the uninjected animal. This was all the more necessary, as the excretions following the test drugs were seldom very strikingly greater than the normal daily excretions.

The test drug in similar volume was given the following day.

Syringes used were "Accosan" tuberculin syringes 1 ml capacity, graduated in 0.01 ml, and mounted with a sharp 2.54 cm hypodermic needle, gauge 20.

Materials injected.

Specifications of the drugs injected are given under each drug. If necessary, the test drug solution was neutralized (Universal indicator paper), just before injection. This was done to obviate the likelihood of extreme irritation and possibly much pain incident on injecting a too acidic or too alkaline solution, as irritation and pain were feared to be able to influence the sympathin metabolism. Care was taken of course, to see that there was no precipitation or other loss of the active principle of the test substance when the neutralization was carried out.

The volume of injection was limited to round about 0.5 ml. It was feared that a large volume would/

would cause a distension of the subcutaneous tissues and influence the rate of absorption, and possibly discomfort or even pain, which would in turn, influence the sympathin output of the rats. A smaller volume on the other hand, would require the use of a more concentrated solution; any error in measurement would thus become magnified.

General procedure of experiments in rats.

Six male Wistar albino rats of about 250 g body weight were used. Female rats were avoided, since it was considered that variation in the stages of oestrous cycle might influence the sympathin output. This point has not been investigated further.

As the amount of the urinary sympathins in rats was small, the urine of two rats was usually pooled, thus giving three pooled samples for the six rats. Two suitable amounts, usually 10 ml each, were pipetted out, from each of the first two pooled urine samples; one to be hydrolysed (see p. 46), the other not. The remaining third sample was for determining the recovery percentage, using the direct method (see p. 47). For this, the pH was brought to 10 (glass electrode) by the addition of 2N NaOH, the solution then boiled gently for 20 minutes to destroy the sympathins present. After cooling,/

cooling, the pH was brought back to 1.8. Two 10 ml portions were pipetted out, one to be hydrolysed, the other not. To each portion was added a known amount of sympathin, which ideally speaking should be about the same amount as would likely be present in the other four samples. In practice, this was not critical, as the percent recovery proved to be about the same for the level of concentration of sympathin likely to come across in the present work.

The amine added was noradrenaline, except in adrenaline injection experiment when adrenaline was used, and in those experiments where separate determinations of adrenaline and noradrenaline were required, in which case, a known weight of both the amines would be added.

The various urine samples were then analysed according to the method indicated in the later description of the individual experiments, the results for the control and the test samples of urine being corrected by a factor obtained from the recovery experiment, to allow for the losses occurring during the analytical technique.

Results/

RESULTS

Preliminary Experiments

Fluorimetric estimation of adrenaline and noradrenaline.

The results shown in Table 1 demonstrate the similarity in the fluorimetric readings for equal amounts of adrenaline and noradrenaline when the modified Lund's method (p.7) is used to develop the fluorescence.

TABLE 1

Comparison of fluorimetric readings, given by adrenaline and noradrenaline, under identical conditions. Reagent blank value already deducted.

Amount of amine µg	Fluorimetric readings	
	Adrenaline	Noradrenaline
1	82	82
1	78	82
1	81	-
0.75	61	64
0.75	60	65
0.5	39	41.5
0.5	41.5	40.5
0.25	20.5	22.5
0.25	22	25

Table 2/

Table 2 shows that the intensity of the fluorescence of mixtures of adrenaline and noradrenaline is the sum of the separate intensities.

These results indicate that no marked error would be introduced by using one or the other amine as a standard in the measurements of the sympathins (adrenaline and noradrenaline) of the urine extracts, the result obtained being a measure of the sum of the two amines present. As a routine, however, eluate containing noradrenaline is assayed against noradrenaline, and that containing adrenaline against adrenaline.

TABLE 2

Comparison of thefluorimetric readings, given by mixtures of adrenaline and noradrenaline, with the readings expected from the sum of the individual readings. Reagent blank values already deducted.

Amount present (μg)		Fluorimetric readings	
Adrenaline	Noradrenaline	Actual	Expected
0.5	-	41.5	
-	0.5	44.5	
0.25	0.25	44	43
0.25	0.5	67	65
0.5	0.25	67	64

Percentage/

Percentage recovery of adrenaline and of noradrenaline from pure solutions and from urine, by extracting by adsorption on aluminium hydroxide (von Euler's method) and by extraction by adsorption on cation-exchanger, Amberlite IRC 50.

Adsorption on aluminium hydroxide (von Euler's method, p. 21) and assay on rat's blood pressure preparation.

Quantities of (-) adrenaline and (-) noradrenaline of the order expected in the subsequent analysis of urine samples were dissolved in aqueous solution and extracted by von Euler's method, the final assays of the extracts being carried out on rat's blood pressure preparations. The results of these experiments, shown in Table 3, indicated a recovery from pure solution, of some 70% for both adrenaline and noradrenaline, at the level of 2-4 µg.

Similar recovery experiments, using noradrenaline, were carried out on 10 ml samples of pooled urine from normal rats, using the indirect method of determining the recovery (p.47); i.e. by the difference recorded between the results obtained with a/

TABLE 3/

TABLE 3

Recovery of noradrenaline and of adrenaline in aqueous solution, by von Euler's method of extraction.

The amount of amine indicated in 2nd column in both tables, dissolved in 10 ml of distilled water (glass), was treated with appropriate amount of aluminium sulphate solution (20% w/v). The extracts were assayed on rat's blood pressure preparation, against standard (-)-noradrenaline, or (-)-adrenaline as indicated.

A. NORADRENALINE

No.	Amount used µg	Amount recovered µg	Recovery %
1	2	1.41	70.5
2	2	1.38	69
3	3	2.2	73.3
4	4	2.6	65
		Mean	69.5
		S.D.	3.46
	B. ADRENALINE		
1	2	1.42	71
2	2	1.41	70.05
3	3	2.08	69.3
4	4	2.51	62.75
		Mean	68.25
		S.D.	3.75

The difference in the recovery percentages of adrenaline and noradrenaline was not significant at $P = 0.05$ level.

a sample of urine to which a known weight of the catechol amine had been added, and with a like sample to which no amine had been added. Such experiments were mainly carried out with hydrolysed urine samples (p.46), but two experiments were recorded for unhydrolysed samples, as per Table 4. The mean recovery from the seven experiments with hydrolysed samples was 73% with a standard deviation of 4.66. With the unhydrolysed urine (only 2 experiments) a mean of 79.5% was obtained, but the spread of the two estimate was somewhat large (19%).

TABLE 4

Recovery of noradrenaline, added to urine samples, by von Euler's method of extraction. To one of the two 10 ml portions of urine, 2 µg of (-)-noradrenaline were added. 5 pairs of such samples were acid-hydrolysed; 2 pairs were not, and then the sympathin contents extracted accordingly; the extracts were assayed against standard noradrenaline, on rat's blood pressure preparation.

No.	Noradrenaline added µg	Amount recovered µg	Recovery %
	Hydrolysed urine		
1	2	1.4	70
2	2	1.45	72.5
3	2	1.5	75
4	2	1.39	69.5
5	2	1.64	82
6	2	1.38	69
7	2	1.4	70
		Mean	72.57
		S.D.	4.66
	Unhydrolysed urine		
1	2	1.4	70
2	2	1.78	89
		Mean	79.5

Adsorption on cation-exchanger, Amberlite IRC 50, and assay by fluorimetry (p.7) and/or by rat's blood pressure preparation.

Table 5 records the recoveries obtained from pure aqueous solutions, containing 2 µg of (-) adrenaline or (-) noradrenaline, extracted on 10 cm IRC 50 columns (p.25). In two cases, the amine solutions were first subjected to the hydrolysation procedure (p.46) before being extracted. A fairly good agreement between the fluorimetric and biological assays is evident, with a mean recovery of 66% (range 60 - 75%).

TABLE 5

Recovery of adrenaline and of noradrenaline in aqueous solution by IRC 50 resin method of extraction. 2 µg of either amine in 10 ml of aqueous solution. 2 samples were acid hydrolysed. Adsorption on 10 cm columns. The extracts were assayed both fluorimetrically, and on rat's blood pressure preparations. The figures in brackets indicate percent of recovery of the amount of amine used.

	Amount of amine used µg	Amount recovered (fluorimetrically) µg	Amount recovered (Rat's BP) µg
Adrenaline:			
Hydrolysed	2	1.3 (65%)	1.25(62.5%)
Unhydrolysed	2	1.4 (70%)	1.35(67.5%)
Noradrenaline:			
Hydrolysed	2	1.31 (65%)	1.2 (60%)
Unhydrolysed	2	1.3 (65%)	1.5 (75%)

Adsorption on cation-exchanger, Amberlite IRC 50 and/

and assay by fluorimetry only, of added (-) adrenaline or (-) noradrenaline, to urine samples, the catechol amines+ of which, present originally, had been previously destroyed and the recovery percentage was estimated by the direct method (p.47).

Table 6 records the results of 51 experiments. The over all mean recovery was 82 with a standard deviation of about 6. There was no significant difference between the recoveries of adrenaline and of noradrenaline in the range of dosages investigated. The results indicate that the process of hydrolysis as conducted in these experiments (at pH 2 for 20 minutes in boiling water-bath), did not cause any significant change in recoveries.

TABLE 6/

+ For dosage level above 1,000 µg, the urine samples used were normal human urine, untreated with NaOH to destroy the catechol amines originally present, as the amount of sympathin present in 100 ml of the urine was negligible in proportion, compared to the amount of the amines added.

TABLE 6

Recovery of adrenaline and of noradrenaline added to urine, the original sympathin of which had previously destroyed by heating at pH 10. The estimate was given by the amount recovered (direct method, see p.47)

A. Recovery of (-) adrenaline, by the direct method, from 10 cm columns of IRC 50. Fluorimetric assay.

	Amount of amine used µg	Amount of amine recovered µg	Recovery %
Hydrolysed urine	4	3.66	91.5
	6	4.2	70
	8	6.73	84.13
	10	8.0	80
	16	13.1	81.87
	20	16.2	81
	20	17.5	87.5
	30	24.5	81.66
		Mean	82.2
		S.D.	6.25

B. Recovery of (-)-noradrenaline, by the direct method. 10 cm columns of IRC 50. Fluorimetric assay.

Unhydrolysed urine	3	2.26	75.3
	3	2.17	72.33
	3	2.37	79
	3	2.34	78
	3	2.32	77.33
	4	3.23	80.75
	4	3.05	76.25
	4	3.23	80.75
	4	3.1	77.5
	4	3.2	80
	4	3.45	86.25
	4	3.3	82.5
	5	4.0	80
	5	4.34	86.8
	5	4.08	81.6
	8	7.8	97.6
		Mean	80.75
		S.D.	5.83

Hydrolysed/

Table 6 Contd:

	Amount of amine used µg	Amount of amine recovered µg	Recovery %
Hydrolysed urine	3	2.41	80.33
	3	2.13	71
	3	2.29	76.33
	3	2.4	80
	3	2.78	92.66
	3	2.33	77.66
	4	3.0	75
	4	3.14	78.5
	4	3.0	75
	4	3.28	82
	4	3.45	86.25
	5	3.53	70.6
	5	4.76	95.2
	5	4.08	81.6
	6	4.85	81
	6	4.95	82.5
	8	7.72	96.5
	8	7.6	95
		Mean	82.06
		S.D.	8.07

C. Recovery of large doses of (-) adrenaline and of (-) noradrenaline, by the direct method. 20 cm column of IRC 50. The amine added to 100 ml of human urine. Fluorimetric assay. Urine unhydrolysed.

Amine	Amount used mg	Amount recovered mg	Recovery %+
(-)adrenaline	5.0	4.15	83
	3.2	2.68	83.75
	1.6	1.246	78
	0.8	0.624	78
	0.4	0.326	81.5
	0.2	0.167	83.5
		Mean	81.2
		S.D.	2.68
(-)noradrenaline	5.0	4.3	86
	2.5	2.08	83.2
	1.25	1.05	84
		Mean	84.4
		S.D.	1.44

+The normal sympathin content of the urine, being of negligible proportion compared to the amount of amine added, has been ignored in the calculation.

The overall mean for the 51 estimations listed under A, B and C of Table 6, is 81.72 with a standard deviation of 6.22. There is no significant difference in the recoveries of both adrenaline and noradrenaline whether from hydrolysed or unhydrolysed urine.

Quantitative separation of adrenaline and noradrenaline by paper chromatography.

Table 7 records the recoveries obtained when pure aqueous solutions, containing a mixture of adrenaline and noradrenaline were submitted to the paper chromatographic separation (p. 35), and the amine contents of the eluates determined both fluorimetrically and biologically (rat's blood pressure preparation). These results indicated a satisfactory separation and recovery of the amines even in amounts as low as 0.5 µg.

TABLE 7/

TABLE 7

Recovery of adrenaline and noradrenaline from mixtures in aqueous solution after separation by paper chromatographic technique. The eluates were assayed both fluorimetrically and on rat's blood pressure preparation. The figures in brackets indicate percentage of the amine recovered.

Mixture No.	Amine	Amount applied µg	Amount of amine recovered: Fluorimetric µg	Rat's B.P. µg	Rat's B.P. range
I	(-)adrenaline	0.5	0.36(72)	0.5 (100)	0.2 (40) - 0.8 (160)
	(-)noradrenaline	2.0	1.4 (70)	1.95(97.5)	0.9 (45) - 3.0 (150)
II	(-)adrenaline	1.0	0.84(84)	1.13 (113)	0.75(75) - 1.5 (150)
	(-)noradrenaline	nil	< 0.01		< 0.01
III	(-)adrenaline	2.0	2.1 (105)	3 (150)	1.5 (75) - 4.5 (225)
	(-)noradrenaline	0.5	0.5 (100)	0.6 (120)	0.2 (40) - 1.0 (200)
IV	(-)adrenaline	nil	< 0.01		< 0.01
	(-)noradrenaline	nil	< 0.01		< 0.01

Table 8 gives the results obtained with mixtures of adrenaline and noradrenaline added to the urine (rat and human) the sympathin of which had previously been destroyed by heating at pH 10 (p.48). The urine samples were then subjected to extraction on a 10 cm IRC50 column/

column if it was rat's urine, and on a 20 cm column if it was human urine; followed by paper chromatographic separation by the amines in the extracts (obtained with IRC 50). 5 sets of results were with urine samples that had been hydrolysed (p.46). The recovery for both adrenaline and noradrenaline, with or without initial hydrolysis of the urine samples, was about 50%, SD about 9). The difference between the two in the table being statistically insignificant.

TABLE 8

Recovery of adrenaline and noradrenaline in mixture, added to urine (rat and human) the original sympathins of which had been previously destroyed by heating at pH 10. Extraction on 10-20 cm columns of IRC 50, followed by paper chromatography. The eluates were fluorimetrically assayed.

No.	Amine	Amount used µg	Amount recovered µg	Recovery %		
	Hydrolysed urine					
1	(-) adrenaline	2	1.09	54.5		
	(-) noradrenaline	2	1.345		67.25	rat's urine
2	(-) adrenaline	2	0.9	45		
	(-) noradrenaline	10	4.875		48.75	rat's urine
3	(-) adrenaline	8	3.24	40.5		
	(-) noradrenaline	8	2.8		35	rat's urine
4	(-) adrenaline	10	4.85	48.5		
	(-) noradrenaline	10	4.95		49.5	human urine
5	(-) adrenaline	15	8.35	55.7		
	(-) noradrenaline	15	9.25		61.66	human urine
	Unhydrolysed urine+					
1	(-) adrenaline	10	4.65	46.5		
	(-) noradrenaline	10	4.56		45.6	rat's urine

Recovery percentage figures for adrenaline and for noradrenaline were statistically not significantly different at P = 0.05 level.

While such recoveries cannot be regarded as entirely satisfactory, evidence is presented in Table 9 to show that by applying the necessary correction with the recovery factors, satisfactory agreement is obtained between the sum of the estimated amount of the separated amine, and the estimated total sympathin. Table 9 records the results of the experiment carried out on 100 ml samples of human urine in a case of pink disease, which had been hydrolysed and then extracted on a 20 cm column of IRC 50. Part of the extract obtained at this stage was assayed fluorimetrically for total sympathins, while part was submitted to paper chromatographic separation and the separated adrenaline and noradrenaline in the respective eluates determined fluorimetrically. A recovery experiment was carried out in parallel with each and the necessary correction made. The figures quoted therefore, refer to the content in the 24-hour sample, and not to that of the sample analysed.

The substantial agreement between the total sympathin figures and the sum of the separated amines indicates that a "total sympathin" determination on the urine extracts made with the cation exchanger IRC/

IRC 50 and estimated by modified Lund's fluorimetric method does in fact, give a true value for the sum of the constituent adrenaline and noradrenaline content of the urine.

TABLE 9

Results of the assays of urinary sympathin extracts obtained by the IRC 50 technique, compared to those of the same extracts after further paper chromatographic separation. Both, fluorimetric assays.

Sample No.	Total sympathin in the extract from IRC 50. µg	The same extract after paper chromatographic separation.		
		Adrenaline µg	Noradrenaline µg	Total µg
1	41	13.9	26.8	40.7
2	21.5	4.1	15.4	19.5
3	41	11.2	26.2	37.4
4	20	3.1	13.6	16.7

The/

The degree of racemization of adrenaline and noradrenaline resulting from acid hydrolysis (at pH 1.8 - 2) in boiling water-bath.

Table 10 records the recoveries from 10 ml portions of aqueous solution containing 2 µg of either adrenaline or noradrenaline. One set consisting of one adrenaline and one noradrenaline sample, was hydrolysed at pH 1.8 - 2 for 20 minutes in boiling water-bath, but the other set was not. They were then subjected to extraction on a 10 cm IRC 50 columns, and the extract so made assayed fluorimetrically and on rat's blood pressure preparation.

The differences between the recoveries from the hydrolysed samples and from the unhydrolysed are not different statistically. The slightly lower percentage recovery was due to the fact that these experiments were done in the early phase of the present work when the technique was not so perfect as at later stages.

The results indicate that acid hydrolysis as carried out has caused some racemization but not much.

TABLE 10/

TABLE 10

The degree of racemization of adrenaline and of noradrenaline, in aqueous solution, following acid hydrolysis at pH 2 for 20 minutes, in boiling water-bath. 2 µg of the amine in 10 ml of aqueous solution. IRC 50 resin extraction, on 10 cm columns. Assay fluorimetrically and on rat's blood pressure preparation. Figures in the brackets indicate percent recovery of the amine used.

