J. Anat. (1966), **100**, 2, pp. 227–243
With 10 figures
Printed in Great Britain

The mechanism of the differential staining reaction for adrenaline- and noradrenaline-storing granules in tissues fixed in glutaraldehyde

By R. E. COUPLAND and D. HOPWOOD
Department of Anatomy, Queen's College, Dundee, University of St Andrews

INTRODUCTION

The value and limitations of formaldehyde as a histological fixative are widely appreciated. During recent years, however, attention has been directed towards other aldehydes and these have been examined from the viewpoints of preservation of cell organelles at the ultrastructural level and relative potency as regards enzyme inactivation (Sabatini, Bensch & Barrnett, 1963; Sabatini, Miller & Barrnett, 1963).

In this laboratory the effects of a variety of aldehydes on biogenic amines, both in chemical solutions and tissues, have been studied (Coupland & Hopwood, 1965; Hopwood & Coupland, 1965). Early work (Coupland, Pyper & Hopwood, 1964) showed that noradrenaline could be localized in both the light- and electron-microscope in tissues fixed initially in glutaraldehyde. The nature of the reaction products which result from the addition of glutaraldehyde to catecholamines has now been examined in more detail and the mechanism of the reaction whereby noradrenaline is differentiated from adrenaline in tissue sections elucidated.

MATERIALS AND METHODS

Chemical

Adrenaline and noradrenaline in the form of bases and as tartaric acid salts were obtained from British Drug Houses or Koch–Light Laboratories Ltd. Glutaraldehyde was a product of Union Carbide Ltd., supplied as an aqueous 25% solution free from stabilizer. During storage, even at 0–4 °C, solutions of glutaraldehyde become acidic due to the formation of glutaric acid. In the present investigation some of the commercial glutaraldehyde was purified by passage through an ion-exchange column (Amberlite I R. 45). The solution obtained showed 97% purity on chromatography and had a pH of 6·8. The purified and commercial samples were compared in both chemical and histological reactions.

Aqueous solutions containing known quantities of adrenaline or noradrenaline were added to glutaraldehyde. The reactions were followed visually and the effect of pH on the precipitate produced was determined.

The insoluble precipitate formed when glutaraldehyde reacts with noradrenaline at pH 7·3 was allowed to dry on Whatman no. 1 chromatography paper and subjected to a variety of spot tests including spraying with aqueous 1% potassium ferricyanide followed by ammonia vapour (James, 1948), 2·5% potassium dichromate

or saturated potassium iodate for catecholamines; 1% sodium molybdate for *o*-dihydroxy-groupings; 1% osmium tetroxide for unsaturated and reducing compounds, etc; 0·2% ninhydrin in acetone (Smith, 1963) for α-aminoacids and amines and 2,4-dinitrophenylhydrazine in dilute hydrochloric acid for aldehyde groups.

The precipitate was also prepared in quantity and after repeated washings with water was tested for solubility in organic solvents in both the wet and dry states. An attempt was made to recrystallize and determine the melting point of the substance.

The reaction between adrenaline and glutaraldehyde was followed by assaying the mixture for free adrenaline using the technique of Euler & Hamberg (1949) with and without adsorption of the adrenaline on alumina and elution with 0·1 M acetic acid (Weil-Malherbe & Bone, 1952).

Histochemical

Slices of *c.* 2 mm thickness of adrenal glands of rat, mouse, cat, rabbit, guinea-pig and domestic fowl were fixed at 0–4 °C for various times in 4–6% glutaraldehyde buffered to different pH's within the range 6·5–7·4 with 0·1 M phosphate or cacodylate buffer. Best fixation and differentiation between the primary and secondary amines were obtained in tissues fixed for 4 h or longer. The tissue blocks were then either sectioned on a freezing microtome or dehydrated prior to embedding in paraffin wax or araldite and cutting. Frozen sections were transferred to saturated potassium iodate, 3% potassium dichromate, 1% sodium molybdate or 1% buffered (pH 7·3) osmium tetroxide before examination. Some aldehyde-fixed tissues were cut with a razor blade into small blocks or thinner slices and then immersed in iodate, dichromate or osmium tetroxide solution before paraffin embedding, sectioning and examination with the light-microscope.

Most tissues for electron microscopy were fixed for 4–24 h in ice-cold buffered glutaraldehyde. After fixing for 5–10 min the tissues were cut into small blocks (*c.* 1 mm^3) or thin slices ($\leqslant$ 1 mm thick) with a stainless-steel razor blade and then returned to the fixative. This procedure ensured penetration and reduced cellular damage as compared with thin slices or blocks prepared from fresh tissues. Fixed tissues were washed for 10 min to overnight in three changes of ice-cold buffer containing 0·1 M (cacodylate) or 0·25 M (phosphate) sucrose, and subsequently transferred to 1% buffered osmium tetroxide (Caulfield, 1957) for 1 h. The material was then rinsed in ice-cold water, dehydrated in ice-cold 70%, 90% and absolute alcohol, reaching the absolute after 10 min; it was finally transferred to two changes of absolute alcohol at room temperature (10 min in each) prior to 1:2 epoxypropane and embedding in araldite.

As part of the investigation into the mechanism of the reaction between glutaraldehyde and tissue catecholamines some tissues were fixed in buffered glutaraldehyde only, or in buffered glutaraldehyde followed by immersion for 1–2 h in (*a*) 3% potassium dichromate at pH 5·8, (*b*) 1% sodium molybdate or (*c*) saturated potassium iodate at pH 5·8 and the results compared with those fixed by the glutaraldehyde/osmium tetroxide régime described above. The sections for electron microscopy were cut on a Cambridge–Huxley microtome, mounted directly on the grid and

stained for 20 min with 1 % aqueous potassium permanganate and differentiated for 20 s in 2·5 % aqueous citric acid, or stained for 10 min with aqueous uranyl acetate followed by 20 min with lead citrate. Sections were examined with an EM 6 electron microscope.

Some tissues for light microscopy were fixed initially in a mixture of 4–6 % glutaraldehyde and 3 % potassium dichromate buffered with 0·2 M acetate to pH 5·8.

