amino acids in myosin and actin, the values calculated from the lysine and leucine figures receiving the greatest and least weights respectively, the average value obtained is about 73 %. The error in this weighted average cannot be calculated simply but is probably about 6–7 %. The ratio of myosin to actin in cod 'natural' actomyosin is therefore between 2 and 4:1; this is also probably close to the ratio between the two proteins in whole cod muscle since cod 'natural' actomyosin makes up practically the whole of the fibrillar protein of cod (Dyer, French & Snow, 1950) and therefore contains all the myosin and actin of the muscle. It appears that the ratio of myosin to actin in cod muscle is similar to the ratio of close to 3:1 found for rabbit muscle by Hasselbach & Schneider (1951). However, the actual quantities of myosin and actin in cod muscle are likely to be greater than those in rabbit muscle since the amount of fibrillar protein is greater in cod than in rabbit (for summary see Bailey, 1954).

SUMMARY

1. The amino acid compositions of cod myosin, cod actin and cod 'natural' actomyosin have been determined.
2. Cod myosin and cod actin closely resemble the corresponding rabbit proteins in amino acid composition.
3. The ratio of myosin to actin in cod 'natural' actomyosin was found to lie between 2 and 4:1 and therefore approximates to the corresponding ratio in rabbit muscle.

We are indebted to Dr J. Leggat Bailey for communicating in advance of publication Drs Moore, Spackman and Stein's method of amino acid analysis.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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A Quantitative Method for the Chromatographic Separation of 17-Oxo Steroid Sulphates from 17-Oxo Steroid Glucuronides: with Observations on the Behaviour of Conjugated Corticosteroids on the Same System

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(Received 12 May 1958)

Steroid metabolites are excreted in the urine mainly as glucuronide or sulphate conjugates. Although it is possible to distinguish between these two forms of conjugation by specific hydrolytic

procedures and to separate them physically by solvent partition after acetylation (Kellie & Wade, 1956), neither process gives the steroid conjugates in unmodified form. The former process liberates free steroids and the latter acetylated glucuronides which can no longer be hydrolysed by β -glucuronidase or oxidized by bismuthate. A method has been evolved in which, by taking advantage of the adsorption of the conjugates on neutral alumina,

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the 17-oxo steroid glucuronides and 17-oxo steroid sulphates are separated quantitatively from each other in unmodified form.

Simultaneous observations have been made of the behaviour of conjugated corticosteroid metabolites, measured as 17-oxogenic steroids (Norymberski, 1952), on the same chromatographic system.

A preliminary report of this work has been published (Barlow, 1957).

METHODS

Extraction of steroid conjugates. Steroid conjugates were extracted from urine by the method of Edwards, Kellie & Wade (1953), in which $(\text{NH}_4)_2\text{SO}_4$ (50 g./100 ml.) is dissolved in the urine and the conjugates are extracted with ether-ethanol (3:1, v/v) mixture ($3 \times \frac{1}{2}$ of the original urine volume). No adjustment of the urinary pH was necessary for quantitative extraction of 17-oxo steroid conjugates but maximum extraction of 17-oxogenic steroid conjugates (85–92%) was obtained by adjusting the urine to pH 2 before adding $(\text{NH}_4)_2\text{SO}_4$ (Edwards & Kellie, 1958). The ether-ethanol extracts were combined and filtered before chromatography.

Hydrolysis of steroid conjugates. To discriminate between the various forms of conjugation the following procedures were adopted.

The glucuronides were hydrolysed with ox-liver β -glucuronidase (Ketodase, Warner-Chilcott Laboratories, New York, U.S.A.) at a concentration of 750 Fishman units/ml. of original urine. (One Fishman unit is the amount of enzyme necessary to liberate 1 μ g. of phenolphthalein from its glucuronide after incubation of the mixture at pH 4.5 and 38° for 1 hr.) In parallel experiments, limpet (*Patella vulgata*) powder was also used as a source of β -glucuronidase (Dodgson & Spencer, 1953), 0.03M- KH_2PO_4 being added to inhibit the sulphatases present. The material was dissolved in acetate buffer containing 0.03M- KH_2PO_4 (pH 5.0 for Ketodase and pH 4.0 for limpet powder) prepared by mixing 0.5M-sodium acetate with the corresponding volume of 0.5M-acetic acid. The enzyme and penicillin were added and the hydrolysis was carried out at 37° for 40 hr. According to Wotiz, Lemon, Marcus & Savard (1957) Ketodase contains no sulphatase.