Amine	Sample No.	Amount of amine used µg	Method of assay	With hydrolysis µg	Without hydrolysis µg
(-)adrenaline	A_1	2	Fluorimetric	1.3 (65%)	1.4 (70%)
			Rat's B.P.	1.25(62.5%)	1.35(67.5%)
(-)noradrenaline	N_1	2	Fluorimetric	1.3 (65%)	1.3 (65%)
			Rat's B.P.	1.2 (60%)	1.5 (75%)

The differences in the 'hydrolysed' and the 'unhydrolysed' samples were statistically not significant. With fluorimetric results, $P > 0.4$; with biological result, $P > 0.1$.

Release/

Release of free sympathin in urine by acid hydrolysis at pH 0.5 and at pH 2.

In this experiment, 4 portions of 100 ml of the same human urine were taken. The pH of two samples were adjusted to 0.5; that of the other to 2.0 (all by glass electrode). They were then put into the same boiling water bath for 20 minutes, and the pH kept constant by adding more 2N H_2SO_4 at intervals when found necessary after testing with paper indicator from time to time.

They were then subjected to extraction on 20 cm IRC 50 resin columns, and the extracts so made were assayed fluorimetrically.

Table 11 records the results. Those for pH 2, namely samples 3 and 4, were straightforward and gave identical results. The set at pH 0.5 gave extracts that contained some materials quenching the fluorescence (p. 12). The extent of the masking or quenching was about 40%. The results shown in the table are corrected values, after allowance had been made for the effect of masking. To ensure accuracy, these extracts had been further assayed on rat's blood pressure preparation. The fluorimetric values (after the correction) agree with the biological values.

TABLE 11/

TABLE 11

The effect of acid hydrolysis for 20 minutes in a boiling water-bath, at pH 0.5 and pH 2. 100 ml portions of human urine; pH of 2 samples brought to 2; pH of the other 2 to 0.5 (both glass electrode) by adding 2N H_2SO_4(A.R.) After hydrolysis, extraction on 20 cm columns of IRC 50. Fluorimetric assay and on rat's blood pressure preparation, against standard (-)-noradrenaline.

pH	Sample No.	Fluorimetric assay µg	Rat's B.P. assay with range. µg	Remarks
0.5	1	4.35+	4.5 (range 3-6)	+About 40% masking. Figures represent values after correction for masking.
	2	4.23+	3.5 (range 3-4)	"
2.0	3	2.2°		° No masking.
	4	2.2°		

The difference between the mean values for pH 0.5 and pH 2 is significant. $P < 0.001$.

Table 12 records the results of experiments with (-) adrenaline or (-) noradrenaline added to 100 ml of normal human urine, and then extracted on 20 cm cation-exchange resin IRC 50 column, and the extract/

extract assayed fluorimetrically. The results indicate that a 20 cm resin column could handle 5 mg of either of the two amines.

TABLE 12

The results of experiments to gauge the loading capacity of IRC 50 resin columns, 20 cm x 1.1 cm internal diameter. Amine added to 100 ml human urine, except Sample 1. Rate of adsorption 1.5 ml/minute; rate of elution 0.5 ml/minute. Volume of eluate collected 40 ml. Fluorimetric assay on the eluates directly.

No.	The amine used and amount	Amount of the amine recovered.	Recovery* %
	(-)-adrenaline		
1	6.4+	6.1	95.3
2	5.0	4.86	97.2
	(-)-noradrenaline		
3	5.0	4.9	98.0
4	2.5	2.49	99.0
5	1.25	1.24	99.6

+Dissolved in 100 ml of salt solution, containing 3 g NaCl and 1 g Na $H_2PO_4.2H_2O$. Solution treated as urine.

*The normal amine contents of the urine, being of negligible proportion compared to the amount of amine added, have been ignored in these calculations.

The effect of caging on the output of urinary sympathin was investigated. For this, pooled urine samples of 3 male rats (210-230 g) for 24-hour period were used. They were extracted after acid hydrolysed at pH_2 on 10 cm IRC 50 columns, and the extracts assayed fluorimetrically. The results are recorded in Table 13. It will be seen that the urinary sympathin was comparatively higher on the first few days of confinement, but fell to the normal range by the 6th day. Consequently, all experiments on rats were carried out on only those rats that had been conditioned to the metabolism cages for at least one week.

TABLE 13

The effect of caging of a rat on the output of its urinary sympathin. 3 male rats (210-230 g) age about 7 months were individually caged in the urine collecting apparatus (p.52). 24-hour hydrolysed samples for 1st, 4th, 6th and 10th day of confinement were analysed on the urine pooled for the day. IRC 50 resin method of extraction. Fluorimetric assay, against standard noradrenaline.

No.	Day in confinement	No. of rats used.	Total sympathin µg/day/rat
1	1st	3	5.0
2	4th	3	4.1
3	6th	3	1.9
4	10th	3	1.9

The/

The determination of the sympathin content in the normal and pathological urine by the techniques already discussed.

The results quoted in this section have all been corrected with the recovery percentage figures obtained in analysis, run in parallel with the test samples.

The excretion of sympathin in the urine of normal rats.

Urine samples from normal male rats were collected as described on page 52. Samples in most cases were pooled from three rats, were extracted by von Euler's method, and the extracts assayed on rat's blood pressure preparation. The results are given in Table 14 which show that the mean total sympathin output amounted to 1.9 µg/day/rat, of which some 1 µg was present in the free form, while the remainder existed in a form from which the free amines were liberated by the acid hydrolysis described (p. 46).

TABLE 14/

TABLE 14

Daily urinary output of sympathin of normal rats. 3 male rats (250-260 g, age about 8 months). Urines were pooled. Extraction by von Euler's method. Assay on rat's blood pressure preparation, against standard (-)-noradrenaline. Figures in brackets denote lower and upper limits of the estimates.

Expt No.	No. of rats employed	Sympathin µg/day/rat	
		Hydrolysed urine	Unhydrolysed urine
1	3	1.7 (1.3 - 2.1)	1.2 (0.7 - 1.4)
2	3	1.9 (1.8 - 2.0)	1.1 (0.8 - 1.4)
3	3	1.9 (1.6 - 2.2)	
4	3	1.4 (0.8 - 1.6)	
5	3	2.1 (1.4 - 2.8)	
6	3	1.0 (0.7 - 1.3)	
7	1	3.6 (2.7 - 4.5)	
	Mean	1.94	Mean 1.15
	S.D.	0.82	S.D. 0.07
	Fiducial limits - (P=0.05)	1.19-2.69µg	Fiducial limits - (P=0.05) 0.52-1.78µg

Comparable figures (Table 15) were obtained when the urine samples from the same rats, were extracted by the cation-exchange resin technique, and assayed fluorimetrically and on rat's blood pressure preparation.

TABLE 15/

TABLE 15

Daily urinary output of sympathin in normal rats. Male rats (250-260 g; age about 8 months). Pooled urine mostly. Extraction on 10 cm columns of IRC 50. Assay, fluorimetric and on rat's blood pressure preparation, against standard noradrenaline. The figures in brackets denote the limits of the estimate.

A. Hydrolysed urine.

Expt No.	No. of rats used	Sympathin µg/day/rat	
		Fluorimetric	Rat's B.P. with range
1	3	2.0	1.1 (1.0 - 1.2)
2	6	-	2.0 (1.0 - 4.4)
3	3	2.4	2.0 (1.5 - 2.5)
4	1	1.28	1.35(0.9 - 1.8)
5	1	1.98	2.1 (1.5 - 2.7)
	Mean	1.92	1.71
	S.D.	0.47	0.45
Fiducial limits (P=0.05)		1.17-2.77µg	1.21 - 2.21 µg

B. Unhydrolysed urine.

1	3	1.0	0.9 (0.6 - 1.2)
2	3	0.9	0.8 (0.6 - 1.0)
3	3	-	0.5 (0.4 - 0.7)
4	3	-	0.8 (0.6 - 1.0)
5	3	1.0	0.9 (0.8 - 1.0)
	Mean	0.95	0.78
	S.D.	0.06	0.16
Fiducial limits (P=0.05)		0.81-1.11µg	0.58 - 0.98 µg

Separate determinations of the urinary output of adrenaline and of noradrenaline in 24-hour samples of normal rats were made by extraction of the urine by/

by the cation-exchange resin technique, followed by paper chromatography of the extracts; the adrenaline and the noradrenaline contents of the eluates from the paper were determined fluorimetrically. The results are shown in Tables 16 and 17. Results in Table 16 represented those from the pooled urine of 6 male rats, given in µg/day/rat. Table 17 shows the results in µg/day/rat for the urine of individual rats.

TABLE 16

Daily urinary output of adrenaline and of noradrenaline in normal rats. All male rats, body weight 250-260 g, age about 8-9 months. Samples from pooled urine of 6 rats analysed. Extraction on 10 cm columns of IRC 50, followed by paper chromatographic separation. Fluorimetric assay. Figures in brackets denote percent of the total amines.

	Noradrenaline µg/rat/day	%	Adrenaline µg/rat/day	%	Total (noradrenaline plus adrenaline)
Hydrolysed urine	1.1	(62)	0.67	(38)	1.77
Unhydrolysed urine	0.81	(66)	0.41	(34)	1.22

TABLE 17/

TABLE 17

Daily urinary output of adrenaline and of noradrenaline in normal rats. All male rats, body weight 250-260 g, age about 9 months. Individual sample of urine analysed. Extraction on 10 cm columns of IRC 50, followed by paper chromatography Fluorimetric assay.

Figures in brackets denote per cent of total amines.

No.	Treatment of urine sample.	Noradrenaline			Adrenaline			Sympathin (adrenaline + noradrenaline).		
		µg/day and	% of sympathin	% of the amine in free state.	µg/day and	% of sympathin	% of the amine in free state.	Total µg/day	Free µg/day	% of sympathin in free state.
1	Hydrolysed	1.19 (62)		95	0.72 (38)		32	1.91		71
	Unhydrolysed		1.13 (83)			0.23 (17)			1.36	
2	Hydrolysed	1.95 (75)		67	0.64 (25)		28	2.59		57
	Unhydrolysed		1.3 (88)			0.18 (12)			1.48	
3	Hydrolysed	1.12 (74)		66	0.4 (26)		100	1.52		78
	Unhydrolysed		0.74 (63)			0.44 (37)			1.18	
4	Hydrolysed	1.86 (79)			0.48 (21)			2.34		
	Unhydrolysed		(not done)			(not done)			(not done)	
	Mean	1.53 (73)			0.56 (27)			2.09		
			1.06 (78)			0.28 (22)			1.34	
	S.D.	0.44 (7.3)			0.15 (7.3)			0.47		
			0.28 (13)			0.14 (13)			0.15	
	Fiducial limits (P = 0.05)	0.83-2.23 (61-84)			0.32-0.8 (16-39)			1.34-2.84		

The effect of the subcutaneous injection of 0.5 ml of 0.9% w/v NaCl solution on the urinary output of sympathin in rats has been investigated. Male rats of 250-260 g were used. 0.5 ml of the saline was injected under the skinfold over the nape of the neck of each rat, and the 24-hour sample of the urine collected, and those of 2 rats were pooled. Out of this pool, two 10 ml portions were taken; one portion was hydrolysed; the other not. They were then subjected to extraction on a 10 cm IRC 50 column, and the extract so obtained was assayed fluorimetrically. The results are given in Table 18. The values stated represent the amount of urinary sympathin in µg/day/rat, and are statistically greater than the normal daily excretions. The increase appears to be mainly in the free form.

TABLE 18/

TABLE 18

Sympathin content of 24-hour sample of rat's urine following a subcutaneous injection of 0.5 ml normal saline/rat. Each analysis on pooled urine of 2 rats (250-260 g). Extraction on 10 cm columns of IRC 50. Fluorimetric assay. Figures quoted are µg/rat/day.

No.	Hydrolysed urine	Unhydrolysed urine	Amount of conjugated sympathin.
1	4.3	4.9	(-0.6)
2	4.5	4.2	+0.3
3	2.9	2.4	+0.5
4	3.2	3.0	+0.2
5	2.4	2.5	(-0.1)
6	2.2	2.0	+0.2
7	2.8	2.2	+0.6
	Mean 3.185 = 3.19	Mean 3.028 = 3.03	0.16
	S.D= 0.89	S.D= 1.10	

These figures for the urinary sympathin output following single subcutaneous injection of 0.5 ml 0.9% NaCl were used as control figures for the experiments with subcutaneous injection of adrenaline and of noradrenaline. In all other experiments to be cited hereafter, control values, either with saline (often more than one injection per rat were given

to/

to match the number of injections in the test series) or with some other solvent, were obtained for the rats immediately prior to their being used for the particular drug experiment.

Urinary sympathin excretion in male rats following subcutaneous injections[+] of adrenaline and of noradrenaline.

Subcutaneous adrenaline injection.

As early as 1908 Jackson demonstrated that the pressor effect of adrenaline disappeared in spite of clamping the aorta; thus showing that its excretion through the kidneys was not the only means of inactivation of adrenaline.

Burn (1952) considered that the destruction of adrenaline in vivo depended on the action of amine oxidase.

It would be interesting as well as informative, therefore, to see how much of the injected adrenaline could be recovered from the urine of the injected animals. In this connection, Schayer (1951b) using β-labelled/

[+]As control figures for the urinary sympathin output in these experiments, the values in Table 18 were used. These values represented the output following subcutaneous injections of 0.5 ml of 0.9% w/v NaCl.

β-labelled dl-adrenaline, reported that 0.04 μg adrenaline/g body weight of rats, given intravenously, produced no increase of free or conjugated sympathin in the urine. With 0.3 μg/g body weight, the amount excreted in the free state was equivalent to 6% of that injected, while another 3% in the form of acid hydrolysable conjugated compounds.

In the present work, a subcutaneous dosage level of 1 μg/g body weight was chosen for a pilot experiment. In deciding on this dosage, Trendelenberg's work (1924) and Bovet and Bovet-Nitti's work (1948) were consulted. According to Trendelenberg, the toxic dose for rats subcutaneously was 10 to 20 mg/kg (10 to 20 μg/g), while Bovet and Bovet-Nitti reported the same to be half this range. Our dosage level of 1 μg/g was found to produce marked clinical effects. The rats were observed to be rather ill after the injection, and refused food for two days. Their hairs stood erect, giving a swollen appearance. However, they survived the injection.

Four male rats (230 to 255 g; mean 241 g) were used for this experiment. 24-hour samples of individual rats were hydrolysed and then subjected to extraction on 10 cm IRC 50 resin columns, followed by paper chromatographic separation. The final adrenaline and noradrenaline eluates were assayed fluorimetrically/

fluorimetrically, and Table 19 records these results.

TABLE 19

Urinary excretion (µg/rat) of adrenaline and noradrenaline in the 24-hours following the subcutaneous injection of (-)-adrenaline, 1 µg/g body weight of rat. All male rats (230-255 g., mean 241 g). Mean amount of (-)-adrenaline injected 241 µg/rat. Urine samples hydrolysed, adsorbed on IRC 50 (10 cm column), followed by paper chromatographic technique. Fluorimetric assay. All the rats were rather ill after the injection.

Rat No.	Amount of adrenaline injected. µg	Sympathin in urine for 24-hours following injection.		
		Adrenaline fraction µg	Noradrenaline fraction µg	Total µg
1	255	20	0.54	20.54
2	230	19.7	0.61	20.31
3	240	21.1	1.0	21.10
4	240	23	1.1	24.10
Mean	241	20.95	0.81	21.76
S.D.		1.49	0.28	1.75

Mean value of 7 saline control injections (Table 18).	3.19
Amount of sympathin in excess	18.57
Mean amount of adrenaline injected.	241
% of adrenaline recovered	7.7

Fiducial limits (P=0.05) of noradrenaline in normal rats = 0.83 to 2.23 µg/day/rat (Table 17).

It would be noted that at the dosage level

of/

of 1 μg/g body weight subcutaneously, the rat's kidneys were able to excrete an amount of adrenaline equal to some 8% of the injected adrenaline. The remainder of the injected adrenaline must have been dealt with by other mechanisms of inactivation. No marked change was observed in the noradrenaline output (c.f. Table 17).

In a second experiment, the dosage was reduced to 0.1 μg/g body weight, subcutaneously. The urines of 2 rats were pooled to make the volume big enough to give 2 portions, one for hydrolysed sample and the other unhydrolysed. They were then subjected to the extraction technique on 10 cm IRC 50 columns, and the extracts so obtained were assayed fluorimetrically. The results are given in Table 20. The rats were a little quieter for about one hour after the injection; otherwise, they appeared quite normal.

TABLE 20/

TABLE 20

Urinary sympathin excretion (µg/rat) in the 24 hours following subcutaneous injection of (-)-adrenaline (0.1 µg/g body weight). All male rats (265-270 g). Mean body weight 267.5 g. Mean weight of adrenaline injected 26.75 µg/rat. Urine of 2 rats pooled. Extraction on 10 cm columns of IRC 50. Fluorimetric assay, against standard (-)-adrenaline.

	Hydrolysed urine µg	Unhydrolysed urine µg
	6.65	4.03
	6.25	4.55
	6.32	6.42
	7.42	7.55
	Mean 6.66 (SD 0.54).	Mean 5.64 (SD 1.64).
Mean saline control figure	3.19	3.03
Difference	+3.47	+2.61
Recovery percent	12.9%	9.7%

Subcutaneous noradrenaline injection.

Burn and Robinson (1951) reported that noradrenaline was more easily oxidized than adrenaline by amine oxidase. Trendelenberg (1953) working on the comparative hyperglycemia producing activities of adrenaline and noradrenaline, explained on the same ground, the lower activity of noradrenaline, (being only 18% of that of adrenaline).

Rapid/

Rapid destruction by amine oxidase might therefore explain at least in part, the decreased glycogenolytic activity of the injected noradrenaline. If this assumption be correct interpretation for the phenomena observed, then one should expect very little of the injected noradrenaline to come out in the urine, or proportionately less than that in the case with adrenaline.

The present experiment was designed to find out (a) how much of the injected noradrenaline could be recovered from the urine; and (b) how this excreted amount compared with that of adrenaline of the same dosage level, given by the same route. The rats used were the same rats used in adrenaline experiments, and all the procedures were similar and comparable. The results are given in Table 21.

TABLE 21/

TABLE 21

Urinary sympathin excretion (µg/rat) in the 24 hours following the subcutaneous injection of (-)-noradrenaline, 0.1 µg/g body weight. All male rats, 265-270 g, mean body weight 267.5 g. Mean weight of noradrenaline injected 26.75 µg/rat. Urine of 2 rats pooled. Extraction on 10 cm columns of IRC 50. Fluorimetric assay, against (-)noradrenaline.