RESULTS

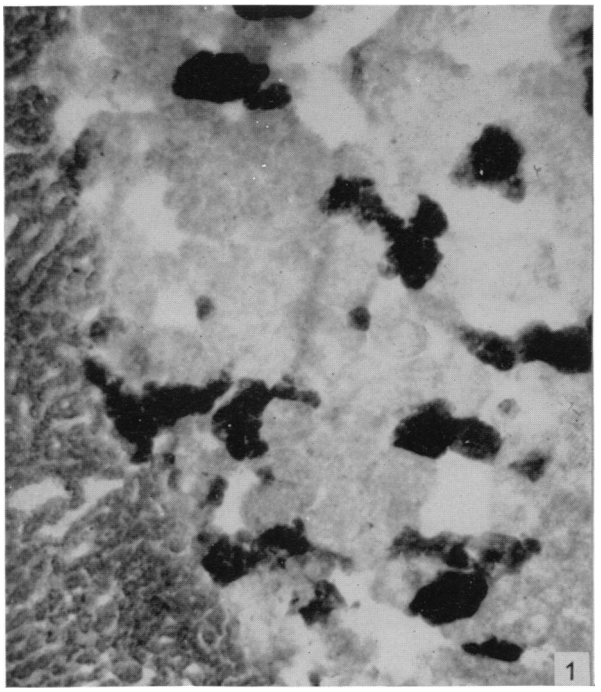
Histochemical

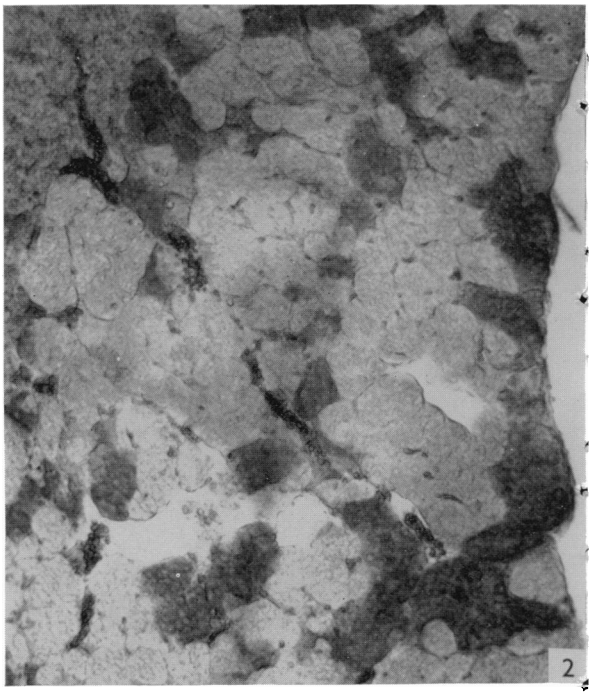
Light microscopy

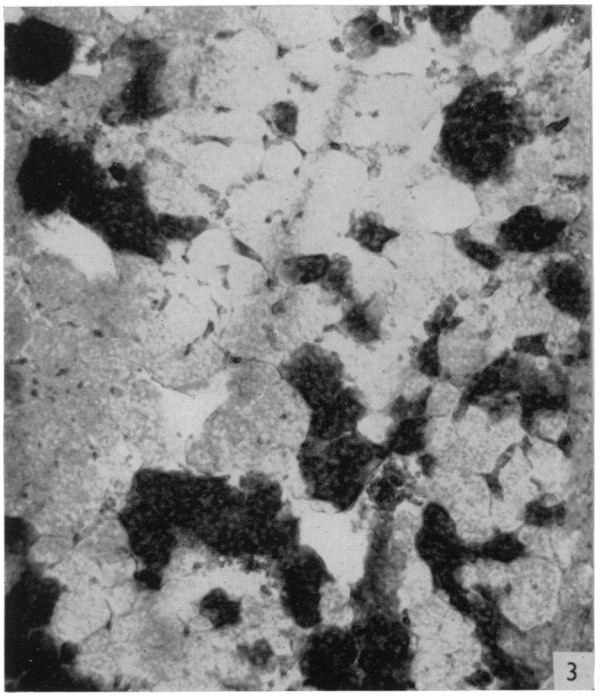
Frozen sections of glutaraldehyde-fixed adrenal medulla of rat, mouse and cat show islands of cells which are more deeply straw coloured than the remainder of the gland; the difference in intensity of the two is, however, insufficient to be of histochemical value. When the glutaraldehyde-fixed frozen sections are immersed in solutions of 3 % potassium dichromate (pH 5·8), 1 % sodium molybdate, 1 % osmium tetroxide or saturated potassium iodate, the same cell islands become highly coloured (Figs. 1, 2, 3). Islands of glutaraldehyde-fixed noradrenaline-storing cells appear yellow after treatment with potassium dichromate, golden brown after sodium molybdate, brown-black after osmium tetroxide and brown after potassium iodate. Similar reactions have been seen in the fowl's adrenal (Fig. 4) while no reaction occurred in the chromaffin cells of the central part of the rabbit's and the guinea-pig's adrenal medulla. The general pattern of the colour reaction produced by the above oxidizing agents is similar to that seen when slices of fresh adrenal glands of cat, rat, mouse and fowl are immersed in a saturated solution of potassium iodate (Hillarp & Hökfelt, 1953). The reaction has not been observed in adrenals of Wistar and Sprague–Dawley rats removed from animals 24 h after intravenous injections of reserpine (5–15 mg/kg), but occurs in animals which have received convulsive doses of insulin. These findings are consistent with the coloration being due to the presence of noradrenaline in the reacting chromaffin cells, since reserpine depletes both adrenaline and noradrenaline elements while insulin depletes the adrenaline-storing cells only (Coupland, 1965*a*). The reaction is still obtained after fixing tissues for some months in glutaraldehyde.

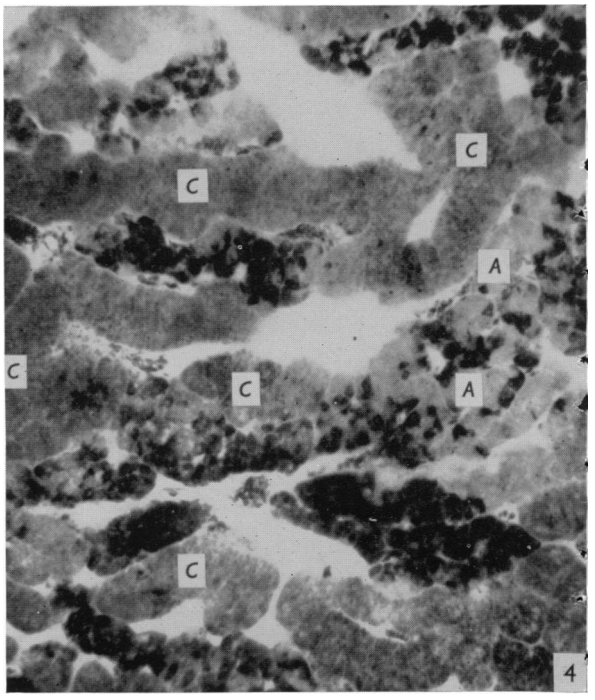
In glutaraldehyde-fixed paraffin-embedded sections a weak-to-moderate colour reaction develops on immersion in osmium tetroxide or potassium dichromate, but this is of little practical value. A good reaction is, however, observed when small blocks (*c.* 2 mm^3) are fixed in glutaraldehyde and immersed in osmium tetroxide or potassium dichromate for 1–2 h prior to dehydration and embedded in paraffin. Sections fixed only in glutaraldehyde show no differential staining of adrenaline- and noradrenaline-storing cells after Giemsa stain or haematoxylin and eosin.

When sections are fixed initially in a mixture of 3 % potassium dichromate and 4–6 % glutaraldehyde buffered to pH 5·8 a positive colour-reaction (chromaffin reaction) occurs in both the adrenaline- and noradrenaline-storing cells. In paraffin-embedded sections of this material the noradrenaline-storing elements show a strong golden-yellow colour while adrenaline-storing cells have a less intense and relatively dull yellow-grey colour.


