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CYTOCHEMISTRY OF 5-HYDROXYTRYPTAMINE AT THE ELECTRON MICROSCOPE LEVEL

I. STUDY OF THE SPECIFICITY OF THE REACTION IN ISOLATED BLOOD PLATELETS[1]

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Blood platelets obtained from normal rabbits and those isolated from reserpine-treated animals and subsequently incubated *in vitro* with 5-hydroxytryptamine, norepinephrine and histamine were assayed for amine content or processed for examination under the electron microscope. With the glutaraldehyde-dichromate reaction for unsubstituted catechol- and indoleamines, reactive granules were observed in normal platelets. Formaldehyde fixation prior to the glutaraldehyde-dichromate reaction resulted in a similar image under the electron microscope. In platelets obtained from animals treated with reserpine a decrease of the amine content with a corresponding reduction in the number of dense granules was observed. Following incubation with 5-hydroxytryptamine the concentration of the amine increased markedly and the number of dense granules that reacted with both techniques became practically normal. In norepinephrine-incubated platelets dense granules were demonstrated with the glutaraldehyde-dichromate reaction, but no reactive products were observed using prefixation with formaldehyde. Histamine was also incorporated into depleted platelets but gave no reaction. It is concluded that prefixation with formaldehyde renders negative the reaction with catecholamines, leaving unaffected indoleamine-reactive sites. The previous assumption that the dense granules contain 5-hydroxytryptamine has been confirmed by such a cytochemical approach. The possibility that these organelles constitute a common storage site for different amines is discussed.

The solution of many basic problems concerning the synthesis, storage and metabolism of biogenic amines depends on their accurate localization in cellular and subcellular structures. The introduction of fluorescence microscopic methods for the cellular demonstration of monoamines (11, 12, 18) has provided a valuable tool in the study of central and peripheral monoaminergic systems. However, the ultimate goal in the cytochemical detection of these compounds is the development of specific techniques for their identification at a fine structural level. Following the introduction of glutaraldehyde as a fixative for electron microscopy (24), several authors were able to localize norepinephrine (NE)[2] stored in adrenomedullary cells at the electron microscope level (9, 10, 29, 35, 36). The methods developed were based on the fact that the insoluble complex resulting from the interaction between glutaraldehyde and unsubstituted catecholamines (CA) reacts with metal-containing oxidizing agents and produces a dense precipitate. Model experiments demonstrated that both unsubstituted CA (NE and DA) and 5-HT gave a dense precipitate in the test tube when treated with glutaraldehyde and potassium-dichromate (33). Consequently, indolic derivatives could be identified under the electron microscope in conditions similar to that used for the demonstration of CA in adrenomedullary cells (32).

Light microscopic studies have demonstrated that fixation with formaldehyde renders negative the chromaffin reaction given by CA, not affecting the reaction displayed by cells containing 5-HT such as enterochromaffin cells (13, 21). Recent light microscopic studies showed that formaldehyde fixation was also effective in blocking the reaction given by CA when performed

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[2] The abbreviations used are: NE, norepinephrine; CA, catecholamine(s); DA, dopamine; and 5-HT, 5-hydroxytryptamine.

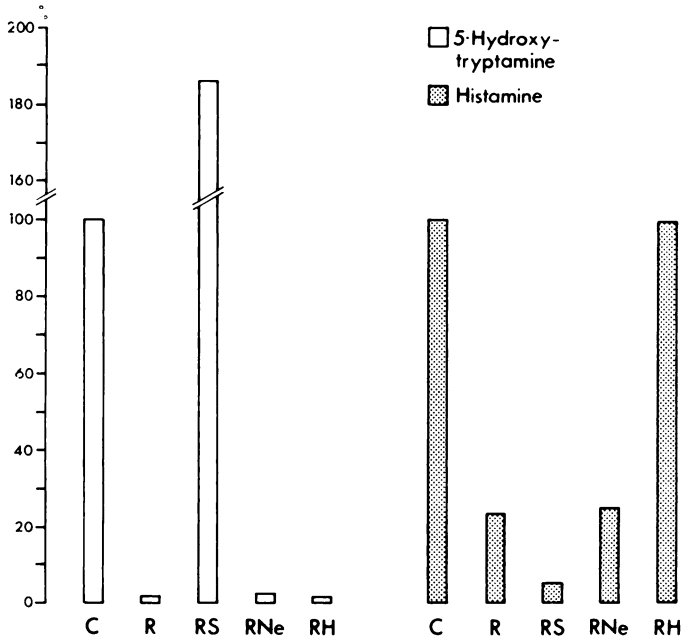


FIG. 1. 5-Hydroxytryptamine content of control (C) and reserpinized (R) platelets, as well as of reserpinized platelets incubated with 5-HT (RS), norepinephrine (RNe) and histamine (RH). Values are expressed as percentages of the amine content of normal platelets. Absolute values for normal platelets are: 11.72 $\mu g/10^9$ cells for 5-HT and 8.54 $\mu g/10^9$ cells for histamine.

prior to glutaraldehyde-dichromate treatment, without impairing the reactivity of indole-containing structures (34).