	Hydrolysed urine µg	Unhydrolysed urine µg
	7.1	7.4
	7.6	7.5
	7.01	3.14
	6.37	4.52
	6.25	3.9
	5.18	6.1
	6.28	6.13
	Mean 6.54 (SD 0.78).	5.52 (SD 1.64)
Mean saline control figure	3.19	3.03
Difference	+3.35	+2.5
Recovery percent	12.4%	9.3%

It would appear from the results of these experiments that in the rats, the proportion of the subcutaneously injected adrenaline and noradrenaline excreted in the urine, at the level of 0.1 µg/g body weight, is much the same, amounting to some 12% of the injected amine. There was therefore, no evidence of any difference between the two amines in regard to their/

their treatment by the intact rats. The results for both adrenaline and noradrenaline are at variance as to whether the additional amount of the amine appearing in the urine, is wholly in the free state, or partially in a combined form, since in both the sets of experiments, the results of two urine samples showed the presence of supranormal amount of conjugated amines, while the others showed the absence of any conjugated amines. The reason for this difference is not known.

With the higher dosage level of adrenaline (1 µg/g as against 0.1 µg/g), a lower proportion of urinary sympathin excretion was observed. It is possible that this is a reflection of the prolonged renal vasoconstriction consequent to the injection subcutaneously of a large dose of adrenaline, preventing normal urinary secretion, and resulting in an increase in the proportion detoxicated by other mechanisms. And also, the rats were rather ill after the injection, and so possibly the various mechanisms of inactivation might not be functioning in the normal way.

Urinary/

Urinary sympathin excretion in rats following subcutaneous injection of morphine hydrochloride, B.P.

In much of the past work on the effects of morphine in animals, its effects on the sympathetic nervous system has been assessed by the change produced in the blood sugar level. The researches of Elliott (1912), Ross (1918) and Stewart and Rogoff (1922) pointed to the release of adrenal medullary hormone by the stimulating action of morphine, as the cause of hyperglycemia. The two last named workers showed that splanchnicectomy abolished this effect. Bodo, CoTui and Benaglia (1937) demonstrated that denervation or demedullation of the adrenals also abolished this effect in cats and dogs. Chon and Chin (1943) showed that adrenalectomy produced the same effect in rabbits.

This stimulating effect of morphine, resulting in the release of medullary hormone, was studied by Sato and Ohmi (1933) in another direction. Working on non-anaesthetized dogs, they found that the maximum output of the hormone was within 10 minutes of an intravenous injection, and 30 minutes of a subcutaneous injection. But prolonged administration of morphine established tolerance and caused a marked reduction in the release of the hormone,

(Wada/

(Wada et al, 1938).

Working on the effect of morphine on the sympathin contents of adrenal glands, Outschoorn (1951b) showed that with four repeated subcutaneous injections of morphine hydrochloride of 2 mg/100 g body weight of rat, at hourly intervals, there was a depletion of adrenaline in the adrenal glands to 43% of the original control level, at about 4 hours after the first injection. There was no significant change in the noradrenaline level. With a single dose of 2 mg/100 g, there was not much effect on either amine.

In the present study, a subcutaneous dosage of 2 mg/100 g body weight of rats, of morphine hydrochloride, B.P. was used. The drug was freshly prepared just prior to injecting, in aqueous solution. The effect of a single dose was first studied; then of 4 repeated doses given at hourly intervals. Results were recorded in Tables 22 and 23 respectively.

TABLE 22/

TABLE 22

Urinary sympathin excretion (μg/rat) in 24 hours following a single subcutaneous dose of morphine hydrochloride B.P., 2 mg/100 g body weight. Morphine was freshly prepared in aqueous solution. Male rats (260-270 g). Urine of 2 rats pooled. Extraction on 10 cm columns of IRC 50, and assayed fluorimetrically against standard (-)-noradrenaline.

		Sympathin excreted μg/rat/day	
		after morphine	control (saline injection)
Sample 1.	H	1.78	2.22
	U	2.2	2.91
Sample 2.	H	4.85	2.97
	U	3.4	3.45
Mean	H	3.32	2.6
	U	2.8	3.18
S.D.	H	2.17	0.53
	U	0.85	0.38

The differences between the sympathin excretion following morphine and that following saline injections were not significant at $P = 0.05$.

H = hydrolysed urine.

U = unhydrolysed urine.

TABLE 23

Urinary sympathin excretion (μg/rat) in the 24 hours following the first of a series of 4 subcutaneous injections of morphine hydrochloride, B.P., 2 mg/100 g. at hourly intervals. Male rats, 265 - 275 g. Analysis by extraction on 10 cm columns of IRC 50, and assay by fluorimetric method, against standard(-)-noradrenaline.

	Sympathin excreted μg/rat/day					
	After morphine		Control (After saline)		Additional excretion	
Sample 1.						
(H	6.94		3.42		3.52	
(U		5.7		3.1		2.6
Sample 2.						
(H	8.6		4.79		3.81	
(U		8.35		4.68		3.67
Mean (H	7.77		4.1		3.67	
(U		7.03		3.89		3.14
S.D. (H	1.17		0.97			
(U		1.87		1.12		

H = Hydrolysed urine.

U = Unhydrolysed urine.

The sympathin excretion following a single injection of 2 mg morphine hydrochloride/100 g body weight, in rats, showed no statistically significant difference from the control values. However, following the injections of the same dose level, repeated at hourly intervals for 4 hours, the sympathin excretion in the urine showed a definite rise mainly related to an increase in the free sympathin output. Statistically, this rise is significant.

Urinary sympathin excretion in rats following subcutaneous injection of Marsilid (1-isonicotinyl-2-isopropyl hydrazide), Roche.

In 1952, Zeller and his colleagues reported inhibitory action in vitro, of Marsilid on the monoamine oxidase. Zeller and Barsky (1953) reported in vivo inhibition of liver and brain amine oxidase in pigs, rats and guinea-pigs. Schayer and Smiley (1953) reported similar inhibitory action in vivo when tested on rats, using adrenaline as substrate. The inhibition of amine oxidase probably also explains the observations of Schayer (1953) that a Marsilid injection resulted in an increase in the urinary output of tyramine administered to mice. His data showed that following a subcutaneous injection of 100 µg/

100 µg/g of Marsilid, 31% of the subcutaneously injected tyramine could be recovered from the mouse urine; while at a dosage level of 150 µg/g body weight of mice, 45% could be recovered. The control excretion of tyramine was about 20% of the injected dose.

In the present work, experiments were performed on male rats (265-275 g) to see if Marsilid would increase the excretion of the endogenous or the exogenous sympathins. The dosage of Marsilid chosen was 150 µg/g body weight, injected subcutaneously, a dose level found by Schayer (1953) to produce a marked effect on the tyramine excretion in mice.

Table 24 shows the results obtained on the effect of subcutaneous Marsilid on the endogenous sympathin excretion in 24 hours subsequent to the injection of the drug. No significant increase in the excretion over the control level was observed.

TABLE 24/

TABLE 24

Urinary sympathin excretion in rats in the 24 hours subsequent to the subcutaneous injection of Marsilid, 150µg/g body weight. All male rats (265-275 g). Analysis by adsorption on 10 cm. IRC 50 columns and assay by fluorimetric methods, against standard (-) noradrenaline.

		Sympathin excreted µg/rat/day				Remarks
		After Marsilid+		Control After saline		
Sample 1.	(H	3.36		2.43		
	(U		2.78		2.48	
Sample 2.	(H	3.28		2.14		
	(U		2.43		2.48	
Sample 3.	(H	2.02		3.03		
	(U		1.5		2.27	
Sample 4.	(H	3.2		1.85		
	(U		1.62		1.84	
Mean	(H	2.97		2.11		
	(U		2.08		2.27	
S.D.	(H	0.64		0.24		
	(U		0.62		0.3	

+About 40% of masking of the fluorescence was encountered during the assays. The figures quoted are after having been corrected to allow for this.

There is no significant difference in the urinary sympathin excretion after Marsilid injection.

The same rats were used for the study of the effect of Marsilid on the exogenous sympathin excretion. For controls, 3 rats were injected subcutaneously with 0.5 ml 0.9% w/v NaCl, followed in one hour by a subcutaneous injection of (-)-adrenaline, 0.1 µg/g body weight of the rats. Another 3 rats were similarly treated with saline injection, but followed by 0.1 µg/g of (-)-noradrenaline subcutaneously. The urines of the six individual rats for 23 hours were collected, acid hydrolysed (p. 46) and extracted on 10 cm IRC 50 columns. The extracts were then fluorimetrically assayed.

For the test, the first 3 rats were given subcutaneous Marsilid, 150 µg/g body weight, followed in one hour, by subcutaneous (-)-adrenaline 0.1 µg/g. The second set of 3 rats received 150 µg Marsilid/g, similarly, but followed by 0.1 µg/g (-)-noradrenaline. The urine of individual rats for 23 hours was collected, acid hydrolysed, and extracted and assayed as for the controls.

Table 25, A, records the results of the experiment for Marsilid with adrenaline, while 25, B, records those for Marsilid with noradrenaline. The results indicate that some 6% of the injected adrenaline and 8% of the injected noradrenaline were recovered/

recovered in the urine, and the increase in both the cases are statistically significant.

TABLE 25

A. Comparison of the urinary sympathin output in the 23 hours following a subcutaneous injection of (-) adrenaline, 0.1 µg/g body weight in rats which had one hour previously a subcutaneous injection of 0.5 ml of normal saline; with that in the same rats, done one week later, following a similar dose of adrenaline, but having had one hour previously a subcutaneous injection of Marsilid, 150 µg/g body weight. 3 male rats (255-270 g). Urine hydrolysed. Extraction of individual sample on 10 cm column of IRC 50. Fluorimetric assay against (-)-adrenaline. Mean amount of adrenaline injected 26 µg.

	Control (saline + adrenaline) µg/rat/day	Test (Marsilid + adrenaline) µg/rat/day	Difference
Sample 1	4.68	6.42	+1.74
Sample 2	3.43	5.42	+1.99
Sample 3	4.42	5.15	+0.73
Mean	4.176	5.66	+1.49
S.D.	0.66	0.67	

Mean amount of adrenaline injected/rat. 26 µg

Recovery of injected adrenaline. 5.8%

B./

Table 25 Contd:

B. Comparison of urinary sympathin output (µg/rat) in the 23 hours following a subcutaneous injection of (-) noradrenaline, 0.1 µg/g body weight in rats which had one hour previously a subcutaneous injection of 0.5 ml normal saline; with that in the same rats, done one week later, following a similar dose of noradrenaline, but having had one hour previously a subcutaneous injection of Marsilid, 150 µg/g body weight. 3 male rats (255-270 g). Urine hydrolysed. Extraction on 10 cm columns of IRC 50, of individual sample. Fluorimetric assay against standard (-) noradrenaline.

	Control (saline + noradrenaline) µg/rat/day	(Marsilid + noradrenaline) µg/rat/day	Difference.
Sample 1.	5.38	7.57	+2.19
Sample 2.	4.37	6.2	+1.83
Sample 3.	5.25	7.5	+2.25
Mean	5.0	7.09	
S.D.	0.55	0.77	

Mean amount of noradrenaline injected/rat. 26 µg

Amount of noradrenaline recovered. 8.05%

Schayer on the basis of his work (1953) referred to above, considered Marsilid to be a potent inhibitor of amine oxidase. The present results do not appear to fully bear this out. No increase was observed/

observed in the urinary excretion of the endogenous sympathin after the subcutaneous Marsilid. The injection of Marsilid prior to the injection of adrenaline or noradrenaline did, however, appear to increase the urinary sympathin excretion, but not to a marked extent.

It should however, be mentioned at this point that the fluorimetric assays of the urinary extracts in this study are open to some degree of doubt, since some masking (quenching) of the fluorescence of the added adrenaline or noradrenaline was noted with these extracts. The figures quoted are the corrected values, based on the estimate of the degree of masking, which ranged from 30 to 40%.

Urinary sympathin excretion in rats following the subcutaneous injection of insulin, B.P.

Much of the previous work on insulin has been in connection with its influence on the sympathin contents of the adrenal glands, and on the blood sugar level.

Burn, Hutcheon and Parker (1950b) using 0.2 unit/100 g body weight of cat, subcutaneously, found a 50% depletion of the total amount of the medullary hormone in the adrenals after 8 hours. West (1951) also showed a depletion of sympathin contents of

adrenals/

adrenals in rabbits, following insulin injections. Outschoorn (1952) using 1 unit/100 g body weight, subcutaneously, on rats that had been fasted overnight and during the experiment, found that the adrenaline contents of adrenals became 20% of the control at the 12th hour after injection, and 10-20% at the 16th hour. The normal level was restored in 24 hours. The noradrenaline contents of the adrenals showed only a depletion, amounting to less than 50% at the 12th hour, normal levels being reached again, in 16 to 20 hours after the insulin injection.

Holzbauer and Vogt (1954), working on man and dogs, demonstrated that the concentration of plasma adrenaline rose in the venous blood when hypoglycemia was induced with intravenous insulin. No plasma noradrenaline was detected with a method sensitive enough to show up a concentration of 1µg/litre.

It might be argued that if the adrenals poured out their medullary hormone, the plasma contents would then increase which in turn would very likely result in an increased sympathin excretion in the urine. That such was in fact the case, was demonstrated by von Euler and Luft (1952). They reported that in man, insulin at the dosage of 0.1 unit/kg body weight, injected intravenously, increased the/

the urinary adrenaline output by tenfold, but the noradrenaline output was moderately lowered.

To see if such an effect of insulin could be demonstrated in the rats, the following experiments were performed.

Male rats (240-260 g) were fed at 6 p.m. the previous day, and no more food was given until the experiment was over. Drinking water was however provided. For the control, on the day of experiment a single 0.5 ml dose of 0.9% NaCl was given subcutaneously, and the urine of each rat was collected for the following 24 hours, and acid hydrolysed, and extracted by the resin technique, and assayed fluorimetrically. For the test, a single dose of soluble insulin (Burroughs Wellcome) was injected subcutaneously, dosage being 0.5 unit/100 g body weight. During the experiment, signs of hypoglycemia were watched for. In fact, all the rats developed convulsions 2.5 to 3 hours following the injection of insulin. For this, 20 ml 6% glucose per rat were given intraperitoneally. The urine excreted during the 24 hours following the insulin injection was collected, acid hydrolysed, extracted on 10 cm resin columns, and the total sympathin contents of the extracts assayed fluorimetrically against standard noradrenaline. The results of this experiment are shown/

shown in Table 26. They indicate a statistically significant rise in the urinary sympathin output following insulin injection, even though the injected insulin had but three hours to act properly.

TABLE 26

The effect of subcutaneous soluble insulin, 0.5 unit/100 g body weight on the urinary sympathin excretion (µg/rat) in fasting rats, in 24 hours subsequent to the injection. Male rats (240-260 g). No feeding overnight and the day of experiment. Drinking water provided. Urine of individual rat collected for 24 hours, acid hydrolysed and subjected to extraction on 10 cm columns of IRC 50. Fluorimetric assay, against (-)-noradrenaline. Control experiments with 0.5 ml normal saline, done similarly.

Expt No.	Rat No.	Sympathin excreted µg/day/rat	
		Control (after saline injection).	After subcutaneous insulin.
I	1	3.16	6.61
	2	4.95	6.45
	3	3.33	5.25
II	4	-	6.32
	5	-	7.58
	6	-	6.82
		Mean 3.81	6.505
		S.D. 0.99	0.75

The increase in excretion of sympathin after insulin is highly significant. $P < 0.001$.

Part/

Part of the extract from experiment I, rat number 1 (Table 26) was subjected to the paper chromatographic separation process, and the eluates containing the separated adrenaline and noradrenaline assayed fluorimetrically. The results are shown in Table 27.

TABLE 27

Relative proportion of adrenaline and noradrenaline in the extract No.1 of Experiment I, Table 26. Part of the extract obtained by the resin technique was subjected to paper chromatographic separation of adrenaline and noradrenaline. Fluorimetric assay.

Adrenaline fraction µg	Noradrenaline fraction µg	Total µg
4.2	2.4	6.6

It would appear from this pair of figures that there has been a relatively greater increase in the excretion of adrenaline as compared with noradrenaline, judging from the proportions of adrenaline and noradrenaline occurring in the urine of normal rats (Table 17). One must however, consider this single set of results only as indicative, since the results of a number of such experiments, each with appropriate controls/

controls, would be required before any definite assertions can be made on this point. Unfortunately, time has not allowed a further investigation of this.

In the above experiment, the rats had been fasted overnight and no food was given for the day of experiment, with the idea of giving the injected insulin full play to bring down the sugar level of the blood, and so to stimulate the adrenals to release the compensatory amount of medullary hormone for glycogenolysis. It was felt that it would be informative to find out if this still occurred if the rats were fed as usual. To settle this point, another experiment was conducted in which the rats (240-265 g) were fed as usual, and the dose of insulin was 1 unit/100 g body weight, subcutaneously. The urine of 2 rats were pooled to admit of sympathin estimations on both the hydrolysed and unhydrolysed samples of the same specimen. Two 10 ml portions were taken from each pool; one was acid hydrolysed, and the other not. They were then subjected to the extraction on 10 cm IRC 50 columns, and the extracts assayed fluorimetrically.

The results, given in Table 28, show that in normal rats subcutaneous insulin, 1 unit/100 g bodyweight, failed to produce a significant change in the/

the urinary sympathin output, (though there were consistent evidences of a slight increase), contrasting with the significant rise observed when insulin was injected into the starved rats.

TABLE 28

Urinary sympathin excretion (µg/rat) following a subcutaneous injection of soluble insulin, B.P. 1.0 unit/100 g body weight in normal rats in the 24 hours subsequent to the injection. Male rats (240-265 g). Urine of 2 rats pooled. Hydrolysis done on half the number of samples. Extraction on 10cm columns of IRC 50; fluorimetric assay, against standard (-)noradrenaline. Control done on same pairs of rats, with 0.5 ml saline, similarly.

	After control saline µg	After insulin µg	Difference µg
Unhydrolysed urine			
Sample 1.	2.03	2.38	+0.35
Sample 2.	2.21	2.8	+0.59
Mean	2.12	2.59	+0.47
S.D.	0.13	0.30	
Hydrolysed urine			
Sample 1.	2.14	2.48	+0.34
Sample 2.	2.77	3.08	+0.31
Mean	2.46	2.78	+0.32
S.D.	0.45	0.42	

The increase after insulin was uniformly in the right direction, but statistically not significant.

Incidental/

Incidental to these experiments, the effect of starvation for 12 hours prior to and during the 24 hour period of urine collection on the sympathin output of rats was investigated. During the period of starvation drinking water was provided ad lib. A subcutaneous injection of 0.5 ml normal saline was given to each rat at the start of the 24 hour urine collection period. For the control, the same rats were normally fed, and then a subcutaneous injection of 0.5 ml normal saline as for the test rats. In both the control and the test series, the urine of individual rat for 24 hours after the injection was collected, acid hydrolysed, and extracted on 10 cm IRC 50 column, and the extracts so obtained assayed fluorimetrically. Table 29 records the results.