1


2


3


C
C
C
A
C
C
A
C
4

Electron microscopy

In sections of adrenal glands of rat, mouse, cat and fowl, fixed initially in glutaraldehyde and subsequently in osmium tetroxide, the noradrenaline-storing chromaffin cells are characterized by the presence of typical membrane-bound cytoplasmic granules which have a homogeneous intensely electron-dense interior and a distinct peripheral limiting membrane, as illustrated in the rat by Figs. 5 and 6. By contrast the typical membrane-bound granules of the adrenaline-storing cells show only moderate electron density and their contents exhibit an internal granularity.

The adrenaline- and noradrenaline-storing granules may be differentiated quite clearly in tissues fixed in glutaraldehyde only, glutaraldehyde followed by potassium dichromate, iodate or sodium molybdate as well as glutaraldehyde followed by osmium tetroxide (Figs. 5, 6, 7, 8). Hence the differential electron density of the adrenaline- and noradrenaline-storing cells must reflect basically the reaction between noradrenaline and glutaraldehyde. Greater contrast between the two types of granules is, however, observed in tissue subsequently immersed in dichromate, and greatest contrast follows osmication. Although adrenaline- and noradrenaline-storing granules may be identified in unstained preparations differentiation and cytological details are further improved if sections are stained on the grid with aqueous potassium permanganate or with uranyl acetate and alkaline lead citrate. If lead is used the staining time must be restricted to 25–30 min since prolonged staining results in the adrenaline-storing granules also becoming intensely electron-dense.

Apart from granule contrast the main differences in the appearance of sections of tissues subjected to the various procedures relate to the electron density, and hence appearance, of cytoplasmic and nuclear membranes. A demonstration of membranes similar to those observed in tissues fixed initially in buffered osmium tetroxide (Coupland, 1965*a*, *b*) is observed only in those glutaraldehyde-fixed tissues which have subsequently been immersed in osmium tetroxide. It is interesting to note that chromaffin granules of rat adrenal fixed only in glutaraldehyde (Fig. 7) or in glutaraldehyde followed by potassium iodate or sodium molybdate show no evidence of a peripheral membrane, those fixed in glutaraldehyde and post-chromed (Fig. 8) show a peripheral membrane around the noradrenaline-storing granules only, while those fixed in glutaraldehyde and post-osmicated (Figs. 5, 6) show membranes around both adrenaline and noradrenaline granules. The cytoplasmic matrix of

---

Fig. 1. Frozen section of rat adrenal fixed in cacodylate-buffered glutaraldehyde (pH 7·3) and post-osmicated. Noradrenaline-storing cells form dark islands, adrenaline-storing cells pale; reticular zone of cortex to left. × 110.

Fig. 2. Rat adrenal medulla fixed as in Fig. 1, but post-chromed; showing selective reaction of noradrenaline-storing islands which appeared golden-yellow. Adrenaline-storing cells uncoloured. × 110.

Fig. 3. Rat adrenal medulla fixed as in Fig. 1, but frozen section immersed in saturated potassium iodate. Noradrenaline-storing cells appeared as discrete brown islands, adrenaline-storing cells uncoloured. × 110.

Fig. 4. Frozen section of gluraldehyde-fixed and post-osmicated cock adrenal and cords of cortical cells (C, grey) intermingle with those of chromaffin cells. Noradrenaline-storing elements appear black, adrenaline-storing cells (A) uncoloured. × 110.

the noradrenaline-storing cells usually exhibits a greater overall electron density than that of the adrenaline-storing cells.

The absence of the membranous elements in Figs. 7 and 8, together with a reduction in the overall electron density of the cytoplasmic and nuclear matrix, results in unusual pictorial compositions as compared with the more conventional ones of Figs. 5 and 6. The difference is, however, simply a reflexion of the difference in electron density of the various cell components after the various fixatives and it is of interest to note that all the material illustrated in Figs. 5–8 was obtained from the same adrenal gland fixed initially for 4 h in glutaraldehyde and that the difference reflects the superimposition of osmium tetroxide fixation.

In all glutaraldehyde-fixed material the contents of the noradrenaline-storing granules are homogeneously electron-dense; in the rat they are often asymmetrical and may be vacuolated or fragmented. This irregularity is absent or less evident in the other species. When present it may reflect the violence of the chemical reaction between noradrenaline and glutaraldehyde since even in the rat adrenal it is less marked in tissues fixed at 0 °C in glutaraldehyde than at room temperature or in those fixed at a lower pH (namely pH 6·5–7). Prolonged washing after glutaraldehyde fixation does not reduce granule distortion below that observed after 10 min washing in buffer on a turntable or shaker. The use of glutaraldehyde purified on the column has little or no effect on granule distortion even though the purified product has much less tendency to cause mitochondrial damage and in consequence is a better cytological fixative.

Adrenaline-storing granules always show a fine internal granularity and occasionally possess a more dense centre, giving a target appearance. Electron micrographs of sections through the central part of the adult rabbit adrenal gland show only the adrenaline-type of storage granule. In the mouse, rat and domestic fowl both adrenaline- and noradrenaline-storing granules may be identified and are limited to specific cells. No definite evidence of cells containing mixtures of adrenaline- and noradrenaline-storing granules has been observed in adult adrenals, though membrane-bound granular material of only moderate electron density has been observed in the vicinity of the Golgi apparatus in noradrenaline-storing cells in all forms; this may represent an early stage in the formation of either dark bodies or of the binding substance of catecholamines (see Coupland, 1965*b*).

Reactions between catecholamines and glutaraldehyde

The addition of 1 ml of 0·1–2 M glutaraldehyde to a mixture of 1 ml of 0·1 M noradrenaline plus 1 ml of 0·3 M cacodylate or phosphate buffer, pH 7·3, results within seconds in the formation of a dense straw-coloured precipitate and a yellow supernatant. At amine concentrations of < 100 μg/ml no precipitate occurs.

When glutaraldehyde is mixed with adrenaline no precipitate develops, irrespective of amine concentration, and the only visible colour change is a pale pink discoloration after 30 min or more, due to the formation of adrenochrome.

The amount of precipitate formed when noradrenaline reacts with glutaraldehyde varies with pH, and the results obtained by gravimetric determination after 6 and 24 h at pH 4–10 are indicated in Fig. 9.