We have analyzed the selectivity of these histochemical techniques at the electron microscope level, using isolated rabbit blood platelets which constitutes a rather pure preparation well suited for pharmacologic, biochemical and ultrastructural studies. These platelets are known to contain large amounts of 5-HT (20, 22) and histamine (8). These amines may be depleted from their storage sites by reserpine (26, 31). The platelets then may incorporate such amines and CA *in vitro* (6, 20). Platelet stores of 5-HT were indirectly identified under the electron microscope as dense osmiophilic organelles by using a double aldehyde-osmium fixation and pharmacologic treatments (3, 30).

A cytochemical technique is described which enables the study of 5-HT stores at the electron microscope level. The specificity of this technique is demonstrated by a correlation of cytochemical stainings, quantitative determination of the reactive granules and amine content and of pharmacologic treatments that change the amounts of various amines in the platelets.

MATERIAL AND METHODS

Isolation procedures: Adult rabbits fasted for 18 hr were used. Blood was collected by means of a polyethylene cannula placed in the carotid artery and it was mixed 1,9 with a 5% solution of disodium ethylenediaminetetracetate (4). After centrifugation at 300 × *g* for 20 min, the platelets were isolated from the supernatant or "platelet-rich plasma" at 1700 × *g* for 25 min. The pellet constituted an almost pure preparation of platelets. Platelets from animals injected 16 hr previously with 5 mg/kg reserpine[3] intraperitoneally were similarly isolated. The term "reserpinized" platelets that is used hereon refers to platelets isolated from rabbits injected with reserpine. Plastic tubes and pipettes or siliconized glassware were used throughout these procedures which were carried at 2°C. Platelets were counted in a moist chamber under a phase microscope.

***In vitro* action of drugs:** The pellets were resuspended in a modified Tyrode solution[4]: at a concentration similar to that present in platelet-

[3] Serpasol, kindly supplied by Ciba, Argentina.

[4] ClNa, 0.13 *M*; KCl, 0.006 *M*; EDTA, 0.002 *M*; $NaH_2PO_4 \cdot 2H_2O$, 0.001 *M*; $NaHCO_3$, 0.003 *M*; glucose, 0.111 *M*; and sucrose, 0.01 *M*. Final pH 7.4.

rich plasma. The suspensions at a final volume of 2–5 ml were placed in a Dubnoff metabolic shaking incubator under air atmosphere at 37°C for 120 min. The following drugs were added to aliquots of the suspension of platelets obtained from reserpinized animals: 0.4 mg/ml 5-hydroxytryptamine creatinine sulfate, 0.6 mg/ml histamine dihydrochloride and 1 mg/ml DL-norepinephrine clorhydrate (drugs obtained from Sigma Chemical Company, St. Louis, Mo.). The concentration of the drugs is expressed as the free base. As controls, normal platelets and those obtained from reserpinized rabbits incubated in Tyrode were used. After incubation, the suspensions were cooled and centrifuged at 1700 × *g* for 25 min. The pellet was resuspended in ice-cold Tyrode and washed twice. The final pellet was extracted for determination of amines or fixed for electron microscopy.

Quantitative assay of amines: 5-HT was extracted in 0.4 *N* perchloric acid with the addition of ascorbic acid and ethylenediamine tetraacetate (2) and the extract was purified in a small column (2.7 x 20 mm) of Amberlite IRP 64 (1). The eluate (0.9 ml 1 *N* HCl) was processed according to Andén and Magnusson (2) and the fluorescence was read in an Aminco-Bowman spectrofluorophotometer at excitation and emission wavelengths of 300 and 540 mμ respectively. Mean 5-HT recoveries were of 95%. NE and histamine were extracted in 0.4 *N* perchloric acid and purified by column chromatography on Dowex 50W-X4 (column size, 4.2 x 50 mm). The two amines were isolated from the same extract by performing a differential elution with hydrochloric acid. Mean recoveries were 97% for NE and 75% for histamine. NE was measured according to Häggendal (15) and the fluorescence was read at 400 and 515 mμ excitation and emission wavelengths respectively. Histamine was determined by condensation with *o*-phthalaldehyde by using a modification (14) of the original technique of Shore, Burkhalter and Cohn (25) and the readings were done at 365 and 445 mμ excitation and emission wavelengths. The fluorescence of samples was plotted by means of an X-Y recorder.

