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AN IMPROVED METHOD FOR THE LARGE-SCALE ISOLATION OF CHROMAFFIN GRANULES FROM BOVINE ADRENAL MEDULLA

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SUMMARY

Chromaffin granules essentially free of contamination from mitochondria, lysosomes and fragments of endoplasmic reticulum have been isolated in a large scale from bovine adrenal medulla. The homogeneity was judged by electron microscopy and assays of various 'marker' enzymes.

INTRODUCTION

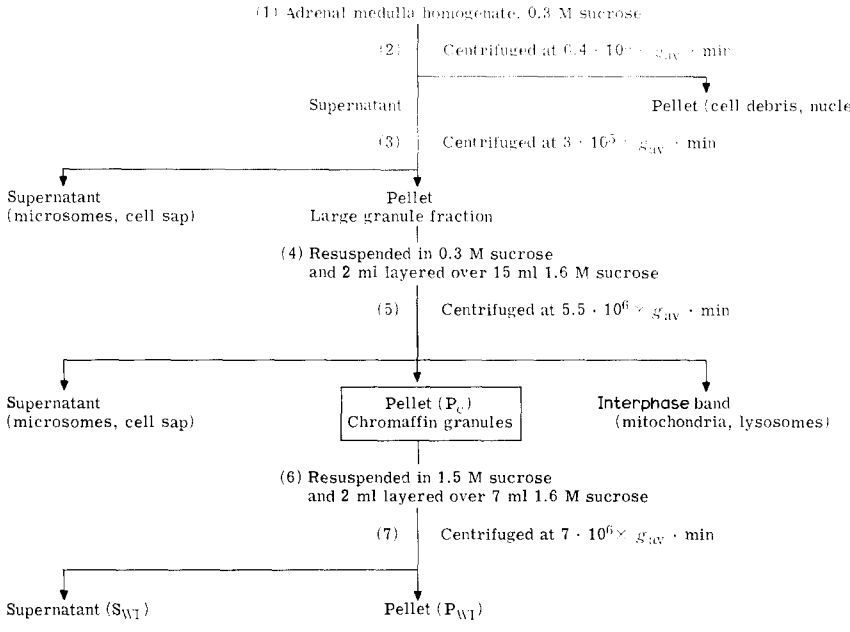
Earlier reports from these laboratories have been concerned with the characterization of chromogranin A[1-4], the major water-soluble protein of the chromaffin granule, and in a recent paper[5] we have reported the presence of an electron transfer chain located in the membrane fraction of this subcellular structure. Essential to the understanding of these observations is the degree of purity of the granule fraction, as even a small contamination of mitochondria and elements of the endoplasmic reticulum with their well-established electron transfer chains would obliterate the interpretation of our findings.

A simple method for the large-scale preparation of chromaffin granules was introduced by SMITH AND WINKLER[6], but the isolated granules were reported to be slightly contaminated by mitochondria and lysosomes. In the present paper an improved modification of their procedure is described. The criteria for purity of the chromaffin granule preparation have been defined morphologically by electron microscopic studies and biochemically by enzymic assays.

MATERIALS AND METHODS

Preparation of chromaffin granules

The method employed for the preparation of chromaffin granules was essentially the method of SMITH AND WINKLER[6] but with the following modifications (Scheme 1): The bovine adrenal medullae were passed through a small stainless steel tissue mincer (Climpex Ltd.) before homogenization. Cell debris and nuclei were removed by centrifugation at $6.4 \cdot 10^3 \times g_{av} \cdot \min$ (Sorvall refrigerated centrifuge RC-2, rotor No. SS-34), and the supernatant thus obtained was filtered through three layers of gauze to remove debris floating at the top. The $6.4 \cdot 10^3 \times g_{av} \cdot \min$ supernatant was then centrifuged $3 \cdot 10^5 \times g_{av} \cdot \min$ (Sorvall centrifuge) to yield a supernatant and a 'large



Scheme 1. Summary of procedure for preparing chromaffin granules from bovine adrenal medulla. Steps 6 and 7 were repeated twice giving the fractions P_{WII}–P_{WIII} and S_{WII}–S_{WIII}.

granule' pellet. This pellet was resuspended by hand homogenization in 0.3 M sucrose and layered in aliquots of 2–3 ml on top of 15 ml of 1.6 M sucrose in the 25-ml tubes of the MSE-Superspeed 40 centrifuge. After centrifugation at $55 \cdot 10^5 \times g_{av} \cdot$min chromaffin granules were obtained as a pink pellet. The supernatant was carefully removed by pasteur pipette to prevent the mitochondrial layer coming into contact with the pellet. The loose pinkish layer (of chromaffin granules) on top of the pellet was allowed to drain off, and the inner surface of the tubes was carefully wiped clean with swabs of gauze. The remaining pink sediment thus obtained served as the source of chromaffin granules in the present work and will be referred to as fraction P_c.