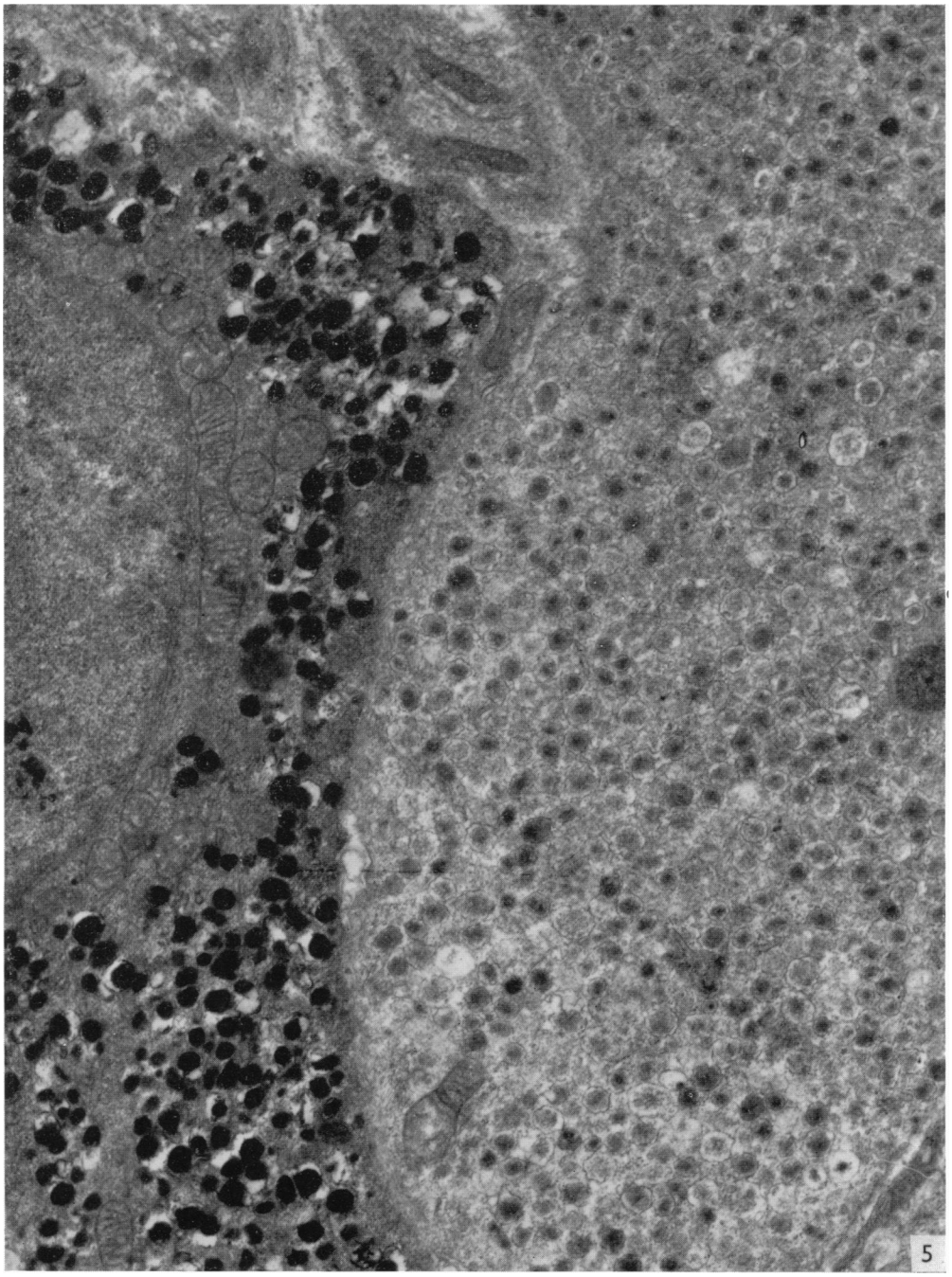


Fig. 5. Electron micrograph of rat adrenal medulla fixed in buffered glutaraldehyde (pH 7·3) and subsequently in osmium tetroxide. Noradrenaline-storing cell with typical granules on left, adrenaline-storing cell with typical granules on the right. Note differences in electron density of granules and cytoplasm. Potassium permanganate; ×15 000.

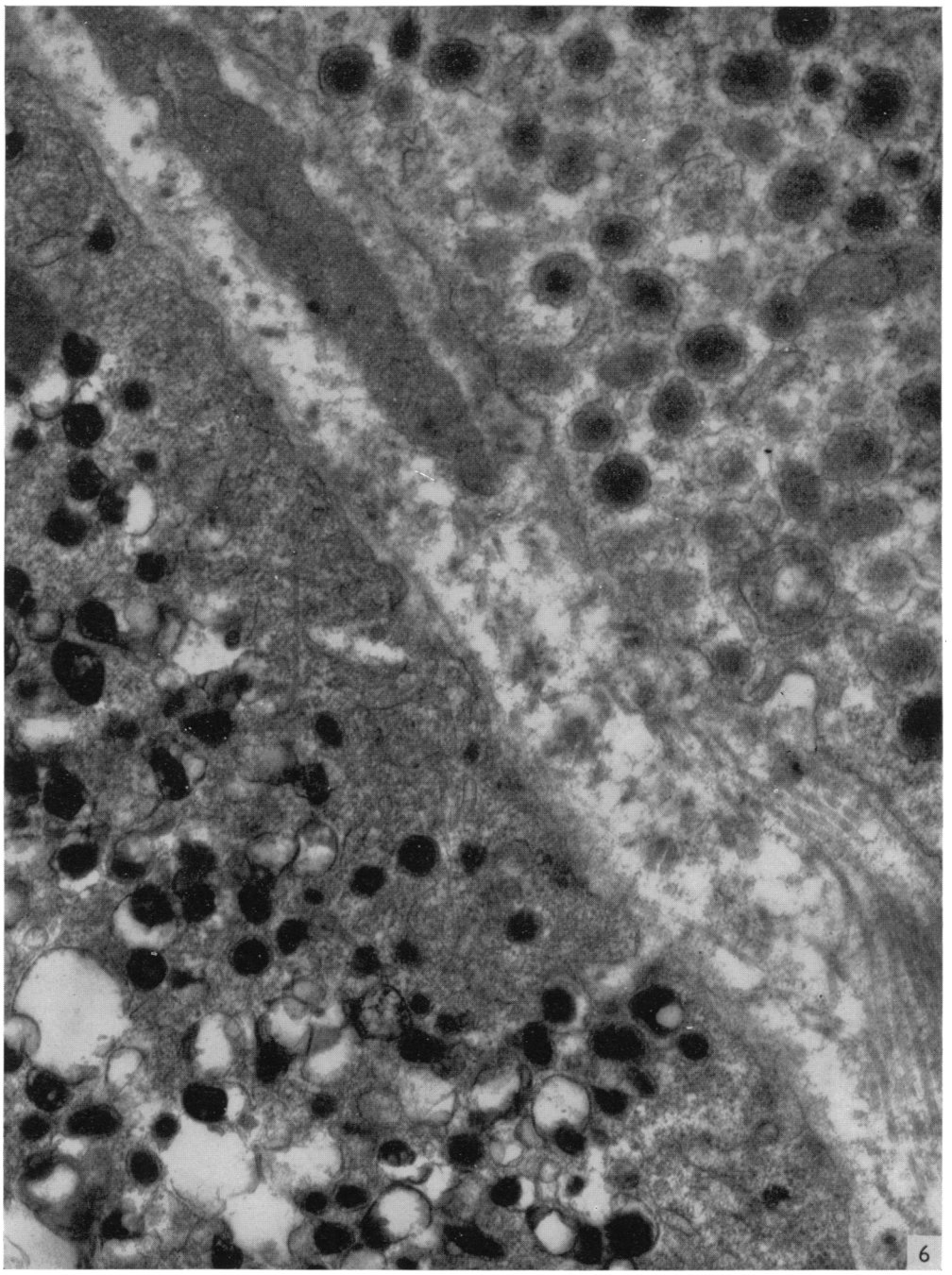


Fig. 6. Higher-power electron micrograph of adrenaline-storing (top right) and noradrenaline-storing (bottom left) granules of rat adrenal medulla fixed in glutaraldehyde and subsequently in osmium tetroxide. Note internal granularity of adrenaline-granules with occasional target appearance and the homogeneous, asymmetrical, highly electron-dense nature of contents of noradrenaline granules. Note the peripheral membrane associated with both types of granule. Potassium permanganate; ×40 000.