Electron microscope techniques: The pellets of normal and reserpinized platelets as well as those from *in vitro* experiments were fixed according to the following schedules.

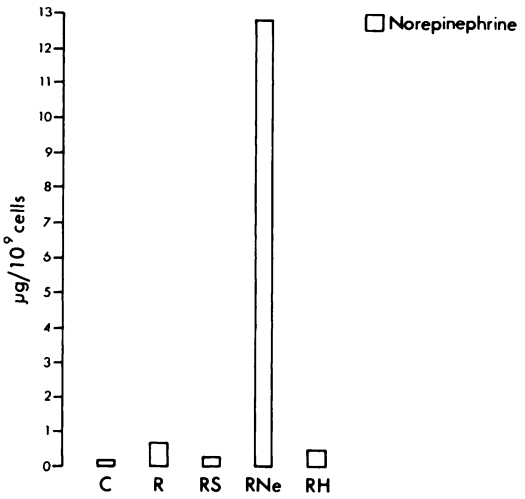


FIG. 2. Norepinephrine content of platelets from control (C) and reserpinized (R) animals; values from reserpinized platelets incubated with 5-HT (RS); norepinephrine (RNe) and histamine (RH) are also represented. Values are expressed as μg/10⁹ cells.

A. Osmium tetroxide, 1.5%, in 0.1 *M* phosphate buffer at pH 7.4 for 90 min at 4°C. This was followed by a 120-min immersion in a 2% acqueous solution of uranyl acetate before dehydration.

B. Karnovsky fixative (17) containing formaldehyde-glutaraldehyde in phosphate buffer at 4°C during 4–24 hr. Washing in 0.3 *M* sucrose in 0.1 *M* phosphate buffer and postfixation in osmium tetroxide followed by uranyl acetate as in A.

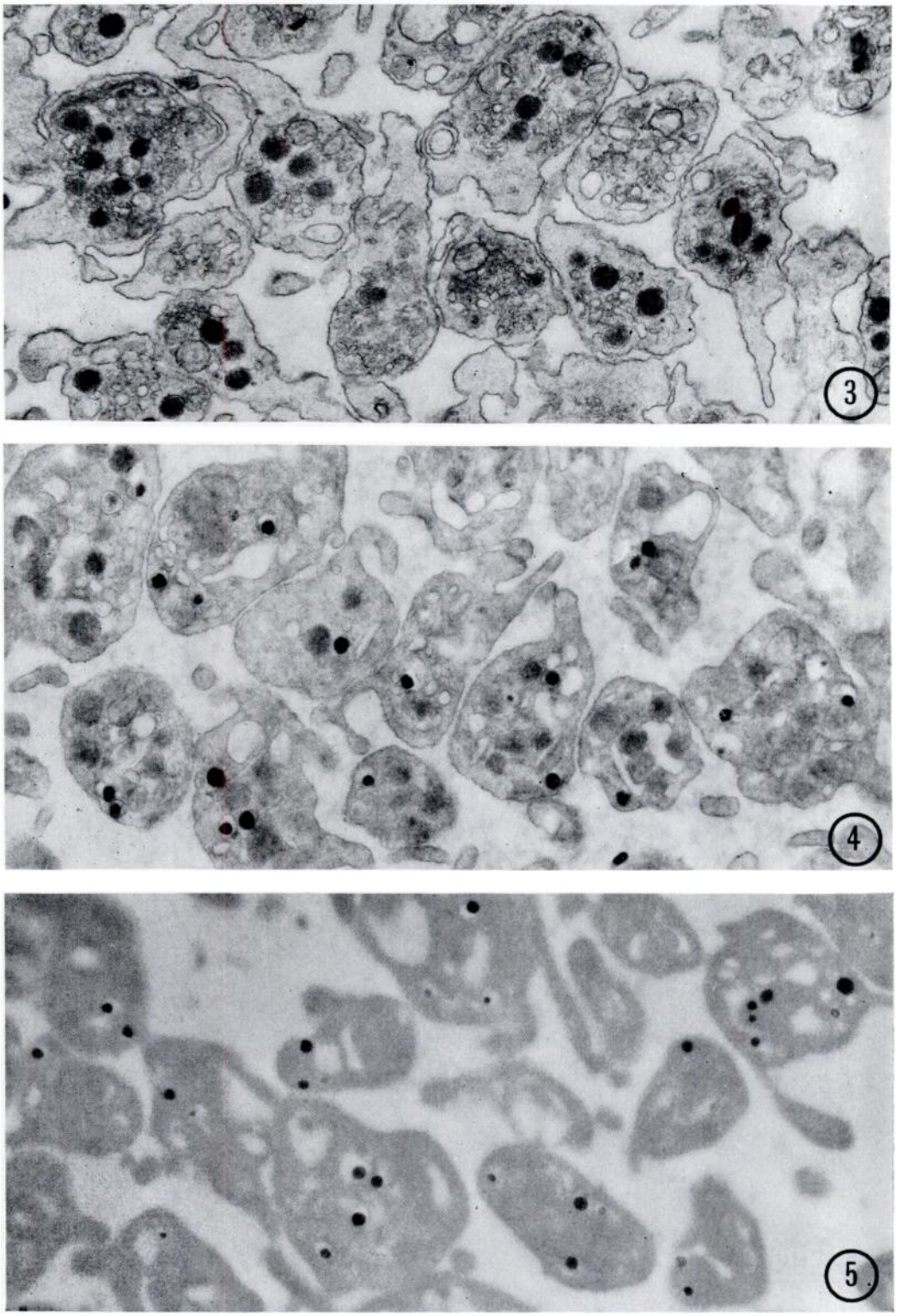
C. Glutaraldehyde, 3%, in 0.2 *M* cacodylate buffer, pH 7.2, for 4 hr at 4°C. Washing in 0.2 *M* buffer with 0.15 *M* sucrose. The blocks were then transferred to a solution containing 2.5% potassium dichromate plus 1% sodium sulfate in 0.2 *M* acetate buffer, pH 4.1, for 4–24 hr at 4°C. This procedure is referred as glutaraldehyde-dichromate (GD).