After hydrolysis, liberated 17-oxo steroids were extracted with benzene ($3 \times \frac{1}{2}$ of the hydrolysate volume) and the combined benzene extracts were washed with N-NaOH ($2 \times \frac{1}{2}$ vol.) and with water to neutrality. Portions of this extract were taken for determination by the Zimmermann reaction (Callow, Callow & Emmens, 1938).

Corticosteroid metabolites liberated by enzyme hydrolysis were extracted with CHCl_3 ($3 \times \frac{1}{2}$ vol.) and the combined CHCl_3 extracts washed with N-NaOH ($1 \times \frac{1}{2}$ vol.) and with water to neutrality. Portions removed for bismuthate oxidation were taken to dryness below 40° and under N_2 .

Steroid sulphates in aqueous solution were hydrolysed by adjusting to pH 1 and continuously extracting with ether in a liquid-liquid extractor for 48 hr. (Lieberman & Dobriner, 1948). The ether extract was washed with N-NaOH ($2 \times \frac{1}{2}$ vol.) and with water to neutrality. Portions were removed for determination by the Zimmermann reaction.

When it was unnecessary to discriminate between the modes of conjugation, hot-acid hydrolysis was used to determine the total amount of 17-oxo steroid. The material was dissolved in, or diluted to, 10 ml. with water in a stoppered tube, and conc. HCl (3 ml.) added, and the tube placed in a boiling-water bath for 10 min. After the solution had cooled, the 17-oxo steroids were extracted by shaking with carbon tetrachloride (5 ml.) and this extract was washed with 3N-NaOH (2 ml.) and with water to neutrality (Drekter *et al.* 1952). Portions were taken for determination by the Zimmermann reaction.

Separation of steroid conjugates after acetylation. The known 17-oxo steroid sulphates are not acetylated by pyridine and acetic anhydride, whereas steroid glucuronides contain three free hydroxyl groups on the glucuronic acid residue which are acetylated by this procedure. Acetylation of these groups converts the previously water-soluble glucuronides into water-insoluble acetylated glucuronides which can be separated from the water-soluble steroid sulphates by partition between benzene and water (Kellie & Wade, 1956). This procedure was used to provide further evidence of the mode of conjugation in the various chromatogram fractions.

The dried residue from each fraction was dissolved in pyridine (2 ml.) and acetic anhydride (2 ml.) and allowed to stand overnight at room temperature. The mixture was diluted with ice-water (25 ml.) and the water-insoluble acetylated glucuronides were extracted with benzene (3×10 ml.). The combined benzene extracts were washed with water (2×10 ml.), and dried over anhydrous Na_2SO_4 and evaporated to dryness. Portions were removed and the amount of 17-oxo steroid present as the acetylated glucuronide was determined.

Determination of 17-oxogenic steroid glucuronides. Brooks & Norymberski (1953) introduced a method of estimating corticosteroid metabolites which makes use of the fact that certain 17 α -hydroxycorticosteroids can be oxidized by sodium bismuthate to 17-oxo steroids and can be indirectly measured in this way. Such steroids are called 17-oxogenic steroids. This method simultaneously measures 17-oxo steroids present before oxidation; the figure obtained after oxidation is therefore the sum of the 17-oxo steroids present before oxidation and those formed during the oxidation (total 17-oxo steroids). To determine the amount of 17-oxogenic steroid present, an independent determination of the 17-oxo steroids is made and the value obtained by difference.

In the present work the procedure used was a modification of the Brooks & Norymberski method (Edwards & Kellie, 1958), and the values quoted are for 'total 17-oxo steroid glucuronides'.

Material (approx. 20 μ g. of 'total 17-oxo steroid') was transferred to a stoppered tube (B19 standard joint, 60 ml. capacity) and dissolved in or diluted with water-ethanol (1:3, v/v; 25 ml.) containing trichloroacetic acid (1 g.). Sodium bismuthate A.R. (2 g.) was added and the mixture agitated in the dark for 2 hr. The reaction was stopped by centrifuging and a portion of the supernatant liquid (20 ml.) pipetted into a separating funnel containing 60 ml. of diluting fluid (3% of Na_2SO_4 anhyd. and 2% Na_2SO_3 anhyd. in water, w/v). The mixture was extracted with dichloromethane (3×10 ml.) and the combined extracts were washed with N-NaOH (5 ml.) and with water until neutral. The extract was evaporated to dryness below

40° and under N₂ and redissolved in 5% (v/v) ethanol in benzene (4 ml.). Alumina (100 mg., moisture content 2–10%) was added and the solution was shaken for 30 sec. The alumina was allowed to settle and a portion of the solution (3 ml.) taken for determination of 17-oxo steroid by the Zimmermann reaction.