TABLE 29

Comparison of the urinary sympathin (µg/rat) following the subcutaneous injection of 0.5 ml saline/rat for the subsequent 24 hours in normal rats with that of the same in starved rats. Male rats (250-260 g). Urine from individual rat. Hydrolysis done. Extraction on 10 cm columns of IRC 50. Fluorimetric assay against standard (-)noradrenaline. "Starved" rats fasted overnight and no food given during the day of experiment. Drinking water was provided.

Sample No.	Normally fed rats	Starved rats
1	2.14	3.16
2	2.77	4.95
3	-	3.3
Mean	2.455	3.8
S.D.	0.45	0.99

Starved rats excreted uniformly greater amounts of urinary sympathin, but the increase was not significant.

The urinary sympathin outputs of starved rats (having had a subcutaneous dose of 0.5 ml normal saline) are consistently greater than the control values, but the difference is not statistically significant.

Urinary sympathin excretion in rats following the subcutaneous injection of thyroxine.

Spinks and Burn (1952) fed thyroid B.P. to rabbits for two weeks at a dose level of 200 mg per day per rabbit, irrespective of body weight, and found that this caused the amine oxidase contents of the liver to diminish. Trendelenberg (1953), studying the effects of thyroid feeding on the hyperglycemia induced by adrenaline and noradrenaline injections, concluded that thyroid feeding caused a fall of liver amine oxidase contents to about 90% of the control value. All three workers sought to explain the hyperactivity of adrenaline and noradrenaline after thyroid administration as being due to this depression of amine oxidase activity.

In contrast, Schayer (1953) reported that thyroxine by subcutaneous injection had no influence on amine oxidase. In his experiment, he saturated the mice/

mice with repeated subcutaneous injections of thyroxine, and then proceeded to estimate the amount of the subcutaneously injected radioactive tyramine recoverable in the urine. He found that the amount of tyramine recovered from the urine was not different from the control values.

If thyroid or thyroxine has indeed an inhibitor effect on amine oxidase, one might expect these drugs to produce, as a result, an increase in the urinary sympathin output. Accordingly, an experiment was designed to test this hypothesis.

The drug used was (-) thyroxine, sodium salt, (British Drug Houses), having a formula $HO(I_2)C_6H_2O.C_6H_2(I_2).CH_2CH(NH_2).COO.Na$, with a molecular weight of 798.92, given subcutaneously at 1 µg/g body weight of the rats, dissolved freshly in 0.01N NaOH. The concentration of the drug was 1 mg/ml of the Na OH solution. To saturate the rats with thyroxine, it was given to each of the six male rats (265-275 g) at 10 a.m. and 8 p.m. for 3 days and once at 10 a.m. on the 4th day. The urines of the six rats were collected over the 24 hours following the last injection. The urines from two rats were pooled, thus giving three pooled samples. The sympathin contents of each pooled sample were extracted by the IRC/

IRC 50 technique, and assayed fluorimetrically, against (-) noradrenaline.

For the control, corresponding volumes of 0.01N NaOH were given similarly 7 times, and the urine similarly collected, extracted, and assayed. This control experiment was, of course, carried out on the rats prior to the thyroxine administration.

The results of this experiment are given in Table 30. They show the absence of any significant influence of thyroxine administration on the urinary sympathin output in rats.

TABLE 30

Urinary sympathin output in rats following subcutaneous injection of (-)thyroxine. Details of the course of injections are given in the text. Male rats 265-275 g. Urines of 2 rats were pooled. Extraction on 10 cm columns of IRC 50. Fluorimetric assay against standard (-)noradrenaline. Mean weight of rat, at the beginning of experiment = 270 g and at the end of the experiment = 240 g.

Sample	Urinary sympathin µg/day/rat.		
	Control	After subcut. thyroxine.	Difference
Unhydrolysed urine			
1	4.2	3.76	-0.46
2	2.5	2.64	+0.14
3	3.12	3.45	+0.33
	Mean 3.27	3.28	+0.01
	S.D. 0.86	0.76	
Hydrolysed urine			
1	3.42	4.04	+0.58
2	2.8	2.98	+0.18
3	3.46	4.37	+0.91
	Mean 3.23	3.79	+0.56
	S.D. 0.37	0.37	

The differences in both the hydrolysed and the unhydrolysed groups are not significant at P=0.05.

The effect of thyroidectomy on the urinary excretion of sympathin in rats.

Thyroidectomy - surgical procedure.

Nine male rats each of about 300 g body weight were used.

Under ether anaesthesia, the rat was tied in a supine position on a corkboard. The head was fixed to the top of the board by a loop of rubber band, passing over the upper incisors.

A midline ventral incision of the skin was made over the front of the neck, 3 cm long, which would extend a little beyond the upper and lower borders of the submaxillary glands. The salivary glands, after being freed by blunt dissection, were displaced laterally. The sternohyoid muscle was divided in the midline, from the hyoid bone to about half the distance to the manubrium sterni. The muscles were retracted laterally, thus exposing the thyroid glands lying on the antero-lateral aspect of the larynx.

The isthmus of the gland was first identified, and divided in the midline. After locating the recurrent laryngeal nerves, a mosquito forceps was applied to the body of one lobe of the gland, and gently pulled upward and outward, thus exposing the attachments/

attachments of the gland which were severed by dissection with a sharp needle or seeker. While doing this, care was taken to avoid damaging the recurrent laryngeal nerve. This was repeated on the other lobe of the gland. Haemorrhage was slight.

The edges of the sternohyoid muscles were replaced, but not sutured. The skin was sutured with silk (No.00).

Inevitably, the parathyroid glands were removed also, in the process. With bigger rats, tetany in the first two or three days of operation was less likely. As a routine precaution, however, calcium gluconate solution in tap water (0.15%) was provided as drinking water for 5 days.

The rats were kept in a warm room at 21-26°C. They were made use of one week after the operation. The mortality as the result of the operation was one out of nine.

For the control group, rats of same sex and weight underwent a sham operation, and then kept similarly in the warm room.

After the experiments to be described below, the rats were killed and sections of the larynx and adjacent areas were made. Microscopic examination/

examination showed the presence of small areas of thyroid tissues in the angle between laryngeal cartilages, and the secretory glands were small in diameter and only a few of them contained the pink staining colloid materials.

Thyroidectomy in rabbits and rats has been reported to cause the amine oxidase activity of the liver to rise (Spinks and Burn, 1952).

Trendelenberg (1953) found that the rise of blood sugar level caused by adrenaline injection was greater in thyroid fed rabbits, and attributed the cause to the fall of liver amine oxidase activity. The rise of blood sugar caused by adrenaline was less in thyroidectomized rabbits, and was explained (by him) by the rise of amine oxidase. In other words, in thyroidectomized rats and rabbits, there was a rise of amine oxidase contents in the liver. It might be expected, therefore, that there would then be a greater destruction of sympathin, which in turn would lead to a decrease in the urinary sympathin excretion.

The object of the present experiment was to verify this hypothesis, by the determination of the urinary sympathin outputs in thyroidectomized rats and in those having had sham-operations.

In this experiment, six thyroidectomized male rats/

rats and six "sham-operated" male rats of about equal weights were used. As it was difficult to be very certain that all the thyroid tissues had been removed, the thyroidectomized rats were made use of 7 days after the operation, on the assumption that if some small bit of thyroid tissue should remain, it would not have the sufficient time to regenerate sufficiently to interfere with the results. The urines of two rats were pooled to give sufficient working amount of sympathin. Thus 3 pooled samples of urine from the thyroidectomized rats, and 3 pooled samples from the control rats were analysed after acid hydrolysis by the IRC 50 and fluorimetry techniques, for total sympathins. In the fluorimetric assay, noradrenaline was used as the standard. The results were as follows (Table 31).

TABLE 31/

TABLE 31

The effect of thyroidectomy on the urinary sympathin excretion (μg/rat) of male rats. Six thyroidectomized rats (300, 320g) and six "sham"-operated rats (325-335g). Urine of two rats of same set pooled. Hydrolysis done. Extraction on 10 cm resin columns. Fluorimetric assay against standard (-) noradrenaline.

Thyroidectomized rats		"Sham" operated rats (controls)	
Sample No.	Sympathin μg/rat/day	Sample No.	Sympathin μg/rat/day
T_1	3.01	S_1	3.06
T_2	2.86	S_2	2.67
T_3	3.05	S_3	2.6
	Mean 2.97 S.D. 0.10		Mean 2.78 S.D. 0.25

The difference in the sympathin outputs of the two groups is not significant at P = 0.05 level.

The results showed no significant difference in the mean urinary sympathin output of the thyroidectomized rats and the "sham-operated" rats.

The effect of thyroxine-Na was then tried on both the thyroidectomized and the "sham-operated" rats, at 1 μg/g subcutaneously, twice daily for 3 days and a final dose at 10 a.m. on the 4th day, as in/

in the thyroxine experiment on normal rats (Table 30). The drug too was freshly dissolved, just prior to injection, in 0.01 N NaOH. The urines over the subsequent 24 hours were collected, and the total sympathin of the acid hydrolysed urine determined as before.

The results of this experiment are given in Table 32, A. and B. These results were consistently in the direction suggested by the theory, and the increase was significant in the case of sham-operated rats (Table 32,B.), and nearly so in the thyroidectomized rats (Table 32,B.).

Table 32

The effect of subcutaneous thyroxine on the urinary sympathin excretion of thyroidectomized rats and "sham-operated" rats. Details of dosage and administration given in the text. Same pair of rats was used for pooling urine, as for the last experiment (Table 31). Hydrolysis done. Extraction on 10 cm columns of IRC 50, and fluorimetric assay against (-) noradrenaline.

A. Thyroidectomized rats.

Sample No.	Untreated (from Table 31) µg/rat/day	After thyroxine µg/rat/day	Difference µg
T_1	3.01	3.47	+0.46
T_2	2.86	3.05	+0.19
T_3	3.05	3.25	+0.20
	Mean 2.97 S.D. 0.10	3.26 0.21	+0.28

The increase after thyroxine injection is not quite significant (P = 0.1 - 0.05).

B./

Table 32 Contd:

B. "Sham"-operated rats

Sample No.	Untreated (from Table 31) ug/rat/day	After thyroxine µg/rat/day	Difference µg
S_1	3.06	3.58	+0.52
S_2	2.67	3.26	+0.59
S_3	2.6	3.35	+0.75
	Mean 2.78 S.D. 0.25	3.39 0.16	+0.62

The increase after thyroxine is significant, ($P = 0.02-0.01$).

Enucleation of adrenal medulla in rats - surgical procedure.

Under ether anaesthesia, the rat was placed on its belly on a corkboard, with a small round rod under the abdominal region, to cause its back to lordose slightly, and then its hind legs were tied onto the sides of the board.

The lower margin of the last rib (the 13th) was felt for, by palpation, and the angle formed by this rib and the vertebral column was defined, also by palpation.

Then a dorsal midline incision of the skin was made,/

made, about 3 cm long, such that it extended 0.5 cm above the angle aforesaid and 2.5 cm below it. The fascial coverings were similarly incised, and reflected. A mass of muscles could then be felt, quite solid to the palpating finger tip, lying on either side of the vertebral column. Slightly lateral to these muscle masses, white striations would be noticed. About 1 to 2 mm lateral to the whole length of this striated area, the feeling of solidness to the finger tip would be absent. This would form the line of incision to gain access to the peritoneal cavity.

The right adrenal gland was first approached. A longitudinal incision (2 cm long), was made at a position one mm lateral to outer margin of the striated area, parallel to the edge of the same, starting about 2 mm from the angle. On retracting the cut edges, the right kidney would come into view. Following the kidney cephaloward, the right adrenal gland would be found, pea-like in shape, and cheesy in look, embedded in mass of fatty tissues, sitting loosely, on the upper pole of the right kidney, but in 3 cases situated more medially towards the vertebral column than expected.

If the incision was right both as to location and length, there would be no difficulty in locating the/

the gland. If difficulty were encountered, it was found better to enlarge the incision carefully than to search for the gland through a too small opening or an incision made too low. A pair of slender curved forceps was then applied to the penduncular side of the gland and gently pulled. However, it must be held firmly enough or else the gland would slip away.

With the peduncular side of the gland thus clamped, the free border would be facing the operator. A scissors cut, slightly greater than half the circumference was then made on the free border. The cut edges of the gland capsule would retract, exposing the dark glandular tissue portion. A slight pressure with the tip of another forceps on the outer side of the gland would dislodge half the adrenal from either of the half capsules which would remain clean and slightly yellowish in colour.

A similar incision was made on the left side. On retracting the cut edges, a dark pinkish mass of spleen would be seen. On its being pushed laterally, the upper pole of the left kidney would come into view. In the fatty tissues antero-medially to this pole, the cheese coloured left adrenal gland would be seen. It was treated as on the/

the right side.

The glands and the fatty tissues were then returned into the peritoneal cavity. The incision edges in the peritoneum and the muscles were kept in apposition as they were (or with a stitch if the wound was large). The skin incision was then closed with 3 or 4 Michel clips.

During the operation, care was needed to avoid damaging the diaphragm and other tissues. Bleeding was slight. Recovery from anaesthesia occurred within 5-10 minutes, after the end of operation.

Postoperatively, each rat was kept in a separate cage in a warm room at 21-26°C (thermostatically controlled). For the first week after operation, the rats were supplied with 0.6% NaCl for drinking. Food consisted of the usual rat cake (see p.53). The wounds healed in 4-5 days, and the Michel clips were removed. Their body weight was taken before the surgical operation, and then weekly. Regain of body weight and then steady maintenance or gain would indicate that the rats had recovered and were behaving normally.

The rats were made use of 6 weeks after the operation.

Mortality/

Mortality of the surgical operation in the present series was nil out of 8.

Vogt (1952b) showed that during ether anaesthesia on intact rats, the plasma adrenaline concentration varied from 1 to 6 ng/ml; while under similar circumstances, 12 demedullated rats showed no detectable adrenaline in the plasma (the threshold of her last method $\leq$ 0.5 ng/ml of plasma). Normally, ether anaesthesia called forth maximal release of adrenaline. Since there was no detectable amount of adrenaline in the plasma of the demedullated rats, in her experiments, it might be inferred that the adrenal medulla was the source of the plasma adrenaline in intact rats; and that possibly, the chromaffine tissue outside the adrenal glands did not release any adrenaline in the rats.

In this work, the urinary sympathin excretion of demedullated rats per se was studied first; then the effect of subcutaneous morphine on the excretion; and thirdly, the effect of ether anaesthesia on the excretion, in which case, its effect on normal rats was also investigated on four male rats of similar weights.

According to Ingle and Griffith (1949), tiny islands/

islands of chromaffine tissues may frequently be found in the regenerated adrenal tissues, but they are never of significant amount to be of functional significance. In the present study, the demedullated rats were killed at the end of the experiments and sections of their adrenals were studied microscopically. No medullary tissues were detected in any of the sections.

Urinary sympathin output in demedullated rats.

Urine was collected for 24 hour period from each of the six male rats which had been demedullated six weeks previously. The urines from two rats were pooled. The three pooled samples were acid hydrolysed, and the sympathin contents extracted on 10 cm IRC 50 columns. Part of the extracts from this were assayed for total sympathins fluorimetrically. Part of the extracts were submitted to paper chromatography, and the separated adrenaline and noradrenaline eluates assayed fluorimetrically. In addition, the adrenaline eluates were also assayed on the isolated uterus of the rat.

The results given in Table 33 show the absence of any detectable adrenaline in the urine by demedullated rats. The noradrenaline values lay within the/

the range of that for normal rats.

TABLE 33

Urinary sympathin excretion, µg/rat/day, of male demedullated rats. 6 male demedullated rats (315-325 g). Urines of 2 rats pooled, and hydrolysed. Extraction on 10 cm columns of IRC 50. Part of this extract was assayed fluorimetrically against noradrenaline (= Result A). Part of the same, subjected to paper chromatographic separation into adrenaline and noradrenaline fractions, and assayed fluorimetrically (= Result B). The adrenaline fraction was further assayed on isolated rat's uterus.

Sample No.	A. Extract from IRC 50 column.	B. Paper chromatographic eluates.		
	Sympathin µg/day/rat (fluorimetric)	Noradrenaline µg/day/rat (fluorimetric)	Adrenaline µg/day/rat (fluorimetric)	Rat's uterus
1	1.05	1.26	< 0.06	< 0.008
2	0.80	0.7	< 0.14	< 0.009
3	0.93	1.0	< 0.19	< 0.009
	Mean 0.93	0.99		
	S.D. 0.13	0.28		

Mean noradrenaline excretion in hydrolysed urine by normal intact rats per day = 1.53 µg/rat.

(Fiducial limits 0.83 to 2.23 µg, P = 0.05). (From Table 17).

Urinary/

Urinary sympathin excretion in demedullated rats, following repeated subcutaneous injection of morphine hydrochloride, B.P.

Six demedullated rats were injected with morphine hydrochloride B.P. (freshly prepared in aqueous solution) at the dose level of 2 mg/100 g body weight, subcutaneously. Four such doses at hourly intervals were given to each rat. Urine was collected for the 24 hours following the first dose. The urines from two rats were then pooled; in so doing, care was taken to pool only the urines of the same pair of the rats as used in the previous experiment. The three pooled samples were then acid hydrolysed and subjected to extraction process on 10 cm IRC 50 columns, and the extracts so obtained were further subjected to paper chromatography. The adrenaline and noradrenaline eluates were assayed fluorimetrically. The adrenaline eluate was, in addition, assayed on the isolated uterus of rat. The results are shown in Table 34. Again, as in the previous experiment, no detectable amount of adrenaline was present in the urine. The noradrenaline excretion showed a small but significant increase over that of the demedullated rats, untreated with morphine.

TABLE 34/

TABLE 34

Urinary sympathin excretion (μg/rat) of demedullated rats in 24 hours following the first of a series of 4 subcutaneous injections of morphine hydrochloride, B.P. (2 mg/100 g body weight), at hourly intervals. The same demedullated rats as used for results given in Table 33. Same pair of rats used for pooling urine too. Hydrolysis done. IRC 50 extraction (10 cm column), followed by paper chromatography. Eluates assayed fluorimetrically. Adrenaline eluate was assayed on isolated rat's uterus in addition.

Sample No.	Noradrenaline fraction μg/rat (fluorimetric)	Adrenaline fraction μg/rat (fluorimetric)	Adrenaline fraction μg/rat (Rat's uterus)
S_1	1.56	< 0.1	< 0.002
S_2	1.67	< 0.1	< 0.002
S_3	1.63	< 0.1	< 0.002
	Mean 1.62		
	S.D. 0.06		

Compared to noradrenaline excretion figures for untreated demedullated rats (Table 33), noradrenaline figures here are significantly greater.