D. Formaldehyde, 10%, in 0.2 *M* cacodylate buffer, pH 7.2. Washing in the same buffer plus 0.15 *M* sucrose. Postfixation in glutaraldehyde, washing and treatment with potassium dichromate solution as in C. This procedure is referred as formaldehyde-glutaraldehyde-dichromate (FGD).

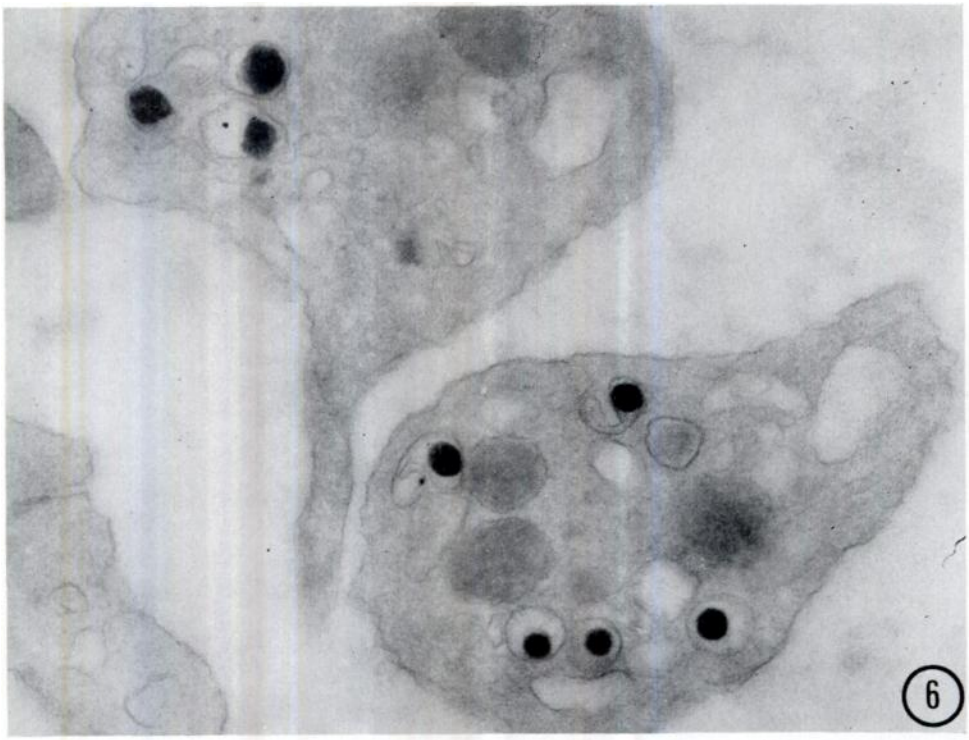
FIG. 3. Isolated normal rabbit platelets displaying the characteristic features following osmium tetroxide fixation. Uranyl acetate and lead citrate stains. ×15,000.

FIG. 4. When using the double fixation (aldehyde-osmium tetroxide), dense granules of 700–1800 Å in diameter are observed inside clear vacuoles. These granules are not present in platelets fixed in osmium tetroxide alone. ×15,000.