This method measures only steroid glucuronides; the steroids conjugated as sulphate remain in the aqueous diluting fluid after extraction with dichloromethane. The treatment of the 17-oxo steroid extract with alumina is not an essential step in all circumstances but helps by removing material which would interfere with the Zimmermann reaction.

Determination of glucuronic acid. This determination was carried out as a qualitative procedure based upon a method described by Paul (1951) (see Mead, Smith & Williams, 1958).

Determination of 17-oxo steroids. 17-Oxo steroids were determined by the method of Callow *et al.* (1938). The method was reduced to one-quarter scale so that dilution of the final solution was made with 2.5 ml. of ethanol. The optical density was read in a Unicam spectrometer (model SP. 600) at 440, 520 and 620 m μ . Dehydroepiandrosterone (3 β -hydroxy-androst-5-en-17-one; 10 μ g.) was used as standard and Allen's (1950) correction was applied.

Chromatographic analysis of the steroid-conjugate extract. The steroid conjugates from 20 ml. of urine were chromatographed on 5 g. of neutral alumina (M. Woelm, Eschwege; supplier L. Light and Co. Ltd.) having a moisture content adjusted to 5% (loss of weight when heated to 100° for 16 hr.). The column (5 cm. $\times$ 1 cm.) was prepared in benzene by allowing the alumina to settle under gravity and then packing tightly with a Martin packer. The slightly pigmented ether-ethanol extract was poured directly on to the column, pigment being adsorbed as a series of bands at the top. The almost colourless filtrate contained less than 5% of the 'total 17-oxo steroids' but contained a large amount of crystalline solid, presumably non-steroid. Any unconjugated steroids present in the extract were found in this fraction.

The column was first washed with absolute ethanol (25 ml.), which eluted more solid material but virtually no 17-oxogenic or 17-oxo steroid.

The eluent was then changed to aqueous 50% (v/v) ethanol (25 ml.), which eluted the 17-oxo steroid sulphates together with some pigment and non-steroid solid. Varying small amounts of 17-oxogenic steroid (1–4%) were also found in this fraction.

Finally, the 17-oxo steroid glucuronides and 95–100% of the 17-oxogenic steroid glucuronides adsorbed on the column were eluted with *m*-acetate buffer, pH 5.0 (40 ml.). Initially the column ran at a moderate rate but slowed considerably when aqueous eluents were introduced. The results were not adversely affected by the application of pressure (up to 10 cm. Hg) to the top of the column as long as the column was not ruptured when pressure was released to introduce new eluents. The procedure up to the collection of the eluates required approx. 1.5 hr.

The results relate to the analysis of steroid conjugates from 20 ml. of urine but the procedure has been carried out on urine volumes ranging from 10 ml. (3 g. of alumina) to 200 ml. (40 g. of alumina) with proportionate adjustment of the volume of eluting solvents. In practice, an ether-ethanol extract was prepared from 40 ml. of urine and one-half of the extract was analysed for steroid glucuronide and steroid sulphate content by conventional procedures of enzyme hydrolysis and continuous ether extraction at pH 1. The other portion of the extract was poured on to the alumina column, which was developed as already described. The composition of the eluates was then determined by differential hydrolysis as above, and also by the acetylation technique.

RESULTS

Analysis of 17-oxo steroid conjugates

The 17-oxo steroid conjugates and the 17-oxogenic steroid conjugates have been dealt with separately. Table 1 shows the results of a typical chromatographic separation of 17-oxo steroid sulphates from 17-oxo steroid glucuronides. On the left of the table is shown the composition of the unchromatographed urinary extract and each subsequent heading represents a column eluate.

Taken together, the ether-ethanol column filtrate and the subsequent ethanol eluate contained only 1.2% of the 17-oxo steroids placed on the column. The determination of the 17-oxo steroids in these fractions was by hot-acid hydrolysis, no attempt being made to determine the mode of conjugation; any unconjugated 17-oxo steroid would be present in the ether-ethanol filtrate.