Urinary/

Urinary sympathin excretion in demedullated rats following the exposure to ether, and a comparison with the excretion of normal rats treated similarly with ether.

Four normal male rats (325-335 g, mean 330 g) were each put into a closed box, normally used for anaesthetizing small animals with ether or chloroform, and exposed to ether fumes. Their reaction to the ether was observed through the perspex lid of the box. From the time the rat fell, and on rolling the box, it did not right itself, 10 minutes were allowed to pass, and the rat taken out and put into the metabolic cage. The urines of each rat for the following 24 hours were collected. The urine of 2 rats were pooled, and acid hydrolysed, and extracted on 10 cm resin columns. The extracts so obtained were next subjected to paper chromatography, and the adrenaline and noradrenaline eluates assayed fluorimetrically against standard adrenaline and noradrenaline respectively. The results are shown in Table 35. The adrenaline excretion was found to be increased and statistically significant.

The noradrenaline excretion was within the range of that for normal rats.

Four demedullated rats (315-325 g) were similarly treated, and the adrenaline and noradrenaline eluates/

eluates, similarly made, were assayed fluorimetrically. In addition, the adrenaline eluate was assayed on the isolated rat's uterus preparation. The results, recorded in Table 36, show the absence of detectable adrenaline, in the urine. The noradrenaline figures are statistically not different from those of non-etherized demedullated rats.

TABLE 35

Urinary sympathin excretion (µg/rat) of normal intact rats in the 24 hours following a 10 minute period of anaesthesia induced by ether anaestheticus B.P. 4 normal male rats (325-335 g). Anaesthesia induced in a closed box. Urines of 2 rats pooled, and hydrolysed. IRC 50 extraction on 10 cm columns followed by paper chromatography. Fluorimetric assay of the eluates.

Sample No.	Noradrenaline fraction µg/rat	Adrenaline fraction µg/rat
1	1.17	0.88
2	1.05	1.06
	Mean 1.11	0.97
	S.D. 0.08	0.13

Daily normal excretion of:

noradrenaline = 1.53 µg (Fiducial limits 0.83-2.23 µg) (P = 0.05)

adrenaline = 0.056 µg (Fiducial limits 0.32-0.8 µg) (P=0.05) (See Table 17).

TABLE 36/

TABLE 36

Urinary sympathin excretion (µg/rat) of demedullated rats in 24 hours following a 10 minute period of anaesthesia, induced by ether anaestheticus, B.P. 4 demedullated rats (315-325 g). Anaesthesia induced in a closed box. Urines of 2 rats pooled and hydrolysed. IRC 50 extraction on 10 cm columns followed by paper chromatography. Fluorimetric assay of the eluates. The adrenaline eluate was further assayed on isolated uterus of rat.

Sample No.	Noradrenaline fraction µg/rat (fluorimetric)	Adrenaline fraction µg/rat (fluorimetric)	Adrenaline fraction µg/rat (Rat's uterus)
1	0.96	< 0.06	< 0.007
2	0.83	< 0.05	< 0.007
	Mean 0.895		
	S.D. 0.09		

The noradrenaline figures are not significantly different from those of non-anaesthetized demedullated rats.

Influence of cohabitation of rats on the urinary sympathin output.

The aim behind this experiment was to find out what effect on the urinary sympathin excretion would be when a male rat and a female rat were kept together in the same metabolic cage. It might be supposed that the cohabitation of the male with the female would result in an increased sympathetic activity in the two animals, and hence lead to a greater/

greater urinary secretion of sympathins.

For this experiment, there were some technical difficulties which had first to be overcome. In all other experiments, only one rat was kept in a cage, and the daily urinary sympathin excretion figures were for rats so confined. In this experiment, two rats were kept together in the same cage, thus introducing the factor of crowding. To obtain some indication of the influence of this factor alone on the urinary sympathin excretion, the following experiment was carried out. Two rats of about equal weight, and of the same sex were kept in one of the metabolism cages for 7 days, to get them used to this crowding. This lengthy period of conditioning was necessary for another reason; namely, male rats did not seem to like each other when first put together in the same cage. They did not actually fight but they were seen to rear up on their hind legs, facing each other, with the front legs touching, on the slightest "provocation", such as one rat treading accidentally the tail or hind part of its cage mate. This happened very frequently on the first day. Female rats did not show this unfriendly behaviour.

On the 8th day, 24 hour samples of urine were collected/

collected; the two samples from the two cages housing two male rats each, were pooled, extracted with IRC 50, and assayed fluorimetrically. Similarly, the urinary sympathin in two samples from the two sets of females was determined. From the results the amount of urinary sympathin excreted in 24 hours by one male rat was calculated. Similarly, the figure for one female rat was also calculated. The sum of these two calculated figures would then form the control figure for the test where one male and one female rat would be kept together. Results for the controls were as follows:

TABLE 37

Urinary sympathin output of rats when two of the same sex were housed together in an individual metabolism cage. 4 male rats and 4 female rats* (240-250g), 2 of the same sex in one cage. Urine collected on the 8th day of confinement, 24 hour sample. Extraction on 10 cm columns of IRC 50 and fluorimetric assay of the extracts against standard noradrenaline. The figures represent calculated value for urinary sympathin in µg/day.

Sex	Hydrolysed urine	Unhydrolysed urine
Per one female rat	1.72	1.56
Per one male rat	1.83	1.65
∴ one male + one female rat	3.55	3.21

*Griffith and Farris (1942).

Lordosis test and ear quivering test for the presence of oestrus (not sure tests) were negative. Ear quivering test is elicited by stroking the rat gently on the head or back. Lordosis test is elicited by pelvic digital stimulation.

On/

On the 9th day, one male rat from one cage was exchanged with one female rat from another cage, thus forming pairs of different sexes. Unlike the control portion of the experiment, there was no conditioning period; for, it was observed that the male rats started their "courtship" (sniffing at female hind parts, licking, playing and caressing) immediately on entering the cage. This was the very period the effect of which, this experiment was intended to measure. 24-hour samples of urine were collected. The urine from two cages were pooled; thus yielding two pooled samples, each from two male and two female rats. This was done in uniformity with the control part of experiment. They were then respectively subjected to resin extraction and the extracts assayed fluorimetrically. The result obtained for each set represented the sum of the urinary sympathin excreted by two male and two female rats. Half this amount represented the sum of sympathin excreted by one male and one female rat. The results were as follows:

TABLE 38/

TABLE 38

Urinary sympathin output of 2 rats of opposite sex when housed together in the same cage.

Calculated value of urinary sympathin in µg/day.

Sex	Hydrolysed urine	Unhydrolysed urine
Per one male + one female rat	3.8 4.3	2.8 3.5
	Mean 4.05	3.15

The mean of control figures, and the mean of the test figures are given for comparison in Table 39.

TABLE 39

Comparison of mean urinary sympathin output of 2 rats of same sex with that of 2 of opposite sex when the pair were housed together in individual cages.

Mean urinary sympathin in µg/day/2 rats.

Pair of	Hydrolysed urine	Unhydrolysed urine
Same sex	3.55	3.21
Opposite sex	4.05	3.15
Difference	+0.50	-0.06

It will be apparent that there was no marked difference/

difference between the urinary sympathin output of a pair of rats of the same sex and that of a pair of opposite sex.

This question was not pursued further, as the number of experiments necessary to yield a sufficient number of results to detect minor differences, if any, would have been too time consuming.

Urinary sympathin excretion in human urine.

Pink disease.

The main clinical features of pink disease are:

(1) Appearance of abject misery, with high irritability.

(2) Loss of appetite.

(3) Photophobia.

(4) Insomnia and restlessness.

(5) Pinkness of extremities when warm; cyanotic when cold.

(6) Non-pitting oedema of extremities, with the skin taut and shiny and often desquamation.

(7) Sensory changes in older children.

(8) Excessive sweating and prickly heat.

(9) Hypotonia.

(10) Tachycardia.

(11) Raised diastolic pressure.

(12) Initial pyrexia.

(13)/

(13) Leucocytosis, polycythemia and hemoconcentration.

Pheochromocytoma, with its paroxysmal hypertension, tachycardia, sweating and cold extremities, resembles pink disease a great deal clinically.

Most of the clinical features of pink disease, notably hypertension, tachycardia, and excessive sweating, seemed to indicate hyperactivity of the sympathetic system. Feer (1925), Blackfan and McKhann (1933) held a similar view, but the latter two workers thought there was involvement of the parasympathetic system as well, because of the increased salivation and lacrimation. Day and his colleagues (1939) observed that the tachycardia could not be increased by emotion, (hence their conclusion that the sympathetic tone was at its maximum); and that atropine caused further increase in the already fast pulse rate, (hence their conclusion that the vagi were functioning).

Histological evidences of lesions in the autonomic nervous system were conflicting. Pehu, Dechaume and Boucomont (1936) believed that there were widespread lesions in the sympathetic nervous system, including the hypothalamus. Lubin and Faber/

Faber (1938) however, disagreed with this view. Further research on this would be necessary before any definite conclusion could be drawn.

Vulliamy's work (1952) appeared to be a useful pointer. He demonstrated that the coldness of the limbs in pink disease was due to excessive vascular tone, causing vasoconstriction of arterioles with capillary dilatation, and not due to any structural defect of the blood vessels, such as occlusion. He considered that excessive vascular tone could be due (1) to the stimulation of the nerve cells at or above the sympathetic ganglia, leading to an increased flow of impulses to the periphery; and (2) to certain vasoconstrictor substances circulating in the blood. In this latter connection, he suggested that adrenaline and noradrenaline might be the substances concerned.

As early as 1928, Kuhl suggested the possibility of hypersecretion of the adrenal medulla. In support of this, Woringer's report (1926) might be cited; he found in one case hypertrophy of adrenal medulla. But the single case we have studied showed no such histological changes. However, it was a noteworthy fact, in this same case, that the sympathin content of the adrenal glands was only 8.3 μg/g of the fresh gland tissue, as compared to some/

some 30 µg/g of fresh tissue, reported by Shepherd and West (1951). This marked depletion of the sympathin contents was quite consistent with the theory of hypersecretion, leading to near exhaustion. But it must be pointed out here that the postmortem for this case was done 12 hours after death, and the depletion might have been due to postmortem changes.

In 1951, Cheek determined the sympathin content in the blood of pink disease cases, but was unable to detect any increase.

In 1951, J.H. Burn suggested estimation of urinary sympathin contents, and G. Burn and his colleagues (1953) conducted the assay on two cases. They found the equivalent of 16 µg and 21 µg noradrenaline/day, respectively, and concluded that there was no increase, since the urinary output of sympathin in normal children of a similar age was found to be equivalent to about 18 µg noradreanline/day.

In the present work, urine samples from twenty normal children, and those from two cases of pink disease were analysed. The normal children group consisted of (1) children who were apparently well and healthy, being inmates of charity homes, and (2) children who were being treated in hospitals for minor/

minor scald or burn and had been declared "cured" and were discharged from the hospital immediately after the urine samples had been collected, for the purpose of this work. Only male children were used, as the collection of urine samples from female children was not satisfactory.

24-hour urine samples were collected by means of a rubber receptacle fixed to the genital area, much like the suprapubic drainage apparatus (described on p. 58), with the drainage tube dipped directly into the collecting bottle which contained 5 ml of 2N H_2SO_4 (A.R.) and 0.5 ml of chloroform (A.R.). The resulting pH was round about 3.5. The urine samples were kept at 5°C until they could be worked on.

One sample of the urine from a male infant of 11 months, was extracted by von Euler's method. 50 ml of the urine were acid hydrolysed, and the extract obtained with this technique was assayed on rat's blood pressure preparation, against (-)noradrenaline as the standard. It was found to contain an equivalent of 11.75 µg per day (range 7.9 to 16.6 µg).

Fourteen samples of 24-hour collection from fourteen male children (6 to 18 months) were analysed by the IRC 50 and fluorimetric techniques. Two portions/

portions of 50 ml were taken of each specimen; one was acid hydrolysed, and the other was not. They were then subjected to extraction process on 20 cm IRC 50 columns and the extracts so obtained were assayed fluorimetrically, against (-) noradrenaline as the standard. The results are recorded in Table 40.

Five samples of 24-hour collection from five male children (age 10-12 months) were also analysed. Two portions of 50 ml were taken of each specimen; one was acid hydrolysed, and the other not. They were then subjected to extraction procedure on 20 cm IRC 50 columns. The extracts so obtained were further subjected to paper chromatography, and the separated adrenaline and noradrenaline eluates were assayed fluorimetrically against standard adrenaline and noradrenaline respectively. The results are shown in Table 41.

TABLE 40/

TABLE 40

Urinary sympathin excretion by 14 normal male infants. 50 ml aliquot of urine. Sympathin extracted on column of IRC 50 (20 cm). Extracts assayed fluorimetrically against standard (-) noradrenaline.

No.	Age (months)	B. wt. (kg.)	Volume of urine (ml)	Sympathin per 24 hours (µg).		% free sympathin	REMARKS
				Hydrolysed urine	Unhydrolysed urine		
1	6	6.5	405	13.9	11.6	84	
2	6	7.45	645	23.4	13.4	58	
3	6	7.2	370	22.8	14.3	63	
4	9	10.3	430	16.3	11.7	72	
5	9	10.0	410	15.4	12.7	83	
6	10	8.8	360	16.7	15.3	92	
7	12	10.4	370	13.3	10.8	81	
8	12	10.4	370	14.4	11.0	77	
9	12	9.6	152	16.8	16.3	97	
10	12	7.25	418	13.1	10.1	77	
11	12	7.75	570	18.1	16.3	90	
12	13	10.0	305	17.6	14.9	85	
13	13	6.4	448	7.6	6.4	84	
14	18	10.9	199	21.5	20.4	95	
Mean	10.7	8.78	389.4	16.49	13.23	81	
S.D.				4.2	3.41	11.45	
Fiducial limits (P=0.05)				14.1 to 18.9	11.3 to 15.2	74 to 88	

TABLE 41/

TABLE 41

Urinary excretion of adrenaline and of noradrenaline by normal male infants. 24-hour urine samples from five infants (age 10-12 months). Extraction on 20 cm columns of IRC 50, and the extracts were further subjected to paper chromatographic separation into adrenaline and noradrenaline. Fluorimetric assay. Figures in brackets denote the relative proportion of each amine.

No.	Age (mths)	B. wt. (kg)	Volume of urine in 24-hours. (ml)	Hydrolysed urine (µg)			Unhydrolysed urine (µg)			Remarks
				Adrenaline	Noradrenaline	Total	Adrenaline	Noradrenaline	Total	
1	10	5.45	475	8.28 (45%)	10.2 (55%)	18.48	3.86 (37%)	6.72 (63%)	10.58	Figures in brackets = %; rounded off to nearest integer.
2	10	5.5	540	8.75 (46%)	10.3 (54%)	19.05	3.74 (32%)	7.9 (68%)	11.64	
3	12	9.1	495	5.9 (34%)	11.4 (66%)	17.3	2.37 (24%)	7.35 (76%)	9.72	
4	12	9.1	485	5.5 (36%)	9.8 (64%)	15.3	3.02 (30%)	7.05 (70%)	10.07	
5	12	9.4	670	8.2 (35%)	15.2 (65%)	23.4	5.53 (30%)	13.0 (70%)	18.53	
Mean	11.2	7.71	533	7.33 (39%)	11.38 (61%)	18.71	3.70 (31%)	8.4 (69%)	12.1	
S.D.				1.51	2.22	2.99	1.18	2.61	3.31	
Fiducial limits (P=0.05)				5.44-9.22	8.6-14.16	14.99-22.43	2.23-5.17	5.16-11.64	7.93-16.29	
				Mean Noradrenaline % = 61			Mean Noradrenaline % = 69			
				S.D. =5.8			S.D. =4.67			
				Fiducial limits 53-68 (P=0.05)			Fiducial limits 63.6-75. (P=0.05)			

According to Burn (1953) the mean noradrenaline equivalent of total sympathins in normal children's urine, aged from 3 months to 10 years (mean 6 years) (9 cases in all), using von Euler's method of extraction and assay on cat's blood pressure preparation, was 18 µg/day. Our results, shown in Tables 40 and 41 are similar. The total sympathin output in the urine of the 14 children in our series (Table 40) is equivalent to about 16 µg/day in terms of noradrenaline, with a standard deviation of about 4 and fiducial limits of 14 to 19 µg/day ($P = 0.05$); of which amount some 80% occurred as free sympathins, (SD about 11; fiducial limits, 74 to 88) ($P = 0.05$).

In the five samples of urine (Table 41) the total sympathin content in the hydrolysed urine was equal to a mean of about 19 µg/day, with a standard deviation of about 3; of which amount some 60% were noradrenaline. In the unhydrolysed urine, the daily sympathin output was about 12 µg/day, with a standard deviation of about 3, and some 70% of it were noradrenaline.

Urinary sympathin excretion in children suffering from pink disease.

A pilot experiment was done on the hydrolysed sample of the urine from a case of pink disease, male/

male, aged one year, using von Euler's method of extraction for the total sympathin content from 100 ml of the urine. The extract so obtained was assayed on rat's blood pressure preparation, against standard (-)noradrenaline. Table 42 records the result of this experiment. The urinary sympathin was found to be considerably in excess of the normal.

TABLE 42

Pilot assay. Urinary sympathin excretion in a case of pink disease.

Patient J.C. Age one year, male, 24-hour sample. 100 ml urine. Hydrolysed and extracted by von Euler's method. Assay of extract on rat's blood pressure preparation against (-) noradrenaline.

Sympathin excretion µg/day (noradrenaline equivalent).	Range of estimate µg
74.24	55.7 - 92.8

More systematic studies were made on a second case. This was a male child of six months. In all, three 24-hour samples of urine were collected, the second and third samples being collected 14 and 16 days respectively after the first. Patient had fever/

fever and loose motion the day after the first sample of urine. Fever subsided four days later. A lapse of ten days occurred before the second sample was collected. The sympathin contents of 100 ml aliquots from each sample, both before and after acid hydrolysis, were determined by adsorption on 20 cm columns of IRC 50, and fluorimetric assay of the extracts. In addition, part of the extracts from the first and second samples was also subjected to paper chromatography, and the adrenaline and noradrenaline eluates assayed fluorimetrically. The results of this study are shown in Table 43.

The sympathin contents of the first and third samples are significantly higher than the normal, while that of the second was about normal.

It would have been good to have more cases and more results to settle the question as to whether there really was such a variation in the urinary sympathin excretion. Unfortunately, no more cases of pink disease were available.