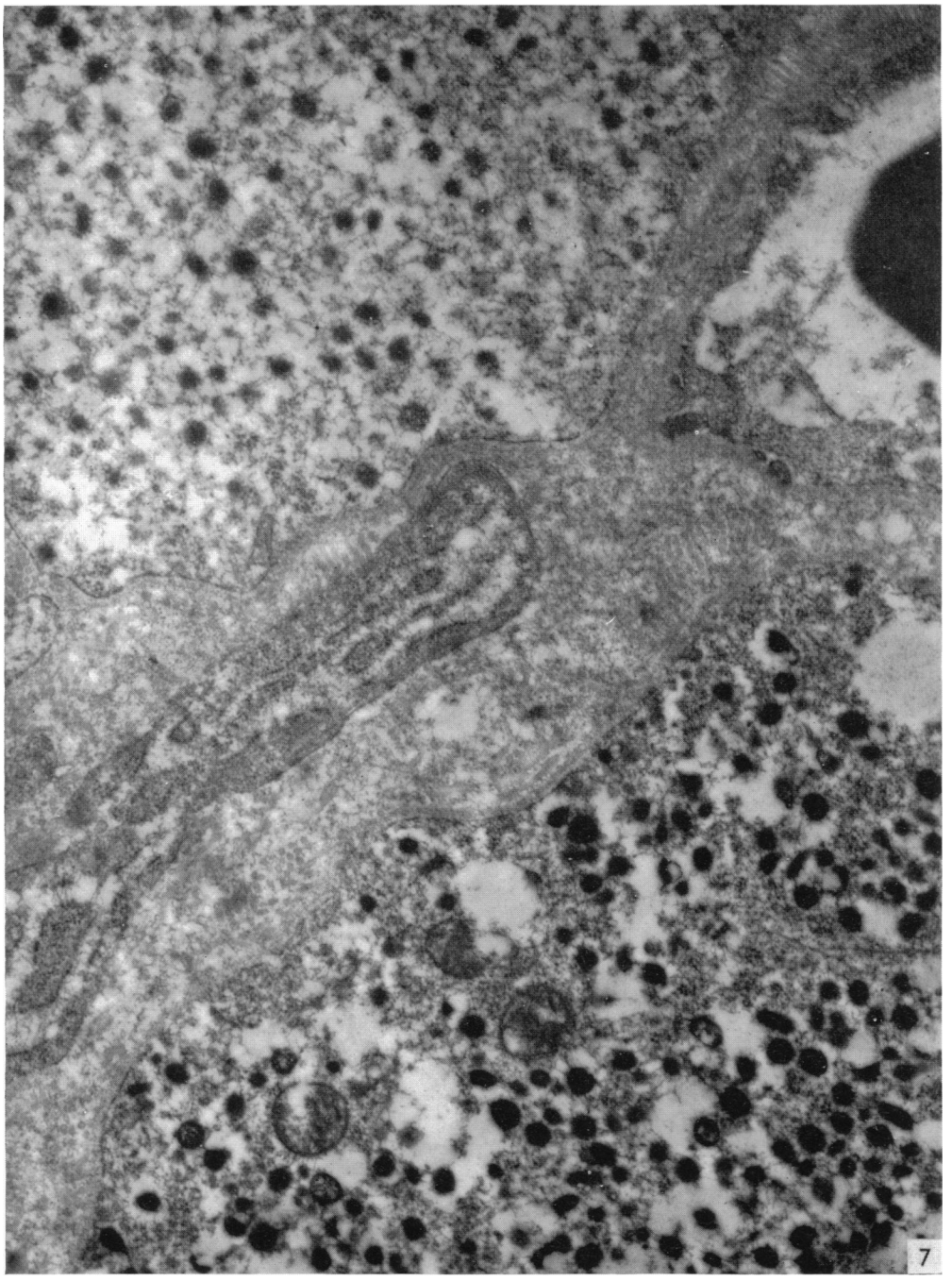


Fig. 7. Electron micrograph of rat adrenal medulla fixed in buffered glutaraldehyde (pH 7·3) only. Cells contain moderately electron-dense adrenaline-storing granules (above) and strongly electron-dense noradrenaline-storing granules (below). Potassium permanganate; × 25 000.