FIG. 5. After formaldehyde-glutaraldehyde-dichromate (FGD) treatment, dense reactive granules are present in platelet cytoplasm. These granules are observed inside clear vacuoles. The rest of the platelet has an amorphous appearance. ×15,000.



FIGS. 3–5



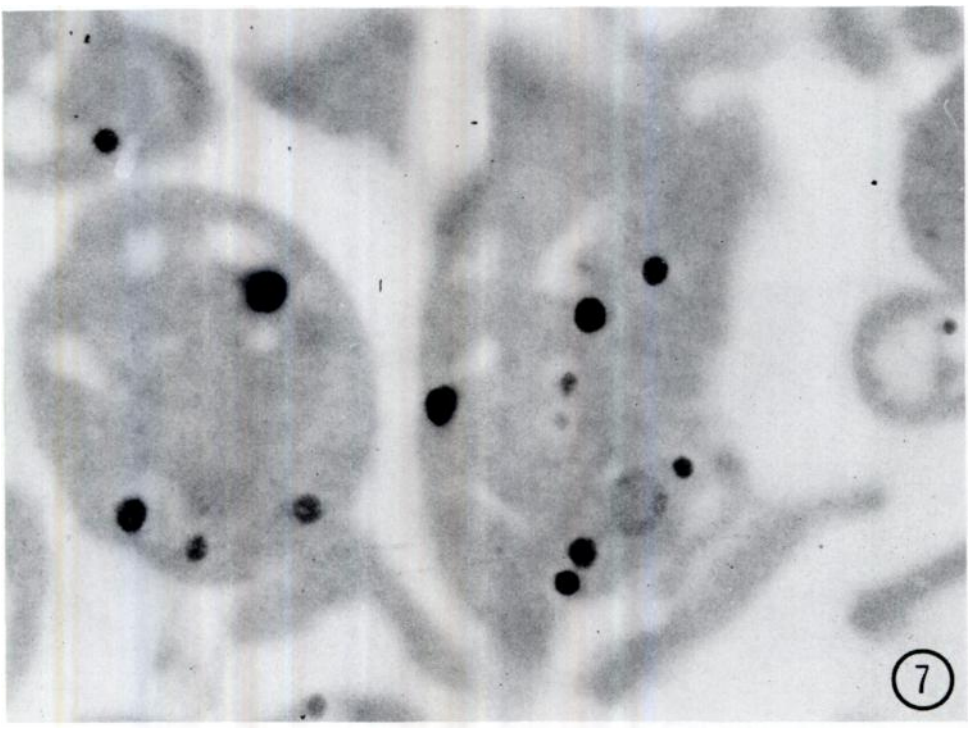


FIG. 6. Higher magnification of same preparation as in Figure 4. Karnovsky fixative followed by osmium tetroxide. ×36,000.

FIG. 7. Higher magnification of same preparation as in Figure 5. Formaldehyde-glutaraldehyde-dichromate. ×36,000.

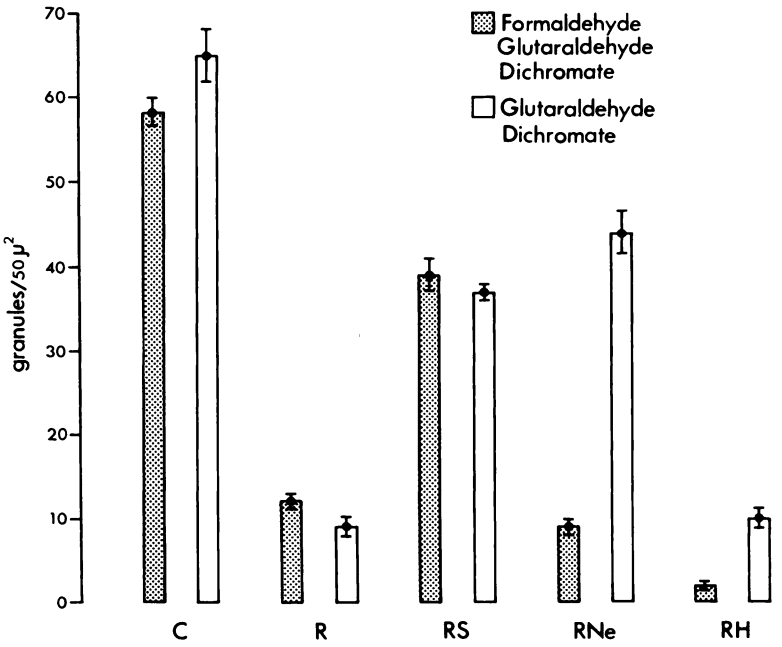


FIG. 8. Reactive granules observed in blood platelets after performing glutaraldehyde-dichromate (GD) and formaldehyde-glutaraldehyde-dichromate (FGD) reactions. Counts, expressed as granules present in 50 μ^2 of platelet surface, are given for control (C), reserpinized (R) as well as for reserpinized platelets incubated with 5-HT (RS), norepinephrine (RNe) and histamine (RH).