TABLE 43/

TABLE 43

Urinary sympathin excretion (μg/day) in a case of pink disease.

Patient I.R., male, aged 6 months; body weight 5.7 kg. No history of ingestion of teething powder, or grey powder, or of use of mercury ointment. Body temperature around 37°C. pulse around 140/minute: blood pressure about 130 mm Hg during the times of this investigation. Three 24-hr sample analysed. 2nd sample and 3rd sample, 14 and 16 days respectively after the 1st sample. 100 ml aliquots of urine extracted on 20 cm columns of IRC 50. Part of the extract was assayed fluorimetrically against (-) noradrenaline; part was further subjected to paper chromatography, and the eluates assayed fluorimetrically.

H = hydrolysed urine. U = unhydrolysed urine.

Sample No.	Treatment to urine.	Extract from IRC 50	Eluates from paper chromatogram		
		Sympathin μg/day	Adrenaline fraction μg/day	Noradrenaline μg/day	Total (adrenaline + noradrenaline). μg/day
1	H	41	13.9	26.8	40.7
	U	41	11.2	26.2	37.4
2	H	21.5	4.1	15.4	19.5
	U	20	3.7	13.6	~~16.7~~ 17.3
3	H	49	(not done)		
	U	42			

Urinary sympathin excretion in patients suffering from coronary thrombosis.

Selzar (1952) reported the presence of hypotension in 69 out of 528 cases of myocardial infarction. The hypotension in these cases appeared to be due to:

(a) Peripheral vasomotor failure; and

(b) Acute left ventricular failure.

These two causes probably acted in varying proportions in a given case.

Of late, treatment of this hypotension with (-) noradrenaline bitartrate has been resorted to with the idea of producing vasoconstriction of the peripheral blood vessels but without increasing the cardiac output (Goldenberg et al. 1949; Miller and Baker, 1952; Sampson and Zipper, 1954).

Clinically, hypotension associated with coronary thrombosis might be divided into four groups:

(a) immediate and brief:

(b) immediate and irreversible:

(c) coinciding with, and apparently due to cardiac arrythmias; and lastly,

(d) delayed, associated with congestive heart failure.

In any given case of hypotension, varying degrees/

degrees of fault might be found in the central pump action of the heart, and in the peripheral vessel tone. In cases especially those that righted themselves, with or without the conventional treatment, it was not unreasonable to suppose that the body defence mechanism would step in to correct the hypotension by increased release of sympathin. It might be expected that this increased sympathin production would be reflected in an increased sympathin excretion in the urine; and in fact, Forssman et al (1952) reported this increase in all the fourteen of their cases.

The aim of this experiment was to verify whether or not there was this sympathin increase.

Urine of coronary thrombosis cases was collected as soon as practicable after the case was admitted into the hospital. Samples were collected for 24-hour periods for three to six consecutive days, and the sympathin contents determined by extraction with IRC 50, and the extracts assayed fluorimetrically against standard noradrenaline. For some unknown reasons, the extracts for the second case contained materials that caused quenching of the fluorescence of the added sympathin, and so the extracts were also assayed on rat's blood pressure preparation for/

for confirmation of the results obtained fluorimetrically.

Cases were classified on clinical basis into three groups for the purpose of the present investigation. Group 1 - cases showing mild clinical manifestations:

Group 2 - moderately severe cases:

Group 3 - seriously ill and in "shock".

Salient points in the conventional treatment given in these cases were: rest in bed, heparine, morphine and phenobarbitone. The actual dosages of each drug will be indicated later.

So far, only three cases have been studied, one from each group. The results were given in Tables 44, 45 and 46.

According to Burn (1953) using von Euler's method of extraction and assay on cat's blood pressure preparation, the mean urinary excretion for adults of 50 years (SD 6-7 cases) is about 80 µg/day (SD 38.6) in terms of (-) noradrenaline. The results in case (1) of some 65 µg/day (SD 32) are comparable to Burn's figure. In the second case, (Table 45) the mean daily urinary sympathin excretion is about 250 µg with a standard deviation of 80 for the/

the first four days of observation. With congestive heart failure setting in on the fifth day, the urinary sympathin excretion became some 50 μg/day. In the third case, 145 μg and 101 μg/day were excreted for second and third day of observation. That for the first day, while patient was in a condition of shock, was only 23 μg/day.

TABLE 44/

TABLE 44

Urinary sympathin excretion (μg/day) in a patient suffering from coronary thrombosis.

Case 1. Clinically, mild attack. No shock, no fever, pulse rate normal; blood pressure, little change. Patient aged 75 years, male, body weight 68 kg. Had three previous attacks. During period of this investigation, has had morphine 15 mg (only one dose, on admission); phenobarbitone 30 mg t.i.d. Heparine 20,000 i.v., i.m., only 3 doses at 8 hourly intervals on admission. Rest in bed. Urine collection started 7-8 hours after onset of symptoms. Six of 24-hour samples analysed. 100 ml aliquots, extracted on 20 cm columns. Fluorimetric assay against (-)-noradrenaline.

Amount of sympathin in μg/day.

Day	Volume of urine for the day. (ml)	Unhydrolysed urine	Hydrolysed urine	% free sympathin
1	542	38.2	41.2	93
2	1440	113.8	113.0	100
3	1365	68	59.3	(114)
4	890	34.9	39.8	88
5	1305	94.5	98	96
6	836	43.1	47	92
	Mean 1063	65.4	66.4	93.8
	S.D.	32.76	31.43	4.5
	Fiducial limits (P=0.05)	31.8-99	34.98.8	

TABLE 45/

TABLE 45

Urinary sympathin excretion (µg/day) in a patient suffering from coronary thrombosis.

Case 2: Clinically, "moderately severe". Developed congestive heart failure on 4th/5th day of investigation, with diminution of urine output to 210 ml/day. During the period of investigation, has had morphine 15 mg on admission. Heparin 20,000 i.u., i.m., only 3 doses at 8-hourly intervals. Phenobarbitone 30 mg t.i.d. (Digoxin on 5th/6th day). Rest in bed.

Urine collection and extraction similar to Case 1.

Five samples of 24-hour periods analysed. Assayed fluorimetrically and on rat's blood pressure preparation, against (-) noradrenaline.

Fluorimetric assay figures are values after being corrected for masking (about 20%). The result for the 5th day has been excluded from statistical calculation.

Figures in brackets denote limits of estimate.

Day	Volume of urine for the day. (ml)	Unhydrolysed urine µg/day		Hydrolysed urine µg/day		% free sympathin+
		Fluorimetric	Rat's B.P.	Fluorimetric	Rat's B.P.	
1	970	296	303 (202-404)	314	301 (151-452)	94
2	740	156	202 (112-292	152	154 (70-224)	100
3	1050	179	177 (88-265)	230	241 (110-351)	78
4	720	175	173 (87-260)	322	300 (150-450)	54
	Mean 870	201.5	214	254.5	249	81.5
	S.D.	63.8	-	80	-	-
	Fiducial limits (P=0.05)	100.5-302.5	-	127.5-381.5		
5	210	49.5	54 (25-79)	(not done)		

+Based on fluorimetric results.

TABLE 46

Urinary sympathin excretion (μg/day) in a case of coronary thrombosis.

Case 3: clinically, "in shock". B.P. 140/106 on admission; 96/78 2nd day; 90/70 on 3rd day. Heart rate 120-160/minute.

Patient, male, aged 43 years. Body weight 60.5 kg. During the period of investigation, has had morphine 30 mg on admission; pethidine 100 mg the same night (1st day's urine relates). Digoxin 1 mg i.v. on 2nd day + 0.25 mg of same orally q.i.d. Also pethidine 100 mg; seconal 180 mg; sodium amytal 180 mg; mephentermine sulphate (Wyeth)* 60 mg + 30 mg + 30 mg + 45 mg (2nd day's urine relates). Digoxin 0.25 mg q.i.d. seconal 180 mg; pethidine 100 mg twice; sodium amytal 180 mg x 2 doses. Mephentermine sulphate 60 mg (3rd day's urine relates).

Three samples of 24-hour urine analysed. 100 ml aliquot, hydrolysed and extracted on 20 cm columns of IRC 50, and the extracts assayed fluorimetrically. Part of the extracts were subjected to paper chromatographic procedure, and the proportion of adrenaline and noradrenaline determined.

Day	Urine volume for the day. (ml)	Sympathin in hydrolysed urine. (μg)	Proportion of adrenaline. (%)
1++	130	23	38
2	955	145	37
3	730	101	47
	Mean 842.5	123	42
	S.D.	31.1	

*N - methylphenyl-tertiary-butylamine sulphate, made by Wyeth Inc., U.S.A. Said to have pressor action approximating "ephedrine effect on blood pressure", but did not appear to be effective in this case.

++1st day's result, not included in the statistical calculation.

DISCUSSION

In this discussion, the term "sympathin" has been used to indicate mixtures of adrenaline and noradrenaline in varying proportions.

Methods of assay of adrenaline and noradrenaline in urinary extracts.

Two types of method for the assay of adrenaline and noradrenaline in urinary extracts have been used in the present study, namely, chemical and biological.

The chemical method (Lund, 1949, 1950) depends on the formation of adrenaline and noradrenaline by oxidation (MnO_2) of the parent amines to adrenochrome and noradrenochrome, and subsequent intramolecular rearrangement of the 'chrome' molecules under the influence of strong alkali. The 'lutines' fluoresce under ultra violet light, and the quantitative transformation of adrenaline and noradrenaline to the corresponding lutines and the measurement of the fluorescence in a fluorimeter affords a method of determining the concentration of these amines in solution. In the present study, a modification of Lund's method has been used, so that equal concentrations of adrenaline and noradrenaline yield, to all/

all intents and purposes, the same intensities of fluorescence. This has the advantage that the total amine concentration of solutions, containing mixtures of adrenaline and noradrenaline can be determined, no matter what the relative proportions of the two amines is. With pure solutions of the amines, concentrations of the order of 0.01 µg/ml in the solution in which the fluorescing substance was produced yielded instrument readings about double that of the "blank" values, while concentrations as low as 0.0025 µg/ml showed readings definitely greater than the "blank" value. Over the range of concentration from 0.01 µg/ml to 0.2 µg/ml, Crawford (p.11) found a linear relationship between concentration and instrument reading, and the standard deviation of a single estimate to be 10% (27 observations) for adrenaline, and 9% (27 observations) for noradrenaline.

In the application of this method to the assay of adrenaline and noradrenaline, or mixtures of these amines in extracts from biological material, one must bear in mind the possibility of errors arising from the presence of other substances. These substances may interfere either as a result of their being fluorescent themselves, or as a result of/

of exerting a potentiating or inhibitory effect on the adrenaline or noradrenaline fluorescence. As regards interference from substances in the first category, the method is rendered more specific to the catechol amines by the fact that the fluorescent lutines, in the absence of a reducing agent e.g. ascorbic acid, are rapidly oxidized in alkaline solution to non-fluorescent products. Allowance can therefore be made for the presence of other substances which are stable in alkali by developing the lutines from the chromes by treatment with alkali both in the presence and in the absence of ascorbic acid. The fluorescence intensity in the absence of ascorbic acid when it has reached a steady level will serve as the "blank" value.

Such a procedure does not, of course, eliminate errors due to the presence of fluorescent substances having similar stability characteristics as adrenaline and noradrenaline. One substance, possibly present in urine extracts and producing a fluorescent compound which behaves similarly to adrenaline and noradrenaline, is dopamine (Euler, Hamberg and Hellner, 1951). Dopamine, however, produces a fluorescent intensity only about 0.6% of adrenaline and noradrenaline (Crawford, unpublished). No marked interference from this source has been met

with/

with in the present work. As evidence of this, may be cited the fact that with extracts of both human and rat urine, sympathin (adrenaline + noradrenaline) estimations have agreed well with the sum of the estimates of the separated amines in paper chromatographic eluates (Tables 9 and 33). Moreover, the pooled eluates of 8 paper strips in between adrenaline and noradrenaline strips, failed to show the presence of any activity. The paper chromatographic technique would eliminate most or all of any dopamine present in the extracts, since this substance takes up a position on the chromatogram (as carried out) intermediate between adrenaline and noradrenaline, and consequently is eliminated wholly or partially on the strip of paper which lies between the adrenaline and noradrenaline strips, and which is discarded. This agreement may be extended to indicate the absence of any other similar substance exerting any marked effect on the estimates of total sympathin, unless of course, it occupied the same position on the paper as adrenaline or noradrenaline. That this is unlikely, at least in the rat urine extracts, is indicated by the substantial agreement between the results of parallel quantitative assays on the eluates from the adrenaline and noradrenaline fractions/

fractions of a paper chromatogram of the rat urine extract, as per table below:-

TABLE 47

Parallel assays on adrenaline and noradrenaline eluates from a paper chromatogram of a rat urine extract. µg/ml of the extract. The figures in brackets denote the range of the estimate.

	Fluorimetric µg/ml	Perfused rabbit's ear.+ µg/ml	Rat's blood pressure µg/ml	Rat's isolated uterus. µg/ml
Noradrenaline fraction	0.39	0.4 (0.3-0.5)	0.52 (0.2-0.6)	0.45 (0.3-0.6)
Adrenaline fraction	0.12	0.13 (0.08-0.18)	0.15 (0.1-0.2)	0.15 (0.1-0.2

+Fingl and Gaddum (1953).

Errors in sympathin estimates arising from the possible presence in the urine extracts, of substances potentiating or inhibiting the fluorescent intensity, have been guarded against (p.12) by the addition of a known quantity of adrenaline or noradrenaline to the extract aliquot before developing and determining the fluorescence. The amount of the amine/

amine added was usually of the order of that being determined. If the fluorescent intensity is in excess of that due to the extract, by an amount equivalent to the added adrenaline or noradrenaline, the absence of potentiation or inhibitory substances was demonstrated. In the present study, every determination of sympathin, or of adrenaline or of noradrenaline, in an extract obtained from urine was checked in this way. No potentiating substances were ever detected; and the presence of substances exerting an inhibitory effect was not at all frequent. One notable instance of this inhibitory phenomenon was in connection with the experiment with Marsilid on rats, where about 40% of such effect was observed. Wherever such an inhibitory effect was noted, the estimates given in the table of results, were values after the correction allowing for this, and the fact of this correction was reported. The correction of the sympathin estimate by a factor obtained from the observed and the expected values for the added amine, probably yields a reasonably accurate figure for the sympathin estimate, as the biological confirmatory estimate for the same extract agreed closely. Nevertheless, the need for the application of a correction factor reduces considerably/

considerably the confidence which can be placed on an estimate when not accompanied by another confirmatory test.

The biological method most frequently employed was the rat's blood pressure preparation. Using rats of 200-250 g body weight, the threshold dose of noradrenaline was about 20 ng; that of adrenaline about 50 ng. Pretreatment with hexamethonium bromide (1 mg/100 g body weight, intravenously) had a marked effect on the threshold dose of noradrenaline, reducing it to 1-2 ng; while that of adrenaline was usually reduced only by a slight extent (to about 30 ng), but with some preparations it remained unaltered or even slightly increased. The inequality of the responses of blood pressure to the same doses of adrenaline and noradrenaline make any estimate of the sympathin content of an extract dependent on which amine is used as the standard and on the relative proportions of each amine in the extract. Without making a special study of the point, the impression gained was that this inequality of the response was lowest at dose level just above the threshold. Consequently, for sympathin assays, doses producing just supra-threshold responses were used in order to obtain the most accurate estimate possible./

possible. Noradrenaline, being the major constituent in the sympathin of normal urine, was used as standard.

There was the additional advantage in using low dose levels. The quantities of interfering substances possibly present and introduced with each dose of the urine extract would also be reduced to a minimum. The depressor action of any histamine present was eliminated by pre-treatment of the preparation with mepyramine maleate. Interference due to 5-hydroxytryptamine, also a depressor of blood pressure with a threshold value of 1 μg (Amin, 1953), was not specifically guarded against. On the basis of the figures quoted by Twarog and Page (1953), the 5-hydroxytryptamine content of an extract of 100 ml human urine would be about 1.8 μg. Since the highest dose of extract injected into a rat preparation never exceeded one-thirtieth of the total extract, the amount of 5-hydroxytryptamine present as impurity in each injection of the test substance would amount to only 0.06 μg, a quantity too low to produce any interference in the sympathin assay. Paper chromatography of extracts of rat's urine failed to demonstrate the presence of 5-hydroxytryptamine/

5-hydroxytryptamine, although it was detected in similar extracts of human urine.

During the course of the present study, the fluorescence method has been the one of choice, the blood pressure and rat's uterine preparations being used from time to time to confirm the results obtained by fluorimetry. The fluorescent method has the advantage that it is the more accurate, and does not have the vagueness of the biological method. In the latter, the estimate is based on the mean of doses of the standard producing responses just less than and just greater than that of the dose of test solution. Frequently, the discrimination of the preparation is such that the upper bracketing dose of the standard is double that of the lower bracketing dose, and consequently, the estimate may be anything from 50% too high to 25% too low. With the fluorimetric method, doing estimates in duplicate, the error is of the order of 7% (standard deviation).

Further, accurate estimates of sympathin content in a urine extract were not possible on biological preparations because of the inequality of the response to equal amounts of adrenaline and noradrenaline. Such is not the case with the fluorimetric method as employed in the present study. Here, the/

the fluorescence intensity is much the same for equal amounts of the two amines, and with mixtures of the amines, as the intensity of fluorescence of each is additive.

Both methods are liable to be subject to error from the presence of possible interfering substances in biological extracts. This question has already been discussed.

Methods of extraction of sympathin from urine.

Before the assay of sympathin in urine can be attempted, ordinarily, a preliminary treatment is necessary with the aim of concentrating the amines and of removing substances likely to interfere with the assays.

Two methods have been examined.

The first is von Euler's method (1948; subsequently elaborated, von Euler and Luft, 1949; von Euler and Hellner, 1951), in which the amines are adsorbed on aluminium hydroxide which is formed by the addition of aluminium sulphate to the urine and subsequent adjustment of the pH to 7.5, by the addition of NaOH. After dissolution of the separated precipitate in sulphuric acid, salts in solution/

solution are precipitated at pH 3.5 by the addition of acetone-ethanol mixture. The filtrate is evaporated to dryness under reduced pressure, and the residue taken up in normal saline. This extract containing adrenaline and noradrenaline is assayed biologically, on a rat's blood pressure preparation. Experiment showed a recovery of adrenaline and of noradrenaline in aqueous solution and in urine, to an extent of about 70%. Unfortunately, it cannot be used with the fluorimetric method of estimation, since according to Crawford (p.20), inhibition of the fluorescence is met with, particularly with extracts from urines which, prior to extraction, have been submitted to acid hydrolysis to split conjugated excretion products of adrenaline and noradrenaline. This interference is possibly to be traced to the presence of pigments which are noticeably present in great concentrations in extracts from hydrolysed urine.