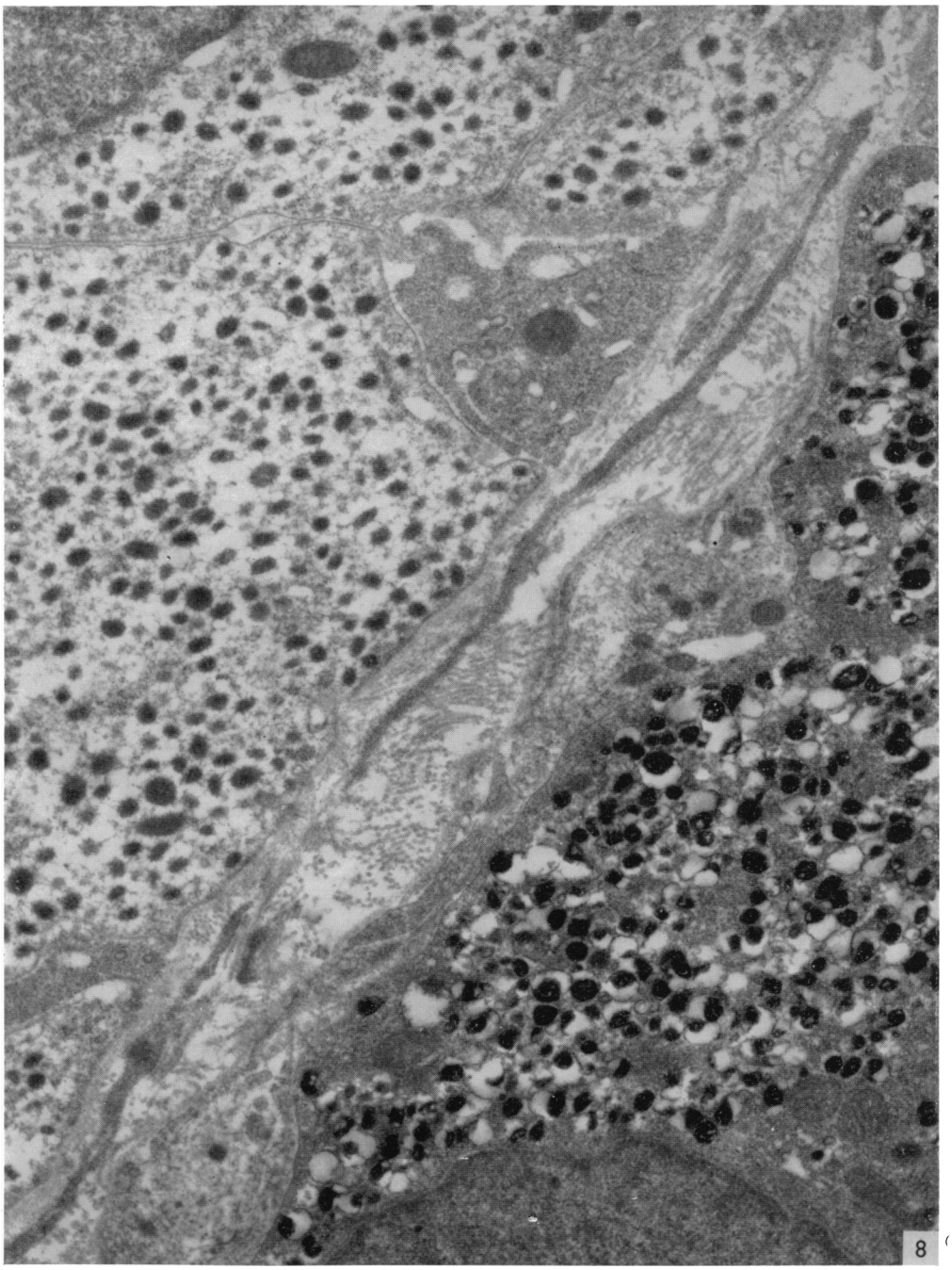


Fig. 8. Electron micrograph of rat adrenal medulla fixed in buffered glutaraldehyde (pH 7·3) and subsequently in 3% potassium dichromate (pH 5·8). Adrenaline-storing granules above, noradrenaline-storing below. Note difference in electron densities of two types of granule, the asymmetry of contents of noradrenaline-granules and their peripheral limiting membranes. No membrane is seen in relation to the adrenaline-granules. Potassium permanganate; ×15 000.

Observations on the noradrenaline–glutaraldehyde complex

The precipitate produced by the reaction of commercial and purified glutaraldehyde with noradrenaline at pH 7·3 was centrifuged, and washed repeatedly with distilled water. Spots of the precipitate as well as of aqueous solutions of adrenaline and noradrenaline bitartrate and glutaraldehyde, all initially at pH 7·3 were allowed to dry on Whatman no. 1 paper and sprayed with a variety of test substances. The results are indicated in Table 1. Noradrenaline base gave the same results as the bitartrate.

The precipitate was also tested for solubility in various organic and inorganic

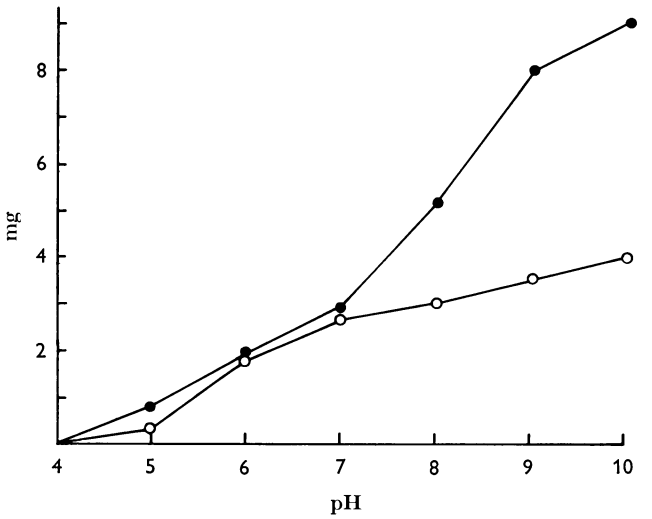


Fig. 9. Effect of pH on amount of precipitate formed after addition of 0·1 M noradrenaline to 2 M glutaraldehyde. Ordinate: mg precipitate at 6 hr (○—○) and 24 hr (●—●). Abscissa: pH.

Table 1

	FCY	MOLY	Os	KDC	NIN	DNPH	FLUO
Adrenaline	P+++	Y+++	B+++	B+	MP++	0	A
Noradrenaline	M+++	Y+++	B++	B+	MP++	0	A
Glutaraldehyde	0	0	0	0	0	Y++	0
Noradrenaline–glutaraldehyde complex	B+++	Y+++	B+++	B+	Y+	0	A

A = absorption
B = brown
M = mauve
0 = no colour
P = pink
Y = yellow
+++ = strong
++ = moderate
+ = weak

FCY = potassium ferricyanide
MOLY = sodium molybdate
Os = osmium tetroxide
KDC = potassium dichromate
NIN = ninhydrin
DNPH = 2, 4-dinitrophenylhydrazine
FLUO = fluorescence

solutions. These included distilled water, IN hydrochloric acid, methanol, ethanol, *n*-butanol, glacial acetic acid, pyridine, ether, acetone, benzene, chloroform and dioxane. Immediately after precipitation and washing the complex was soluble in hydrochloric acid, ethanol, butanol, glacial acetic acid, pyridine and dioxane, and showed greatest solubility in the last. However, after drying, the precipitate was insoluble in all solvents tested. The dried precipitate decomposed on heating between 190–220 °C.

Reaction of adrenaline with glutaraldehyde

No precipitate results when aqueous solutions of adrenaline are mixed with equimolar or excess aqueous glutaraldehyde solution at pH 4–10 even after standing for some days, and the yellow colour which develops when noradrenaline and glutar-

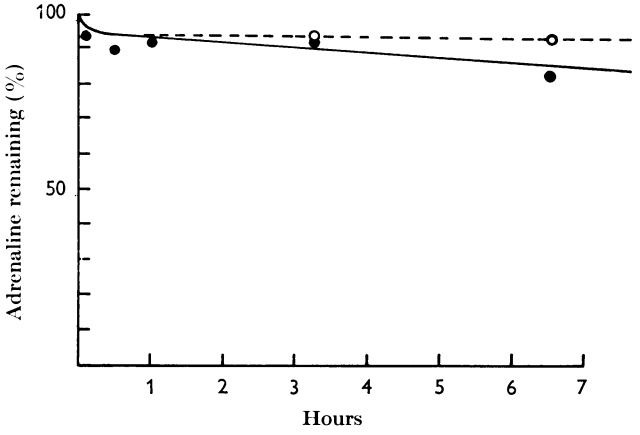


Fig. 10. Amount of adrenaline persisting in mixture of glutaraldehyde and adrenaline at pH 7·3. —, total adrenaline; – – –, adrenaline present after correction for loss due to oxidation.

aldehyde interact does not appear. Mixtures of adrenaline and glutaraldehyde were assayed for catecholamine over a period of 7 h, using the method of Euler & Hamberg (1949). Direct assay of the fluid for adrenaline or assay after adsorption of catecholamine on alumina and elution with acetic acid indicates that a substantial amount of the free-base adrenaline remains for some hours following the addition of glutaraldehyde. The effect of time on the concentration of adrenaline in a mixture of adrenaline and glutaraldehyde at pH 7·3 is illustrated in Fig. 10, which shows the absolute concentration and the concentration after correction for loss due to oxidation. It is of interest to note that the presence of glutaraldehyde does not interfere with the Euler–Hamberg method for the assay of adrenaline.