Table 1. *Chromatography of 17-oxo steroid conjugates*

Conjugates were extracted from normal male urine (20 ml.) and chromatographically separated on neutral Woelm alumina (5 g.; moisture content 5%). Except for the last row of the table, figures are expressed as μ g. of 17-oxo steroid conjugate.

Material	Placed on column	Eluted from column			
		Ether-ethanol	Ethanol	Aq. 50% ethanol	<i>m</i> -Acetate buffer
Sulphate	68.0	2.6*	1.0*	72.0	3.1
Glucuronide	228.0			4.0	215.0
Acetylatable 17-oxo steroid	—	—	—	3.0	219.0
Glucuronic acid	+	Nil	Nil	Nil	+
Wt. (mg.) of solid matter present in solution	110.2	51.2	13.3	27.8	12.2

* Obtained by hot-acid hydrolysis as described in the text.

The aqueous ethanol fraction was taken to dryness under reduced pressure below 40°, ethanol being added to facilitate evaporation. The residue after incubation with β -glucuronidase gave 4 μ g. of benzene-soluble 17-oxo steroid but subsequent hydrolysis by continuous ether extraction at pH 1 liberated 72 μ g. of 17-oxo steroid, which corresponded closely to the 17-oxo steroid sulphate (68 μ g.) present in the unchromatographed extract.

The M-acetate buffer eluate was diluted with an equal volume of water and incubated with β -glucuronidase. With Ketodase no adjustment of pH is usually necessary but with limpet β -glucuronidase the pH was adjusted to 4.0 and 0.3M-KH₂PO₄ added. Enzyme hydrolysis liberated 215 μ g. of 17-oxo steroid compared with 228 μ g. in the parallel extract and subsequently an additional 3.1 μ g. was freed after ether hydrolysis at pH 1. No free 17-oxosteroid was extracted from this eluate by benzene before enzyme hydrolysis. The 17-oxo steroid and 17-oxogenic steroid glucuronides can be quantitatively recovered from the acetate buffer eluate by extracting this fraction by the procedure described for urine. The extract obtained in this way from the buffer fraction contained all the steroid glucuronides; on evaporation to dryness it contained between 10 and 15 % of the weight of solid matter placed on the column in the original ether-ethanol extract of urine.

Analysis of 17-oxogenic steroid conjugates

Data relating to the behaviour of 17-oxogenic steroid glucuronides on the same column are given in Table 2. The figures quoted are for total 17-oxo steroids and relate only to material conjugated as glucuronide, for 17-oxogenic steroid sulphates, if excreted, would not be measured by the method

used. With this sample 86 % of the 17-oxogenic material present in the urine was extracted by the ether-ethanol procedure. Of the total amount placed on the column 4.5 % was eluted in the ether-ethanol filtrate; unconjugated steroids appear in this fraction and unconjugated cortisone has been identified by paper chromatography as being present. Very little 17-oxogenic steroid was present in the ethanol fraction and only 15 μ g. of 'total 17-oxo steroid' glucuronides (3.5 %) was found in the aqueous ethanol eluate.

Substantially all the 17-oxogenic steroid glucuronides were found in the acetate buffer fraction and subsequently hydrolysis with β -glucuronidase liberated 96.5 % of this material. Several attempts were made to determine 17-oxogenic steroid sulphates in the aqueous ethanol and acetate buffer by comparing the figures obtained for 17-oxo steroid sulphates (ether extraction at pH 1) before and after oxidation with sodium bismuthate. The 17-oxo steroids determined before oxidation have been equal to or greater than the value after oxidation and no evidence for the presence of sulphate-bound 17-oxogenic steroid has been found.

Application of the method

The procedure offers a rapid method of determining the ratio of 17-oxo steroid sulphates to glucuronides and, at the same time, supplies sufficient material for differential analysis of the 17-oxo steroids of each fraction. Small portions of the aqueous ethanol and acetate buffer eluates are hydrolysed by the hot-acid procedure and determined in the usual way. A comparison of results obtained by this method with those obtained by the more laborious procedure of enzyme hydrolysis and ether extraction at pH 1 are shown in Table 3.