The second method which has been extensively examined, depends on the adsorption of the amines on columns of a weak cation-exchange resin, Amberlite IRC 150, at a pH of 7-7.5, and elution of the adsorbed amines by sulphuric acid. Salts are removed at pH 3.5 by precipitation with acetone-ethanol/

ethanol mixture, and the filtrate evaporated to dryness under reduced pressure. The residue is taken up in water or saline, and the sympathins may be estimated by the fluorimetric method and/or by biological methods. In the form as finally evolved and used, this resin method of extraction gave about 82% (standard deviation about 6) recoveries of adrenaline and of noradrenaline from 10-100 ml of rat and human urine for amounts in urine sample, extending from 2 to 5,000 µg. Incidentally, it may be remarked here that a 20 cm resin column could handle not less than 5 mg of either adrenaline or noradrenaline.

As has been already pointed out, this extraction technique permits the use of a fluorimetric assay and thus a more accurate method of estimation of the sympathin concentration of the urinary extract is possible than when biological methods are used.

Furthermore, as the extract obtained by this method could be efficiently assayed chemically, and does not require a biological assay, it should prove invaluable to workers in clinical laboratories, for the urinary sympathin estimation for the purpose of diagnosing cases of phecchromocytoma. In such laboratories, facilities for carrying out biological assays/

assays are usually lacking.

Against these advantages must be weighed the fact that the IRC 50 resin method is more time-consuming than von Euler's method. This extra time is consumed in the preparation of the resin columns and the relative slowness of the process of adsorption of the sympathins from the urine on to the columns.

Control recovery experiments.

It is unfortunate that neither method of extraction yields recoveries which are sufficiently close to the 100% level, to avoid the necessity of applying a correction factor to the results in order to obtain a true evaluation of the urinary sympathin concentration. In addition, the recoveries tend to show variations, as indicated in the standard deviations, so that it was considered unwise to apply a constant correction factor. Consequently, throughout the present study, a control recovery experiment was incorporated with each batch of analysis, and the result of this experiment used in correcting the other analytical figures. Although this increased the labour, it was considered worthwhile, since it was felt that more confidence could then be placed on the results which had been corrected/

corrected by a recovery factor obtained under conditions as identical as humanly possible, to those to which the samples were subjected, than would be the case if the analytical results were corrected by a mean recovery figure, calculated from results obtained at different times and showing a not insignificant spread.

Two methods of performing control recovery experiments were initially employed. The first involved the parallel analysis of two aliquots from the same urine sample to one of which had been added a known quantity of adrenaline or noradrenaline of the order expected in the urine aliquot. The difference in the assay figures represented the recovery of the added amine. In the second method, the sympathin originally present in the urine was destroyed by gentle boiling of the urine at pH10 for 20 minutes. After cooling and acidifying, a known quantity of adrenaline or noradrenaline was added, and the sample subjected to the analytical technique. The assay result represented the recovery of the added amine. Initial experiments (p 47) failed to show any significant difference between the two methods. Since the second method required the processing of only one sample, as against/

against two in the first, it has been used routinely.

Separate determination of adrenaline and noradrenaline.

For the separate determination of adrenaline and noradrenaline in the extract from urine, the amines were first separated by paper chromatography, using a mixture of phenol (100 g) and 0.1N HCl (15 ml) as solvent, in a nitrogen atmosphere. The separated amines were then eluted and assayed in the eluates either fluorimetrically or by biological assay against an appropriate standard. Crawford (unpublished), using the fluorimetric assay method, has reported recoveries of 80-100% for each amine from mixtures of 0.25-10 μg of each amine applied to the paper in pure solution. In the present study, recoveries of 70-100% were obtained, in the range of 0.5-2 μg of each amine (Table 7), when mixtures of the two amines were applied in pure solutions. Recoveries of the order of 50% (Table 8) were obtained from mixtures of adrenaline and noradrenaline (2-15 μg of each amine) added to urine and extracted by the IRC 50 resin method, and the extracts then submitted to paper chromatography. Assuming a recovery of 80% from the resin columns, the/

the final recovery figure of 50% appeared lower than one would expect, as judged by the recovery figure from paper chromatography with pure solutions of the amines. No explanation can be offered yet for this loss. However, it is satisfying to note that experiments (in which the sympathin in the extract from the resin column and the individual amines after separation on paper were estimated) gave results which after the application of the appropriate recovery correction factor, showed good agreement between the sympathin amount and the sum of adrenaline and noradrenaline amounts (Table 9, p. 77). These results, it is believed, provide evidence of the validity of the application of these correction factors and show that the sympathin results do in fact represent the sum of the adrenaline and the noradrenaline constituents.

A discussion has already been given (pp.33-35), of the advantage of preliminary separation of the amines before determination over methods that determine the amines without actual separation, and involving parallel assays, either biological (Bergström, von Euler and Hamberg, 1950; Gaddum and Lembeck, 1949) or chemical (Euler and Hamberg, 1950; Schuler/

Schuler and Heinrich, 1948, 1949a and b).

The presence of conjugated sympathin in urine.

Evidence for the excretion of sympathin in urine partly in a conjugated form has been presented by a number of workers, (p.43). To free the amines from their conjugates prior to determining total sympathin excretion, von Euler treated the amine at pH_2 for 20 minutes at 100°C. While he demonstrated that this procedure caused a rise in the amount of sympathin over that estimated in the untreated urine, there was no indication that such treatment liberated all the conjugated sympathin. In the present work, it was in fact demonstrated that a sample of normal human urine showed a considerably higher free sympathin content after hydrolysis in a boiling water-bath at pH 0.5 for 20 minutes than a similar aliquot of the same urine after hydrolysis at pH_2 for the same duration, (Table 11, p. 81). Unfortunately, the more drastic conditions of hydrolysis (at the lower pH) frequently led to the production of extracts containing materials inhibitory to the full development of the fluorescent intensity of the sympathin, with the resulting/

resulting decrease in the confidence in the estimates. This fact, together with the lack of evidence of the quantitative breakdown of the conjugates even with hydrolysis at pH 0.5, greatly influenced the decision to conform to the conditions already used by other workers (von Euler and Hellner, 1951; Burn, 1953); namely, hydrolysis at pH_2, in order that the results obtained may be comparable with those reported in the literature.

In passing, it might be mentioned that in view of the fact that the conjugates of sympathin in urine are likely to be glucuronides, (p 43), hydrolysis of the conjugates in the urine by β-glucuronidase might present a possible means of avoiding drastic acid hydrolysis. It has however, not been investigated.

Reference to total sympathin excretion, in all the discussions and results in the present work, refers to the amount of sympathin estimated to be present after subjecting the urine sample to heating at pH_2 for 20 minutes in a boiling water-bath.

The/

The urinary sympathin excretion in rats under normal and experimental conditions.

Before commencing the discussion of the experimental results and conclusions drawn from them, it should be pointed out that it has not always been possible to repeat experiments as frequently as might be thought desirable, and conclusions have therefore sometimes been drawn from rather small numbers of results but only when the calculation of the statistical index 't' indicated significant differences ($P < 0.05$).

Normal rats. The rats used in the present study were males, weighing about 250-260 g. On being transferred from the large stock cages to the individual metabolism cages, the rats were restless and refused food for the first few days. During this time, the urinary sympathin excretion was high, and decreased after about six days to a more or less steady level, (Table 13). The initial higher figures of 4-5 µg/day/rat in this table, presumably reflect the restlessness, occasioned by being first confined. The subsequent fall to a lower level could be the natural consequence of the rats having become conditioned/

conditioned to the new surroundings. Rats thus conditioned showed a mean total urinary sympathin excretion of about 2 µg/day/rat of which some 50-70% was excreted in the free form (Table 15, p. 86).

These findings pointed to the necessity of using conditioned rats for the purpose of studying the effect of drugs or other deviations from the normal, on the sympathin output.

Separate determination of adrenaline and noradrenaline in the urine of normal rats that had been so conditioned, showed noradrenaline to predominate in both the total and free sympathin, to the extent of some 75% of the sum of the two amines (Table 17). The proportion showed a considerable spread from animal to animal, extending from about 60% to about 90%. The adrenal medulla as the source of the adrenaline in the urine of normal rats is indicated by the fact that the adrenaline excretion dropped to undetectable levels after demedullation (Table 33, p. 132). No significant difference could be detected in the noradrenaline excretion of normal and demedullated rats (Table 33), so that the main if not the sole depot of supply of noradrenaline in the rat must be the sympathetic nerves or other extra-adrenal chromaffin tissues.

Simple/

Simple subcutaneous injection of saline (0.5 ml 0.9% w/v NaCl) was found to lead to a significant increase ($P < 0.05$) of sympathin excretion in the 24 hour sample following the injection, in these conditioned rats, particularly in the output of the free sympathin. The mean total sympathin was 3.19 µg/day/rat (SD 0.87; 7 estimations), while the mean free sympathin was 3.03 µg/day/rat (SD 1.1; 7 estimations). This treatment led to a substantial reduction in the proportion of the conjugated sympathin excreted, in fact, the mean figures for the total and the free sympathin do not differ significantly. Inspection of the individual results however, show the presence of some conjugated sympathin in five out of the seven results. In view of the smallness of the amount and of the normal variability, it is difficult to be dogmatic as to whether the absolute amount of the conjugated sympathin has altered. At all events, one can conclude that the injection of saline had led to an increased excretion of sympathin in rats, the increase being located in the free sympathin fraction. One might surmise then that the handling of the rat during injection and the injection per se caused a stimulation of the sympathetic system for a brief period. Part of the increased circulatory sympathin/

sympathin found its way into the urine, by an overflow process. The fact that no increase in the conjugated sympathin was observed would indicate a minor role for this mechanism of detoxication of supranormal amounts of circulating sympathin. One might expect that evidence to show the correctness or otherwise of this hypothesis would be provided by studying the excretion of the conjugated and the free sympathin, following the injection of adrenaline or of noradrenaline. Unfortunately, the experimental results did not provide an unequivocal answer, (see below).

The fact that the injection of an innocuous fluid like normal saline was sufficient to increase significantly the sympathin excretion of the rat, made it necessary that sympathin excretion following the injection of a drug should be compared not with that of the normal untreated rat, but with that following the injection of 0.9% w/v sodium chloride, or of whatever solvent the drug was dissolved in.

Excretion of urinary sympathin by rats following subcutaneous injection of adrenaline or noradrenaline.

The subcutaneous injection of (-)adrenaline or (-) noradrenaline (0.1 μg/g body weight) resulted in an increase of excretion of urinary sympathin over the following 24 hours. After making allowance for the normal sympathin excretion (in this case, the mean of those following seven subcutaneous saline injections), the mean increase in urinary sympathin excretion corresponded to some 9-10% of the injected dose in the free form while the mean total sympathin corresponded to some 12-13% of the injected dose, irrespective of the amine injected, (Tables 20 and 21). The mean figures would thus indicate some 3% of the injected amine were excreted in the urine in a conjugated form. The individual experimental results do not, however, lead one to such a definite conclusion. Of the four sets of analysis, each on the pooled urine of two rats injected with (-) adrenaline, two indicated the presence of conjugated sympathin to the extent of 27 and 40% of the total excreted while the others failed to indicate the presence of any conjugated/

conjugated sympathin. A similar state of affairs exists in the results for noradrenaline. Of the seven analysis, each on a pooled urine of two rats, injected with (-) noradrenaline, three showed the presence of conjugated sympathin (29%, 38% and 55% of the total sympathin excreted), while the other results indicate an absence of the conjugated forms. It cannot therefore, be dogmatically stated that a subcutaneous injection of adrenaline or noradrenaline leads to an increased excretion of sympathin only in the free form, or partly in the free form and partly in the conjugated form, because the individual results are consistent with both statements.

Schayer (1951b), using β-C^{14} ($\pm$) adrenaline, found that on injection of 0.3 µg/g body weight intravenously into six rats, and analysing the pooled urine, about 6% of the dose was excreted as adrenaline in the free form, and 3% in a combined form. Subcutaneous injection of 2.5 µg/g ($\pm$) adrenaline was found (Schayer, 1951a) to result in an excretion of 7.2% of the dose in the free state, and 0.6% as conjugated adrenaline. A similar level of increased total sympathin excretion was found in the present study, with the subcutaneous dose of 1 µg/g (-) adrenaline (Table 19, p.93) which gave

a/

a mean excretion (with the four rats) of 7.7% of the injected dose. Separate determination of the adrenaline and noradrenaline fractions of the sympathin output in this experiment showed that the increase was to be found entirely in the adrenaline portion. Unfortunately, the fractionation into the free and conjugated forms was not carried out in this experiment.

The present experiments confirm the previous findings for the rat (Schayer, 1951a and b) and for man (Euler, Luft and Sundin, 1954; Euler and Luft, 1951) that only a relatively small fraction of the injected adrenaline or noradrenaline finds its way into the urine, either in the free form only, or partly in a conjugated form. Thus the urinary excretion represent a minor pathway in the detoxication of these amines by the animal body.

Oxidative deamination by the action of amine oxidase has been proposed as an inactivating mechanism for adrenaline and noradrenaline (Burn 1951, 1952). If this were so, one might expect that the administration of an amine oxidase inhibitor to an animal might result in an increased urinary excretion of sympathin. One such substance is Marsilid (1-isonicotinyl-2-isopropyl hydrazide) which was reported by Zeller to inhibit strongly

amine/

amine oxidase in vitro (Zeller et al, 1952), and also when injected into animals to reduce greatly the amine oxidase activity of the tissues, (Zeller and Barsky, 1952).

However, injection of Marsilid, 150 µg/g body weight, subcutaneously into rats did not result in any significant change in the urinary output of sympathin in the 24 hour period following the injection, (Table 24, p.105 in the present study). When adrenaline or noradrenaline, 0.1 µg/g subcutaneously, was injected one hour after a subcutaneous Marsilid, 150 µg/g, the sympathin appearing in the urine in the subsequent 23 hour period showed an increase over the control value (where Marsilid was replaced by a dose of 0.5 ml normal saline). The increase although significant, was not marked, (Tables 25A and B, p.107).

Several explanations suggest themselves for the lack of any marked effect by Marsilid on the sympathin excretion levels. First, the dose of Marsilid may not have been sufficiently large to produce significant effects. It was however, a dose level found by Schayer (1953) to produce quite a marked increase in the urinary excretion of tyramine/

tyramine which was injected into the mice subcutaneously, probably as a result of the amine oxidase inhibition. A second explanation might be that oxidation by amine oxidase, does not in fact, play a major role in the detoxication of noradrenaline or adrenaline in the body, and consequently, a significant rise in the urinary sympathin is not to be expected, on the injection of an amine oxidase inhibitor. A third explanation could be that, while amine oxidase does play a significant role normally, its inhibition by an inhibitor results in the flooding of circulating sympathin, and the increased detoxication of these amines by other metabolic pathways. Evidence that this last explanation is indeed the most likely, is given by the works of Schayer, Smiley and Kaplan, 1952; Schayer and Smiley, 1953; Schayer, Wu, Smiley and Kobayashi, 1954. By studying the paper chromatographic patterns of the radioactive urinary metabolites, following the injection of adrenaline, labelled with C^{14} either at the methyl group or at the β-carbon atom, in rats, injected and not injected with an amine oxidase inhibitor (Marsilid: choline p-tolyl ether), they came to the conclusion that, normally, amine oxidase accounts for the destruction of/

of about one half of the injected adrenaline; but in the presence of an amine oxidase inhibitor, an enzyme system which normally accounts for the destruction of less than one-half the injected adrenaline, takes over almost the entire metabolism. Consequently, amine oxidase inhibition would not lead to any marked increase in the urinary excretion, a conclusion which is in agreement with the results in the present work. The small but significant rise in the sympathin excretion found after the injection of adrenaline and noradrenaline subsequent to Marsilid administration may reflect a slightly decreased efficiency in the detoxication mechanism as a result of the amine oxidase inhibition.

The possibility of using urinary sympathin determinations as a means of detecting supranormal levels (or otherwise) of the blood sympathin was investigated by the administration of drugs reported to have an effect on the release of adrenal medullary hormones.

Morphine

Past researches (Elliot, 1912; Ross, 1918; Stewart and Rogoff, 1922; Bodo et al, 1937; Sato and Ohmi, 1933; Outschoorn, 1951b) pointed to the release of medullary hormones, principally of adrenaline, by the action of morphine.

In the present work, it has been found that a single subcutaneous dose of morphine hydrochloride at the level of 2 mg/100 g body weight did not produce in rats any significant rise in the sympathin excretion in the following 24 hours, (Table 22, p. 101). A more definite rise, mainly related to an increase in the free sympathin output was obtained, following the administration of the same dose four times at hourly intervals, (Table 23, p. 102). Statistically, this rise is significant.

That the adrenal medulla in the rat was the source of much of the additional sympathin, was shown by the fact that in demedullated rats, a similar course of morphine injections failed to produce such a marked alteration in the urinary sympathin excretion (Table 33, p. 132). The findings are consistent with the observation of Outschoorn (1951b) who noticed no significant change in the hormonal contents of rat's adrenal glands/

glands after a single dose of morphine hydrochloride, 2 mg/100 g body weight, but a considerable depletion after four such doses given at intervals of one hour. The depletion was found by him to be due to a loss of adrenaline, no significant fall in the noradrenaline content of the glands being observed.

The experiments on demedullated rats (Table 34, p. 134) showed morphine to produce a small but significant increase in the excretion of noradrenaline over that of the untreated demedullated rats, in spite of the absence of any detectable adrenaline in the excreted sympathin. This would indicate a general sympathetic stimulation by the drug, rather than a specific effect on the adrenal medulla, a finding which is again consistent with past observations, (Bodo, CoTui and Benaglia, 1938).

Ether anaesthesia.

Ether anaesthesia for ten minutes in normal rats appeared to result in an increased urinary excretion of adrenaline, indicating the blood concentration of this hormone had been increased. This finding is consistent with Vogt's observation/

observation (1952b) of increased plasma adrenaline levels under similar conditions. Ether anaesthesia for a similar period on demedullated rats failed to lead to the excretion of any adrenaline in the urine. This also corroborated Vogt's findings (1952b), of the absence of a rise in plasma adrenaline levels following ether anaesthesia to demedullated rats.

It must be mentioned, however, that the increased adrenaline excretion in the normal rats after ether anaesthesia was not at all marked. The two results in this experiment (Table 35, p. 136) showed adrenaline excretion levels slightly in excess of the upper fiducial limits ($P = 0.05$) of the excretion by untreated normal rats.