Dehydration was done through graded series of ice-cold ethanol and embedding was made in Epon 812 (19). Thin sections obtained with a Porter Blum ultramicrotome, using glass knives, were mounted on naked copper grids and examined under a Zeiss EM 9-A or a Siemens Elmiskop I electron microscope. Sections from material processed as described in A were stained with lead citrate (23). The other material was examined without further staining. The granules giving a positive reaction were counted in more than 500 μ of platelet surface in electron micrographs taken at random at an original magnification of 7000 and enlarged 3 times. In each case the count was expressed as number of granules/50 μ^2. Mean values and standard errors were determined.

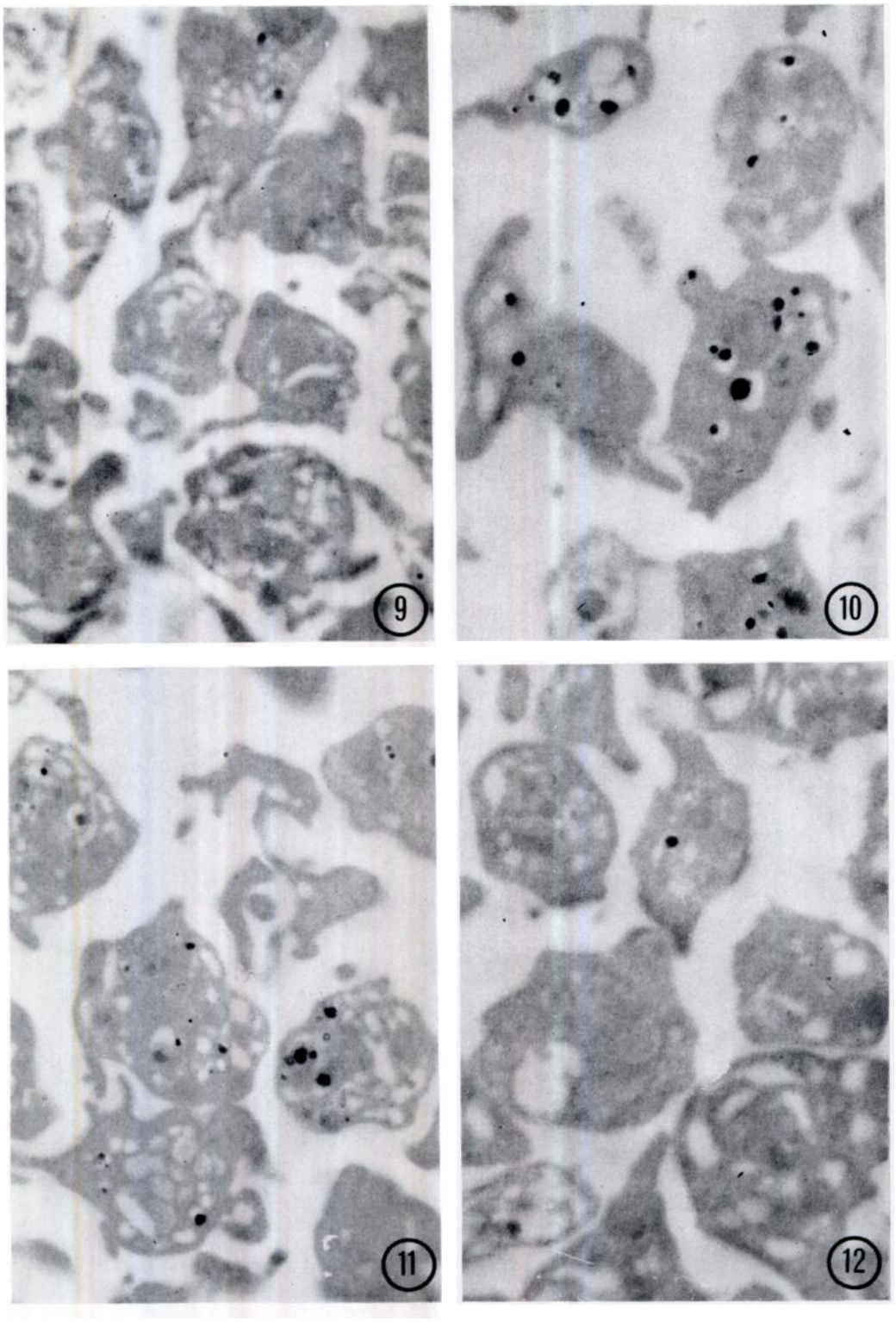
RESULTS

Drug-induced changes in the content of amines in blood platelets: Untreated rabbit platelets contain a considerable amount of 5-HT, averaging 11.72 $\mu g/10^9$ cells. Reserpine reduced this value by 98%. When reserpinized platelets were incubated with 5-HT the amine content was restored and even increased 86% above the control values. After incubation with NE or histamine, the 5-HT content of the reserpinized platelets did not change (Fig. 1).