Table 2. *Chromatography of 17-oxogenic steroid glucuronides*

Conjugates were extracted from normal male urine (20 ml.) and chromatographically separated on neutral Woelm alumina (5 g.; moisture content 5%). The figures quoted are 'total 17-oxo steroid glucuronides', which include 17-oxo steroid glucuronides; they are given in terms of μ g. of 'total 17-oxo steroids' after oxidation with bismuthate.

Placed on column	Eluted from the column				
	Ether- ethanol	Ethanol	Aq. 50 % ethanol	M-Acetate buffer	Hydrolysed by enzyme
443.0	20.0	4.2	15.0	435.1	430.0

Table 3. *Comparison of the glucuronide/sulphate ratio for 17-oxo steroids determined by (a) chromatographic separation followed by hot-acid hydrolysis and (b) β -glucuronidase hydrolysis followed by continuous ether extraction at pH 1*

Results are expressed as μ g. of 17-oxo steroid.

Column fraction	(a) Hot-acid hydrolysis	(b)		
		β -Glucuronidase hydrolysis	Hydrolysis at pH 1	Total
Aq. ethanol	83.0	4.0	72.0	76.0
Acetate buffer	210.0	215.0	3.1	218.1
Ratio glucuronide/sulphate	0.395	—	—	0.349

The results are typical of those obtained in a number of similar determinations on diverse urine samples. The amount of 17-oxo steroid found in the acetate buffer fraction, whether hydrolysed by β -glucuronidase or hot acid, corresponds closely to the amount liberated from urine conjugate extracts by enzyme hydrolysis. Whereas ether hydrolysis of the aqueous-ethanol fraction at pH 1 gives close correspondence with figures obtained by applying the same method to the urine-conjugate extract, hot-acid hydrolysis of this fraction usually gives a higher figure (10–15%). As the difference between the hot-acid hydrolysis and hydrolysis at pH 1 of the aqueous ethanol fraction is not accounted for by the small amount of 17-oxo steroid liberated by β -glucuronidase, these results suggest the presence in this fraction of some type of 17-oxo steroid conjugate other than glucuronide and sulphate.

DISCUSSION

Previous attempts to chromatograph steroid conjugates on alumina have not been encouraging (West, Reich & Samuels, 1951); in spite of the use of very polar organic solvents the recovery of adsorbed material was poor and there was no evidence of separation. As far as 17-oxo steroids are concerned the procedure now described separates sulphates and glucuronides with quantitative recovery. The conjugates obtained in this way show no abnormal behaviour; they are still hydrolysed by the accepted techniques and the liberated 17-oxo steroids behave on paper chromatograms in the normal manner. When separating the conjugates of 17-oxo steroids the moisture content of the alumina is not a critical factor. Neutral alumina with moisture content varying between 0 and 10% has been equally effective.

It is not usually considered practicable to chromatograph corticosteroids on alumina. Reichstein, for his work on adrenocortical extracts, took considerable care to prepare neutral alumina, yet experienced some loss of material (Reichstein & Shoppee, 1949), and there are several reports in the literature of molecular re-arrangements on alumina inside and outside the steroid field. In the present application there was quantitative recovery of adsorbed 17-oxogenic steroid glucuronides in the acetate buffer fraction. That these steroids are still present as glucuronides is shown by quantitative liberation of the free steroids by β -glucuronidase and, furthermore, the side chain of the steroids is still capable of being oxidized by sodium bismuthate to 17-oxo steroid. Paper chromatography of corticosteroids liberated by enzyme hydrolysis from the acetate buffer fraction gives a conventional pattern of urinary-corticoid metabolites. In

an application of the procedure (Ayres *et al.* 1957) these products of enzyme hydrolysis were extensively chromatographed on partition columns yet showed no abnormal behaviour.

The results do not establish that 17-oxogenic steroids are invariably excreted in the urine as glucuronides, because the method employed measures only 17-oxogenic steroid glucuronides; nevertheless, attempts made to measure 17-oxogenic sulphates met with no success and it seems unlikely that this form of conjugate represents more than a very small percentage of the total.