Insulin

All the past researches (Vogt, 1947; Burn, Hutcheon and Parker, 1950; West, 1951; Outschoorn, 1951a; 1952) showed that insulin caused the depletion of adrenal medullary hormones, involving principally adrenaline. Holzbauer and Vogt (1954) showed convincingly with their graphs, the relation between the blood glucose level and the plasma adrenaline concentration. At an intravenous dosage of 1.8 unit/kg body weight of dog, the/

the plasma adrenaline rose as the blood sugar level fell, until at 30 minutes after the injection, the lowest level of the fall of blood sugar and the maximal rise of plasma adrenaline were attained. In other words, the rise of plasma adrenaline bore an inverse relationship to the concentration of blood glucose. This phenomenon was very definite and acute when the subject had fasted before the insulin was given.

In the present work, subcutaneous insulin was given both to the fasted (about 36 hours) and non-fasted rats. In the fasted rats (Table 26, p. 112) the increase in the urinary sympathin was quite marked, the output being 2 to 3 times the control values ($P < 0.001$). The increased excretion was mainly in the adrenaline fraction of the sympathin (Table 27, p. 113). These observations are consistent with the past findings on the depletion of medullary hormones as a result of insulin administration. With non-fasted rats, the increase was there, and uniformly so, but relatively smaller (Table 28, p. 115). Statistically, this increase was not significant.

Thyroxine/

Thyroxine in normal and thyroidectomized rats.

Thyroxine is known to act slowly, hence the procedure in this series of experiments, of giving 1 μg/g body weight of rats, subcutaneously, twice a day for three days and a seventh dose on the fourth morning, in order to saturate the rats with the drug.

Such a course of thyroxine injections produced consistent results in the direction, suggested by the theory (that thyroxine inhibits amine oxidase, resulting in an increase of urinary sympathin excretion). Five out of the six results in Table 30 (thyroxine on normal rats) and all the three results in Table 32A (thyroxine on thyroidectomized rats) showed definite increases, which were nearly significant. The results in Table 32B (thyroxine on 'sham-operated' rats) showed definite significant increases over the control values.

When all nine estimates of the effect of injections of thyroxine on the sympathin in hydrolysed urine are taken together, the mean increase is 0.49 and this is significant ($P = 0.01$).

The general trend of the results obtained in these experiments supported the findings of Spinks and Burn (1952), and Trendelenberg (1953) that thyroid/

thyroid and thyroxine decreased amine oxidase activity in vivo. However, the results of Schayer (1953) definitely pointed to the absence of such an activity on the part of thyroxine.

Thyroidectomy, per se, was apparently without detectable effects on the urinary sympathin levels, the excretion by the thyroidectomized rats being not significantly different from that by control rats (Table 31, p.124). One possible flaw in our experiment is the incomplete removal of all the thyroid tissues (p.121). It has been suggested that thyroidectomy causes a rise in liver amine oxidase activity (Spinks and Burn, 1952; Trendelenberg, 1953). This effect in turn might be expected to result in a decrease in urinary sympathin excretion. No evidence for this was obtained in the present experiment, probably because the proportionate increase in the amine oxidase activity compared with the normal (if indeed there was any increase) was insufficient to influence the quantity of the sympathin metabolised by the enzyme to an extent to be detectable by the estimates of the urinary sympathins. Alternatively, the real explanation may be the incomplete removal of the thyroid tissues.

To/

To conclude the discussion of the results obtained in the experiments reported in this series, on the urinary sympathin levels in rats under various conditions, it seems pertinent to make a few general observations.

It has been experimentally demonstrated that only some 10% of adrenaline or noradrenaline injected into a rat, can be recovered in the urine. Technical difficulties of urine collection have required the estimation of urinary sympathin be carried out on 24-hour samples. These two facts restrict the usefulness of the urinary sympathin estimation in the rats as a means of demonstrating the release of supranormal amounts of sympathin into the blood stream, as a result, for example, of the administration of some drug. If one assumes that the endogenous sympathin is treated by the animal body, in the same way as the exogenous, the mean excretion of about 2 µg/24-hours by the normal rats represents a release into the blood stream of some 20 µg during the interval, or about 1 µg every hour, assuming a constant secretion. The injection of a drug causing the additional release of 5 µg over a one hour period would result only in an additional 0.5 µg/

0.5 µg sympathin in the urine in the 24-hours subsequent to the injection. Owing to the variability of the normal urinary sympathin output (SD about 0.5 µg/day) a mean rise of this order obtained from experiments on two or three rats would not be significant. Therefore, although the drug did bring about a five-fold increase in the circulatory sympathin for a period of one hour, such an effect would not be detected from the urinary sympathin estimations. It is probable that this argument affords an explanation for the failure in some of the present experiments to demonstrate definitely an expected stimulation of the sympathetic nervous system as for example, by ether. The fact that urinary sympathin estimations, under favourable conditions, can act as an indicator of the release of supranormal amounts of sympathin into the blood stream is demonstrated by the increased excretion noted after morphine and insulin injections in the present series of experiments.

Sympathin/

Sympathin content of certain normal and pathological human urines.

Urinary sympathin in pink disease in children.

The urinary sympathin excretion per day in fourteen normal infants (age ranging from six months to eighteen months) was found to be 16.5 µg (SD 4.2; fiducial limits 14-19, P = 0.05).

This investigation of the sympathin excretion level was carried out in order to obtain control figures for normal children, to serve as a basis for comparison of the excretion in infants suffering from pink disease. Unfortunately, the low incidence of this disease in this locality, has made it possible to obtain the urine from only two cases. In the first case, in which only one 24-hour sample was available, the sympathin excretion was estimated to be 74 µg. In the second case, three samples of 24-hour urine specimens were analysed, the second being collected fourteen days; and the third, sixteen days after the first sample. The total sympathin contents of the three urine samples were estimated to be 41, 22 and 49 µg/day, respectively. From the analysis of the urine sample of the second case, it/

it would appear that the sympathin excretion fluctuated from the normal level to about twice the normal level. The analysis of the first case showed a definitely supranormal sympathin excretion. G. Burn et al (1953) analysed 24-hour specimens of two cases of pink disease, and found a sympathin content of 16 and 21 µg.

On the basis of these scanty data, it might be tentatively suggested that, in pink disease, the urinary sympathin excretions show intermittent increases which might or might not have any aetiological significance. Further work on the assay of urine from pink disease patients on consecutive days over a week or two would help to clear up this point. Unfortunately, no patients turned up in the meantime.

Meanwhile, arm-chair discussion of the aetiology of this disease (or syndrome) might be indulged.

Hicks (1951) basing his views on the observations reported by Cheek and Stace (1950) and Cheek and Hicks (1950), considered highly significant the consistent (claimed) subnormal plasma sodium and hemoconcentration in pink disease, and sought to explain the aetiology of this disease as being due to/

to the movement of water from the plasma to the tissue cells, a conception of water and ionic imbalance as the primary cause. He claimed uniformly good and rapid results with salt treatment alone in mild cases, and salt plus intramuscular injection of desoxycortone acetate in severe causes. This led him to suggest that pink disease might have been due primarily to hypofunction of the suprarenal cortex, with its repercussion on the salt and water metabolism.

It must be remembered however, that in pink disease there is a good deal of sweating with the attendant loss of water and salt. The claimed success of Hicks' treatment might in reality have been only symptomatic.

Hicks' view was at once opposed by Williams, MacDonald and Callow (1951). They showed that in the twenty-two cases they investigated, the serum Na level was not lowered significantly than the twenty-two control cases of healthy infants of comparable ages. According to them, moreover, urinary 11-oxysteroid, an indicator of cortical function, was not different from the normal.

Vogt (1944, 1952) showed that adrenaline injections could release ACTH. Long and Fry (1945) and/

and Long (1947, 1952) demonstrated the same. von Euler and Luft (1949) reported in a single case on a human subject that the injection of ACTH lowered the urinary sympathin excretion (which was in reality within the normal limits); but more noteworthy facts in their experiment were (1) the great increase of urinary sympathin on cessation of the ACTH injections, and (2) the preponderance of the adrenaline fraction (to nearly cent per cent) over the noradrenaline fraction. This series of researches indicated that there was a relationship through the pituitary between the medullary and the cortical functions of the adrenal glands.

As the majority of the clinical features of pink disease could be accounted for by the cortico-medullary disfunction or derangement, further work along these lines might prove informative and useful in the elucidation of the aetiology of pink disease.

Urinary sympathin excretion in coronary thrombosis patients.

Forssman et al (1952) reported an increase of urinary output of catechol amines in all their fourteen cases. Out of the fourteen, five cases were regarded clinically as being "very shocked" and in these patients the urinary excretion figures for noradrenaline/

noradrenaline ranged from 72 to 142 µg, and those for adrenaline 8.4 to 30 µg per 24-hours. Curiously enough, the remaining cases that were said to be less severe, gave a range of 30 to 390 µg of noradrenaline per 24-hours per individual. No adrenaline figures were supplied.

In our series, the results had a broad resemblance in the trend. The first case was a male of 75 years, body weight 68 kg. The total sympathin of the urine samples collected over 24-hour periods for six consecutive days following a mild attack of coronary thrombosis, gave a range of 40 to 113 µg per 24-hours, with a mean of 66.4 µg (SD 31.4).

The second case was also male, aged 55 years, body weight 79 kg. The attack was clinically more severe. The total sympathin of the urine samples collected over 24-hour periods for four consecutive days following the attack, was found to range from 152 to 322 µg, with a mean of 255 µg (SD 80). On the fifth day congestive heart failure set in and the volume of urine dwindled to 210 ml in the 24-hours, as against the mean (mode about the same) of 870 ml of the previous days. The urinary sympathin figure too, fell to about 50 µg for that day.

The third case was also a male, aged 43 years, body/

body weight 60.5 kg. The corresponding figures for the first day was only 23 μg per day, contained in 130 mls of urine excreted for the day. The figures for the second and the third days were 145 μg (in 955 ml of urine) and 101 μg (in 730 ml of urine) respectively.

von Euler and Hellner's figure (1951) for the mean 24-hour urinary sympathin excretion for normal young adult males was 40 μg per day. As advancing age was known to cause increased output of urinary sympathins, it was felt better not to use this figure for a comparison, as Forssman did. Instead, it would be more reasonable to use Burn's figures (1953). According to him, the range was 22 to 150 μg per 24-hours, with a mean of 80 μg (SD 38.6, 7 cases) for ages 45 to 63 years (mean 50 years, SD 6.3).

It would be apparent that even with Burn's figures, the values in our second case were definitely high, while those of the third case were still relatively high.

The comparison could be carried out in another way. As the treatment for the first case was similar to the second case in respect of rest, morphine/

morphine and barbiturates administrations, the results of the first could be used conveniently as the control for the second case. The question of the effect of treatments would be equally applicable to both the cases. Hence, the raised urinary excretion of sympathin in the second case was most probably to be associated with the severity of the patient's condition. Such a comparison is not valid in the third case, since the treatment given differed from that of the first case.

In this series of experiments, one important fact was observed. This was the low values in the second case when congestive heart failure set in, and in the third case when the patient was in the initial shock on the first day of the attack of symptoms. These low values were associated with a diminution of the volumes of urine excreted over the corresponding period. It seemed to indicate that the sympathin excretion in the urine was dependent on the volume of urine excreted, at least partially. If for any reason, the kidneys were not functioning properly as in the case of shock, or were not given the chance to do so as in the water-logged condition of congestive heart/

heart failure, the urinary output of sympathins could be correspondingly curtailed.

SUMMARY/

SUMMARY

1. (a) A method for the estimation of urinary adrenaline and noradrenaline has been examined extensively. The amines are removed from the urine by passage of an aliquot of it after three-fold dilution and pH adjustment to 7, through a column (10-20 cm x 1.1 cm diameter) of a weak cation-exchange resin, Amberlite IRC 50, buffered to about 7.5. The amines so adsorbed by the resin are then eluted with dilute sulphuric acid. Salts are precipitated from the eluate after pH adjustment to 3.5, by the addition of acetone-ethanol mixture. The filtrate is evaporated to dryness under reduced pressure, and the residue dissolved in normal saline.

(b) Preparation of the urinary extract in this manner permits the final assay to be carried out by a chemical (fluorescence) method, as opposed to the biological assay, required by the extraction method of von Euler. The fluorescence method was that of Lund, with conditions modified to give equal intensities of fluorescence from equal amounts of adrenaline and noradrenaline. This modification permits the estimation/

estimation of sympathin (adrenaline + noradrenaline) without errors being introduced by variations in the relative proportions of the two amines in the mixture. Results from fifty-one experiments show a mean recovery of about 82% with a standard deviation of about 6, from adrenaline or noradrenaline added to urine in amounts ranging from 2 µg to 5,000 µg in the urine samples analysed. No significant difference was found between the recoveries of the two amines.

(c) When separate determinations of the two amines were required, the urinary extract from the resin column was subjected to ascending paper chromatography on Whatman's No.1 filter paper, (for chromatographic use), using phenol containing 0.1 N HCl as solvent, in a nitrogen atmosphere. The separated amines were then estimated in the eluates from the appropriate paper strips. The recovery of adrenaline and noradrenaline added as mixtures to urine samples which were then extracted, separated, and assayed as outlined above, was about 51, (SD about 9 for 10 estimations, using 2 to 15 µg of either amine).

(d)/

(d) When necessary, biological confirmation of the fluorimetric assay results was obtained by assaying on rat's blood pressure preparation or the rat's isolated uterus preparation.

(e) The specificity and accuracy of these techniques have been discussed.

2. (a) Conversion of sympathin excreted in conjugated form to the free form was effected by preliminary treatment of the urine sample at pH_2 for twenty minutes in a boiling water-bath, (a method used by von Euler). Sympathin assays on urine so treated were considered to yield estimates of the total sympathin; while assays on the untreated urine yielded the estimates of the sympathin excreted in the free form.

(b) There is evidence however, that hydrolysis of urine at pH_2 does not result in the complete liberation of the amines from the conjugates.

3. Using these techniques, a study has been made of the urinary sympathin excretion in normal rats and in rats subjected to various experimental/

experimental procedures. A brief study has also been made of the sympathin excretion in certain normal and pathological human urines.

4. Sympathin excretion/day of normal rats, weighing 250-260 g is as follows:

Sympathin - in hydrolysed urine,

1.9 µg, SD 0.47 (5 cases);
+Fiducial limits 1.17 to 2.77 µg.

- in unhydrolysed urine,

0.95 µg, SD 0.06 (3 cases);
Fiducial limits 0.81 to 1.11 µg.

Adrenaline - in hydrolysed urine,

0.56 µg, SD 0.15 (4 cases)
Fiducial limits 0.32 to 0.8 µg.

- in unhydrolysed urine,

0.28 µg, SD 0.14 (3 cases)
Fiducial limits 0 to 0.65 µg.

Noradrenaline - in hydrolysed urine,

1.53 µg, SD 0.44 (4 cases)
Fiducial limits 0.83 to 2.23 µg.

- in unhydrolysed urine,

1.06 µg, SD 0.28 (3 cases)
Fiducial limits 0.36 to 1.76 µg

5./

+All cases of fiducial limits here and hereafter have been calculated for P = 0.05.

5. Demedullation of adrenal glands in rats was associated with the disappearance of adrenaline fraction in their urine. Consequently, the origin of urinary adrenaline in rats was inferred to be the adrenal medulla.

6. Subcutaneous adrenaline and noradrenaline injections in rats at 0.1 μg/g body weight, were associated with an increased urinary sympathin output, equivalent to some 12 to 13% of the weight of the injected drug, of which possibly about 3% could be hydrolysable - but statistically insignificant.

7. Subcutaneous Marsilid (1-isonicotinyl-2-iso propylhydrazide) 150 μg/g body weight, in normal rats, produced no significant rise in sympathin excretion in the following 24-hours. No marked difference was noted in the sympathin excretions in rats injected with adrenaline and noradrenaline with and without prior administration of Marsilid. Possible reasons for this absence of marked changes in the urinary sympathin levels have been discussed.

8./

8. (a) The trend of urinary sympathin excretion following subcutaneous thyroxine injection in rats, was towards small increases which were significant ($P < 0.01$).

(b) Thyroidectomy in rats, which was reported to cause increased amine oxidase activity of the tissues, failed to produce significant alterations in the sympathin excretion in rat's urine. An explanation for this finding has been offered.

9. Subcutaneous morphine hydrochloride, 4 x one hourly doses of 2 mg/100 g body weight, was followed in the subsequent 24-hours, by an increase in the urinary sympathin output of 3 to 4 µg/rat over the controls in the hydrolysed urine. The rise was significant.

In demedullated rats, similar morphine administration failed to produce a detectable rise in the sympathin excretion over the control level. Adrenaline was absent in these urines. This experiment indicates that the increased blood sympathin levels following morphine was mainly related to adrenaline release from the suprarenal medulla.

10./

10. Subcutaneous insulin, 0.5 unit/100 g body weight, in fasting rats, caused a significant rise in urinary sympathin excretion. The increase appeared to be more pronounced in the adrenaline fraction, and less so in noradrenaline fraction.

11. Ether anaesthesia for 10 minutes in normal rats produced an increased adrenaline level in the urine in the subsequent 24-hours. Adrenaline was absent in the urine of demedullated rats so treated.

12. Sympathin estimations in rat urine as an indicator of increased blood sympathin levels following drug administration, has been discussed in the light of the results referred to above.

13. Daily excretion of urinary sympathin in normal male infants, 6 months to 18 months, body weight 6.4 kg to 10.9 kg is as follows:

Sympathin - in hydrolysed urine,

16.49 µg, SD 4.2 (14 cases)
Fiducial limits 14.1 to 18.9 µg.

in/

- in unhydrolysed urine,

13.23 µg; SD 3.4 (14 cases)
Fiducial limits 11.3 to 15.2 µg.

Adrenaline - in hydrolysed urine,

7.33 µg; SD 1.51 (5 cases)
Fiducial limits 5.44 to 9.22 µg.

- in unhydrolysed urine,

3.7 µg; SD 1.18 (5 cases)
Fiducial limits 2.23 to 5.17 µg.

Noradrenaline - in hydrolysed urine,

11.38 µg; SD 2.22 (5 cases)
Fiducial limits 8.6 to 14.16 µg.

- in unhydrolysed urine,

8.4 µg; SD 2.61 (5 cases)
Fiducial limits 5.16 to 11.64 µg.

14. Urinary sympathin estimates in two cases of pink disease in infants point to intermittent increases of the urinary sympathin excretion in the course of the disease. The results are however, too few in number to permit a dogmatic statement.

15. Urinary sympathin estimates in three cases of coronary thrombosis, indicate a raised excretion in this condition. Lack of time has prevented the more extensive investigation which would be required to settle this point.

+Fiducial limits, calculated at P=0.05.

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