Histamine was found to be concentrated in normal rabbit platelets, the mean value being 8.54 $\mu g/10^9$ cells. After reserpine administration the histamine content of normal platelets was reduced by 77%. This value decreased even more when such platelets were incubated with 5-HT. In this case 5.6% of the histamine content of normal blood platelets remained. When suspensions of reserpinized platelets were incubated with histamine, the concentration of the amine increased, reaching control values. The incubation with NE had no effect on the histamine concentration of reserpinized platelets (Fig. 1).

Although only traces of NE were present in normal rabbit blood platelets, the incubation of reserpinized platelets with this amine resulted in a marked uptake as shown by the increase from 0.70 $\mu g/10^9$ cells in nonincubated reserpinized platelets to 12.82 $\mu g/10^9$ cells in the incubated ones. No significant changes were observed in the NE content of control, reserpinized and reserpinized platelets incubated with 5-HT or histamine (Fig. 2).

Ultrastructural and cytochemical observations on normal rabbit blood platelets: The normal platelet has a diameter of 2–4 μ and a round or irregular contour. The osmium-fixed platelets contain several dense α-granules with a diameter of 3000 Å. Small mitochondria with relatively few cristae as well as vacuoles, microvesicles and tubules are also present in the granulomere (Fig. 3). The double aldehyde-osmium fixation



FIGS. 9–12

disclosed another type of dense granule, with a diameter ranging between 600 and 1800 Å and an extremely dense core with a clear rim interposed between the core and the surrounding membrane (Figs. 4 and 6). Occasionally empty vacuoles were present, suggesting the loss of the dense core. Osmium fixation alone failed to reveal these granular structures and the empty vesicles observed with this procedure may correspond to them. By means of a combined pharmacologic and structural approach these granules were previously identified as the site of storage of 5-HT in blood platelets (3, 30).

When both the GD and FGD cytochemical reactions were performed on normal platelets, granular products were observed. The dense precipitate is commonly present inside a vacuole and is similar in size and shape to the granules described above and interpreted as containing 5-HT (Fig. 5). In each platelet a varying number of granules is observed. The rest of the cytoplasm shows an amorphous aspect (Fig. 7).

Cytochemical reactions following drug-induced changes in amine content: Figure 8 indicates the number of granules having a positive reaction with the GD and FGD techniques. In normal platelets there were respectively 65 ± 3.2 and 58 ± 1.6 granules/50 μ^2. After reserpine administration a considerable decrease was observed, the counts being respectively 9 ± 1.2 and 12 ± 1.0. Morphologically, reserpinized platelets were characterized by an almost complete loss of the granular reactive material, remaining only empty vacuoles (Fig. 9).

After incubation of the platelets obtained from reserpinized animals with 5-HT the normal image was restored (Fig. 10). The dense cytoplasmic granules increased respectively to 37 ± 1.0 and 39 ± 2.1/50 μ^2 with the GD and FGD techniques.

When NE was added to the incubation medium, the dense granules reappeared in the reserpinized platelets only after use of the GD technique (Fig. 11). The granule counts were 44 ± 2.6. The electron microscopic examination of reserpinized platelets incubated with NE and processed with the FGD procedure failed to reveal any reaction product (Fig. 12). The values obtained, 9 ± 0.9 granules/50 μ^2, were similar to those found in nonincubated reserpinized platelets.

The incubation of a suspension of reserpinized platelets with histamine did not produce changes in platelet morphology. The counts were 10 ± 1.2 with GD and 2 ± 0.05 with the FGD technique.

DISCUSSION

The cytochemical technique for unsubstituted catecholamines and indoleamines involving glutaraldehyde fixation and subsequent dichromate oxidation (GD) demonstrates the presence of dense reactive granules in normal rabbit blood platelets observed under the electron microscope. The fact that platelets contain considerable amounts of 5-HT and histamine and only traces of NE favors the view that 5-HT is responsible for the GD reaction. When platelets are fixed in formaldehyde, prior to the glutaraldehyde-dichromate treatment (FGD), the image observed and the count of granules do not differ significantly from those obtained with the GD technique. This supports the previous assumption that 5-HT is responsible for the cytochemical reaction and confirms previous findings of other authors showing that this amine is stored in a granular form in blood platelets (3, 30). When the 5-HT and histamine content of the platelets is decreased by the *in vivo* administration of reserpine, the number of reactive granules revealed by both techniques diminished considerably.