The mechanism of the differential staining reaction

The colour reaction produced by treating glutaraldehyde-fixed noradrenaline-storing cells with potassium dichromate, potassium iodate, sodium molybdate and osmium tetroxide, and the apparent lack of reaction of the adrenaline-storing

elements, may have been due to (*a*) the formation of different complexes with the two amines in the tissues, (*b*) the formation of a soluble adrenaline-glutaraldehyde complex, or (*c*) the loss of adrenaline from the cells during fixation and dehydration.

Since no precipitate or initial discoloration is produced when adrenaline and glutaraldehyde are mixed and the results of assay suggest that adrenaline does not react in significant quantities with glutaraldehyde during the period normally employed for fixation, it was of interest to determine whether the adrenaline remained *in situ*, bound in the chromaffin granules, or was lost during fixation. This was investigated by fixing slices of adrenal medulla from groups of male

Table 2

Adrenals	Adrenaline content (μg)					
		Ethyl alcohol				
	Fixative	70%	90%	Absolute	Total	% normal
Glutaraldehyde fixed and dehydrated	37	33	21	15	106	88
Glutaraldehyde fixed and dehydrated ←		Pooled			→ 96	80
Fresh	—	—	—	—	120	100

Sprague–Dawley rats (four in each), weight 250–300 g, in ice-cold cacodylate-buffered glutaraldehyde and assaying the fixing and dehydrating fluids for adrenaline. Tissues were fixed for 4 h with intermittent agitation in order to ensure adequate contacts with the fixing fluid. After 4 h the fixing fluid was assayed by the Euler & Hamberg method. The tissues were then dehydrated in 70%, 90% and absolute alcohol for 10 min each. Each of the alcohols was assayed and the results are indicated in Table 2. If the total quantity of adrenaline in the fixing and dehydrating fluid is compared with the adrenaline content of a similar number of rats of the same weight and age, it is apparent that some 80% of the total adrenaline content of the tissues is eluted during fixation and dehydration.

DISCUSSION

Solutions of glutaraldehyde and noradrenaline react at pH 7·3 with the formation of dense precipitate which after drying is insoluble in all common organic solvents and which decomposes on heating. The precipitate is formed immediately after mixing the two solutions providing the amine concentration is in excess of 100 μg/ml, a level which is greatly exceeded in the catecholamine granules of noradrenaline-storing chromaffin cells. The reactions of the dried precipitate with a variety of reagents resemble the reactions of noradrenaline-storing cells in frozen sections of glutaraldehyde-fixed adrenal tissue with the same reagents including osmium tetroxide, potassium dichromate, potassium iodate and sodium molybdate. The brown reaction of the precipitate with sodium molybdate indicates the presence of an *o*-dihydroxy grouping (Pridham, 1959), the brown reaction with ferricyanide (James, 1948)

indicates an alteration of the primary amine, while the pale yellow colour produced with ninhydrin would be in keeping with the noradrenaline changing to tertiary amine, being incorporated into heterocyclic-compounds (Smith, 1963) and/or polymer formation. Having regard to the nature of the reactant and the physical characteristics of the precipitate, including its decomposition at 190–220 ° without melting it would appear that an insoluble polymer is formed by the interaction of the aldehyde and the primary amine. The spot tests combined with histochemical reactions of fixed tissues in normal and depleted adrenals indicate that the presence of an insoluble noradrenaline–glutaraldehyde complex, which still gives a positive reaction for *o*-dihydroxy-groupings (with sodium molybdate), is responsible for the reaction of noradrenaline-storing cells with osmium tetroxide, potassium dichromate or potassium iodate.

In contrast to noradrenaline, adrenaline appears to undergo little reaction with glutaraldehyde and, when assayed by the method of Euler & Hamberg (1949), less than 10 % of adrenaline is lost from a mixture of the two during a period of 7 h. Indeed assay of fixing and dehydrating fluids indicates not only that the adrenaline fails to produce an insoluble complex with glutaraldehyde, but that it diffuses out of the tissue so that, using rat adrenals, some 80 % of the total adrenaline may be accounted for in the fixing and dehydrating fluids.

The formation of the insoluble complex with noradrenaline and the loss of adrenaline during glutaraldehyde fixation accounts for the differential staining reaction observed in adrenaline and noradrenaline-storing chromaffin cells with the present techniques. In sections fixed only in glutaraldehyde, cells containing the glutaraldehyde–noradrenaline polymer are pale straw coloured and though they can be identified with the light-microscope, subsequent treatment with osmium tetroxide, sodium molybdate, potassium dichromate or potassium iodate is necessary to obtain satisfactory localization. After these reagents the noradrenaline–glutaraldehyde complex is more electron-dense and homogeneous than the residual contents of the adrenaline-storing granules, and because of this fact the noradrenaline- and adrenaline-storing granules may be distinguished in tissues treated only with glutaraldehyde. Differentiation in the electron microscope is, however, considerably improved by subsequent treatment with dichromate, or better still with osmium tetroxide.

When potassium dichromate is mixed with glutaraldehyde in the initial fixative, both adrenaline- and noradrenaline-storing cells react and assume a yellow colour, the noradrenaline-storing cells showing a more intense and golden hue while the adrenaline-storing cells show a less intense yellow-grey colour. Adrenaline- and noradrenaline-storing chromaffin cells can, therefore, readily be differentiated in the light microscope by comparing the chromaffin reaction of a tissue slice fixed initially in a mixture of glutaraldehyde and potassium dichromate with the reaction in an adjacent slice fixed initially in glutaraldehyde and subsequently post-chromed.

The loss of adrenaline from tissue sections during fixation implies that the moderate electron density and internal granularity of the adrenaline-storing granules (see also Coupland, 1965*b*) represents binding substance. Analysis of dried catecholamine-storing granules of the ox adrenal by Hillarp (1959) revealed a composition of 20 % catecholamine, 35 % protein, 22 % lipid and 15 % adenosine

triphosphate; in the adrenaline-storing granules which have lost the majority of their catecholamine during fixation and dehydration the moderate electron density probably results from the presence of protein and lipid. Fixation in glutaraldehyde for some days or weeks appears to have no deleterious effect and affords preparations in which the two types of granules can readily be differentiated.

The presence in the rat adrenal of a peripheral membrane on the noradrenaline-storing chromaffin granules after post-chroming and its absence on the adrenaline-storing granules suggests that the two limiting membranes may have different chemical and physical properties. This subject is being further investigated.