Unsuccessful attempts have been made to separate individual glucuronides in the acetate buffer eluate by re-adsorption on alumina, followed by gradient elution. With the sodium acetate-acetic acid buffer system, a gradient of hydrogen-ion concentration at constant molarity and a gradient of molarity at constant hydrogen-ion concentration (pH 5) failed to separate the glucuronides of androsterone (3 α -hydroxy-5 α -androstan-17-one) and aetiocholanolone (3 α -hydroxy-5 β -androstan-17-one), yet some buffers are more effective eluting solvents than others; thus quantitative elution of 'total 17-oxo steroid' glucuronides is accomplished by 0.04M-sodium phosphate-citric acid buffer (pH 5), whereas 0.04M-sodium acetate-acetic acid buffer (pH 5) eluates approximately 70% of the 'total 17-oxo steroid' glucuronides. Furthermore, 17-oxogenic steroid glucuronides and 11-oxy-17-oxo steroid glucuronides can be eluted quantitatively by 0.1M-acetic acid, whereas under these conditions most of the 11-deoxy-17-oxo steroid glucuronides remain on the column until sodium acetate is introduced. The use of acetate buffer (pH 5) is preferable to that of phosphate-citrate because β -glucuronidase (Keto-dase) can be added directly for enzyme hydrolysis and also because the former eluates less pigment from the column. According to Abul-Fadl (1957) citrate is a mild inhibitor of β -glucuronidase.

SUMMARY

1. A method has been described whereby urine extracts containing steroid conjugates can be chromatographed on neutral alumina.

2. 17-Oxo steroids conjugated as sulphates have been separated from those conjugated as glucuronides; determinations carried out on the fractions so obtained agree with values for these conjugates by alternative methods.

3. 17-Oxogenic steroid glucuronides (Norymberski, 1952) are also recovered quantitatively from the column and neither these nor the 17-oxo steroid conjugates appears to have been modified during adsorption.

4. The procedure provides a rapid method for the determination of 17-oxo steroid sulphate/glucuronide ratios and forms the basis of a method for the partial purification of steroid glucuronides.

5. Attempts to isolate individual conjugates by gradient elution on these columns have not been successful.

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Seasonal Changes of the Catechol Compounds Present in Rat Tissues

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(Received 23 October 1957)

Raab (1941*a*, *b*, 1943*a*), using Shaw's colorimetric method of estimation, reported that the concentrations of 'adrenaline-like catechols' in rat tissues were higher in winter than in summer. This has been confirmed in the present experiments with the ethylenediamine method of Weil-Malherbe & Bone (1953); these experiments have also shown that lower amounts of catechol compounds are present in spring and early autumn (Montagu, 1956*a*).

The fluorescence produced in the ethylenediamine reaction was previously attributed to noradrenaline and adrenaline (Montagu, 1956*b*). Further investigation has shown that a third basic compound is sometimes present (Montagu, 1957). Catechol compounds other than noradrenaline and adrenaline have been found in several animal tissues: examples are 3-hydroxytyramine in sheep heart (Goodall, 1950), in adrenals of sheep and cattle (Shepherd & West, 1953; Holtz & Westermann, 1956) and in phaeochromocytoma (Weil-Malherbe, 1956); *isopropyl*noradrenaline in cat adrenals (Lockett, 1954); 3:4-dihydroxyphenylalanine in ox adrenals (Werle & Jüntgen-Sell, 1955) in phaeochromocytoma (Weil-Malherbe, 1956) and

in a human brain (Montagu, 1957). 3:4-Dihydroxyphenylacetic acid is present in urine (Euler, Euler & Floding, 1955; Weil-Malherbe & Bone, 1957).

METHODS

The experiments date from April, 1954. Male rats of the Wistar strain, isolated from females, were used. They received a standard diet (diet 41; Bruce & Parkes, 1949, 1950) and drinking water *ad lib*. They were housed in a separate building 16 yards from the Laboratory, exposed to daylight and kept, from October until May, at 65–70° F. They were killed in the morning by a blow on the head within 15 min. of being brought in from the animal house.

Estimation of 'total catechols'. The heart, brain, spleen, diaphragm muscle, both kidneys and part of the liver and of the leg muscles of a single rat were separately extracted and treated as already described (Montagu, 1956*b*). The fluorescence in the final solution was measured with yellow and blue-green filters after condensation with ethylenediamine dihydrochloride (ED). As catechol compounds other than adrenaline and noradrenaline may be present, the calculation previously used may not be valid. The fluorescence produced by the mixture of catechol compounds was therefore compared with that of a standard solution of adrenaline, and the concentration of 'total catechols' calculated in terms of this compound.

During the 3½ years' period of this investigation three modifications in the method were introduced: (1) in April

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