Blood platelets actively take up 5-HT from the surrounding medium (20). This uptake is present in normal platelets and is decreased by reserpine (28). However, in this case, uptake only occurs at high concentrations of the amine in the incubation medium (7, 30) similar to those used here. The ultrastructural aspect of the reserpinized platelet incubated with 5-HT resembles that of normal platelets because of the replenishment of the empty vacuoles with dense reactive granules. These are demonstrated by both the GD and

FIG. 9. Platelets from a reserpine-treated rabbit. No dense granules are observed and clear vacuoles are the main feature of the platelet. Although this corresponds to a formaldehyde-glutaraldehyde-dichromate preparation, an essentially similar image was given by glutaraldehyde-dichromate technique. ×15,000.

FIG. 10. After incubation with 5-HT granules reappeared in reserpinized platelets, thus restoring the image of normal platelets. Formaldehyde-glutaraldehyde-dichromate technique. ×15,000.

FIG. 11. Platelets from a reserpinized animal incubated in Tyrode with norepinephrine. After glutaraldehyde-dichromate (GD) reaction, dense granules are present in the platelets. ×15,000.

FIG. 12. Reserpinized platelets incubated in Tyrode with norepinephrine. Following formaldehyde-glutaraldehyde-dichromate (FGD) reaction, they show no changes when compared with platelets from reserpinized animals as observed in Figure 9. ×15,000.

FGD techniques. In spite of the increase of 5-HT concentrations above normal values, the counts of the granules do not surpass that of controls. This fact could be interpreted by assuming that part of the amine is retained in the pellet without being removed by the washings or that it is in a free form inside the platelets.

The fact that the histamine-incubated platelets showed no changes when compared with reserpinized platelets suggests that this amine does not interfere with the mechanisms of the cytochemical reactions.

The findings with the GD and FGD techniques in the reserpinzied platelets incubated with NE demonstrate that the prefixation with formaldehyde renders negative the NE-reactive sites while leaving intact those due to 5-HT. In the NE-incubated platelets the amine is stored in large amounts and in morphologic entities similar to those normally containing 5-HT.

The possibility that these organelles constitute a common storage site for different amines must be considered. It has been suggested recently that the extremely dense granules contain 5-HT but not histamine (3), a statement based on the disappearance of the granules after a selective depletion of 5-HT from rabbit platelets by tyramine *in vitro*. Histamine, however, unlike 5-HT, neither precipitates with glutaraldehyde nor reacts with osmium tetroxide in aqueous solutions (30). The lack of a specific ultrastructural cytochemical technique for the demonstration of histamine leaves this question unsolved. Studies now being conducted in our laboratory tend to indicate that both 5-HT and histamine compete for a similar storage site in normal and in reserpinized blood platelets incubated *in vitro*. This view is in accordance with cell fractionation studies indicating that 5-HT and histamine are located in the same granular fraction of blood platelets (27).

While the high concentrations of amines present in normal blood platelets may explain the success of our histochemical study, recent observations from our laboratory, using a similar cytochemical approach, demonstrate that NE and 5-HT, known to be present in the autonomic nerve endings of rat pineal gland, are localized in the granulated vesicles characteristic of those endings (16). This indicates that the reaction is also effective in the presence of smaller amine concentrations. In the autonomic nerve endings of the vas deferens, Bloom and Barrnett (5) had already demonstrated NE with the GD technique.

The possible mechanism of the reaction between the glutaraldehyde-amine complex and the oxidizing agents has been discussed at length (9, 29, 36). The pretreatment with formaldehyde is interpreted as able to block the reaction with CA possibly by forming an isoquinoline derivative in which the amino groups responsible for the reaction with glutaraldehyde and dichromate are bound (32). 5-HT is supposed not to form such a compound, maintaining the amino group free for the reaction. Experiments are planned to determine whether other indoleamines are able to give a similar reaction with FGD technique.

From the evidence presented here we conclude that by using the glutaraldehyde-dichromate technique it is possible to demonstrate both the unsubstituted CA and indoleamines. However, with previous fixation with formaldehyde these two groups of compounds can be differentiated; thus a specific reaction for indoleamines is obtained. Blood platelets provide a sutiable model for further studies on the mechanisms of these reactions by directly analyzing isolated cellular structures. The study of a simple system such as provided by blood platelets may serve as a basis to approach more complex aminergic systems.

ACKNOWLEDGMENTS

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