In adrenals of adult rat, cat, mouse and domestic fowl adrenaline- and noradrenaline-storing granules are limited to specific cells, and mixtures in the same cell do not occur in undamaged preparations. As noted previously, however (Coupland, 1965*b*), in the Golgi zone of some noradrenaline-storing cells membrane-bound collections of granular material do occur and may represent binding substance without catecholamine or an early stage in some other granular body formation.

A differential staining reaction for adrenaline- and noradrenaline-storing granules at the fine structural level was described recently by Wood & Barrnett (1964). In this work tissues were fixed in glutaraldehyde and subsequently immersed in 2·5% potassium dichromate at pH 4·1 or pH 6·5 for 24 h at 4 °C. Some pieces of tissue were subsequently treated with osmium tetroxide. The authors reported that in tissues chromated at pH 4·1 only noradrenaline-storing granules showed strong electron density while at pH 6·5 both types of granule showed strong electron density. These phenomena were explained as being due to the selective oxidation of noradrenaline at pH 4·1. In the light of the present work a more likely explanation of their findings is that dichromate reacts only with the glutaraldehyde–noradrenaline complex at pH 4·1, whereas at pH 6·5 it is more widely and less specifically bound in the tissues, and hence staining at pH 6·5 results in a general increase in electron density which affects both types of granule. As observed above, a similar masking occurs if sections are subsequently stained for long periods (> 30 min) with alkaline lead citrate. It is also worth noting that at pH 4·1 potassium dichromate would react, as an oxidizing agent, much more rapidly and completely with adrenaline than with noradrenaline, hence selective oxidation of the primary amine is very unlikely to be the operative factor.

As a method for differentiating between adrenaline- and noradrenaline-storing chromaffin cells initial fixation in glutaraldehyde followed by subsequent treatment with osmium tetroxide, potassium dichromate, potassium iodate or sodium molybdate has an advantage over the iodate method (Hillarp & Hökfelt, 1953) or the fluorescent method (Eränkö, 1955) in that thin or ultrathin sections may be used and examined with either the light or electron microscope. A further advantage is that the noradrenaline complex may be selectively stained in glutaraldehyde-fixed tissue blocks after months of storage at 4 °C in glutaraldehyde, buffer solutions or water.

Although no significant difference with reactions between adrenaline or noradrenaline and glutaraldehyde has been observed with commercial or purified fixative, better preservation of tissues has been observed at the ultrastructural level with glutaraldehyde purified by a weak anionic resin. Following passage down

a column mitochondrial damage has been abolished in the rat adrenal—a tissue and species particularly susceptible to such changes when the commercial solution is used without removal of glutaric acid.

SUMMARY

Noradrenaline reacts with glutaraldehyde to produce a dense precipitate. This reaction is pH-dependent and the product is a polymer. In tissue sections the polymer is bound *in situ* and is electron-dense. The noradrenaline–glutaraldehyde polymer is rendered more electron-dense and is coloured by osmium tetroxide, potassium dichromate, sodium molybdate or potassium iodate.

Adrenaline undergoes little reaction with glutaraldehyde during a period of 6 h. In tissues fixed in glutaraldehyde this secondary amine diffuses away from its binding site into the fixing or dehydrating fluids.

The loss of adrenaline and fixation of noradrenaline in tissue blocks exposed to glutaraldehyde accounts for the differential staining reactions of adrenaline- and noradrenaline-storing chromaffin cell granules.

Glutaraldehyde fixation followed by osmication or chromation has the advantage over other methods for the differentiation between adrenaline- and noradrenaline-storing cells in that thin or ultrathin sections may be used. Furthermore, blocks still react with osmium tetroxide, potassium dichromate or iodate or sodium molybdate after storage for months in the initial fixative.

We are grateful to Mr A. S. Pyper, Mrs C. Murchie and Mrs E. Lloyd-Davies for technical assistance and to Dr R. Foster of the Department of Chemistry for checking the purity of the glutaraldehyde. This work has been made possible by instruments and financial support provided by the Wellcome Foundation and Scottish Hospital Endowments Research Trust.

REFERENCES

CAULFIELD, J. B. (1957). Effects of varying the vehicle for Os O_4 in tissue fixation. *J. biophys. biochem. Cytol.* **2**, 827-830.

COUPLAND, R. E. (1965*a*). The *Natural History of the Chromaffin Cell.* London: Longmans.

COUPLAND, R. E. (1965*b*). Electron microscopic observations on the structure of the rat adrenal medulla. I. The ultrastructure and organization of chromaffin cells in the normal adrenal medulla. *J. Anat.* **99**, 231–254.

COUPLAND, R. E. & HOPWOOD, D. (1965). On adrenaline- and noradrenaline-storing granules in chromaffin cells—an electron microscopic study. *J. Anat.* **99**, 191*P*.

COUPLAND, R. E., PYPER, A. S. & HOPWOOD, D. (1964). A method for differentiating between noradrenaline- and adrenaline-storing cells in the light and electron microscope. *Nature, Lond.* **201**, 1240–1242.

ERANKÖ, O. (1955). Fluorescing islets, adrenaline and noradrenaline in the adrenal medulla of some common laboratory animals. *Annls Med. exp. Biol. Fenn.* **33**, 278–90.

EULER, U. S. VON. & HAMBERG, U. (1949). Colorimetric determination of noradrenaline in the presence of adrenaline. *Science*, **110**, 561.

HILLARP, N.-Å (1959). Further observations on the state of the catecholamines stored in adrenal medullary granules. *Acta physiol. scand.* **47**, 271–9.

HILLARP, N.-Å. & HÖKFELT, B. (1953). Evidence of adrenaline and noradrenaline in separate adrenal medullary cells. *Acta physiol. scand.* **30**, 55–68.

HOPWOOD, D. & COUPLAND, R. E. (1965). Observations on the reaction of biogenic amines, in particular noradrenaline, with glutaraldehyde. *J. Anat.* **99**, 191 *P*.

JAMES, W. O. (1948). Demonstration and separation of noradrenaline, adrenaline and methyl-adrenaline. *Nature, Lond.* **161**, 851–852.

PRIDHAM, J. B. (1959). Paper electrophoresis and paper chromatography of phenolic compounds. *J. Chromat.* **2**, 605–611.

SABATINI, D. D., BENSCH, K. G. & BARRNETT, R. J. (1963). Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17**, 19–58.

SABATINI, D. D., MILLER, F. & BARRNETT, R. J. (1963). Aldehyde fixation for morphological and enzyme histochemical studies with the electron mocroscope. *J. Histochem. Cytochem.* **12**, 57–71.

SMITH, I. (1963). *Chromatographic and Electrophoretic Techniques*, vol. 1, 2nd ed., p. 95. London: Heinemann.

WEIL-MALHERBE, H. & BONE, A. D. (1952). The chemical estimation of adrenalin-like substances in the blood. *Biochem. J.* **51**, 311–318.

WOOD, J. G. & BARRNETT, R. J. (1964). Histochemical demonstration of norepinephrine at a fine structural level. *J. Histochem. Cytochem.* **12**, 197–209.