For a further investigation of the homogeneity of this fraction each pellet (P_c) was carefully resuspended in 2 ml 1.5 M sucrose by hand homogenization in a small Teflon–glass homogenizer. The resuspended chromaffin granules were layered in 2-ml aliquots on 7 ml of 1.6 M sucrose. After centrifugation at $7 \cdot 10^6 \times g_{av} \cdot$min (Beckman Model L centrifuge, rotor No. 40) the turbid upper phase S_{WI} was removed and saved; the pellet was lettered P_{WI}. This procedure was repeated twice and the respective turbid upper phases and pellets were given the numbers II and III.

Preparation of the membrane fraction of chromaffin granules

The water-insoluble membrane fraction of the chromaffin granules was prepared as described in the accompanying paper[5].

Preparation of granules for electron microscopy

Granule fractions P_c and P_{WIII} were resuspended in 1.5 M sucrose, and aliquots of the suspensions were pipetted into 3-ml plastic tubes to give a small pellet of 2–3 mm

in diameter when centrifuged at $70 \cdot 10^5 \times g_{av} \cdot$min (Beckman Model L centrifuge, rotor No. 40). The supernatants S_{WI} and S_{WIII}, containing material that did not sediment in the 1.6 M sucrose, were diluted to 1.5 M sucrose by slow addition of distilled water by a microliter syringe under constant stirring and centrifuged as described above. The changes in sedimentation velocity brought about by this decrease in density of the suspension medium resulted in a complete sedimentation of the granules when centrifuged at $70 \cdot 10^5 \times g_{av} \cdot$min.

Fixation in glutaraldehyde with post-fixation in OsO_4 was carried out essentially by the method of COUPLAND AND HOPWOOD[7]. After centrifugation the supernatant was removed and replaced by 1 ml of 2.5 % glutaraldehyde in 1.6 M sucrose containing 0.03 M sodium phosphate (pH 6.5). Fixation was carried out at $+2^\circ$ for 24 h. The glutaraldehyde solution was removed and the pellets washed with the buffered sucrose. Fixation in 1 % OsO_4 in the buffered sucrose was carried out for 1 h on ice, and the fixed pellets were washed with ice-cold buffered sucrose and dehydrated by increasing concentrations of ethanol followed by a rinse in aceton. The pellets were divided in four sections and mounted in Durcupan. Ultrathin sections were cut across different regions of the pellet on an LKB Ultrotome III microtome, mounted on the grid and stained with lead citrate. A Siemens Elmiskope I electron microscope was used for the examination of the stained sections.

The percentage distribution of light, predominantly large granules and dark, predominantly small granules was counted in six different electron micrographs of pellet P_c at a magnification of 9000. In each micrograph a minimum of 400 granules was classified.

Preparation of microsomes

A microsomal fraction of the homogenate was prepared from the $4 \cdot 10^5 \times g_{av} \cdot$ min supernatant as described[5].

Preparation of mitochondria

A mitochondrial fraction of the homogenate was prepared from the layer (0.3 M sucrose) over the dense sucrose (1.6 M) as described[5].

Enzyme assays

Succinate oxidase activity was determined polarographically as previously described[8]. Native bovine heart cytochrome *c* (Cy I)[8] was added at a concentration which gave maximum stimulation of mitochondrial O_2 consumption.

Acid phosphatase (EC 3.1.3.2) activity was assayed using a modification[5] of the *p*-nitrophenol method of LOWRY *et al.*[9].

Glucose-6-phosphatase (EC 3.1.3.9) was assayed by the method of DE DUVE *et al.*[10]. The P_i liberated was determined by the method of RATHBUN AND BETLACH[11] with slight modification[5].

Protein determination

Protein concentration was determined by a biuret reaction according to the method of YONETANI[12].

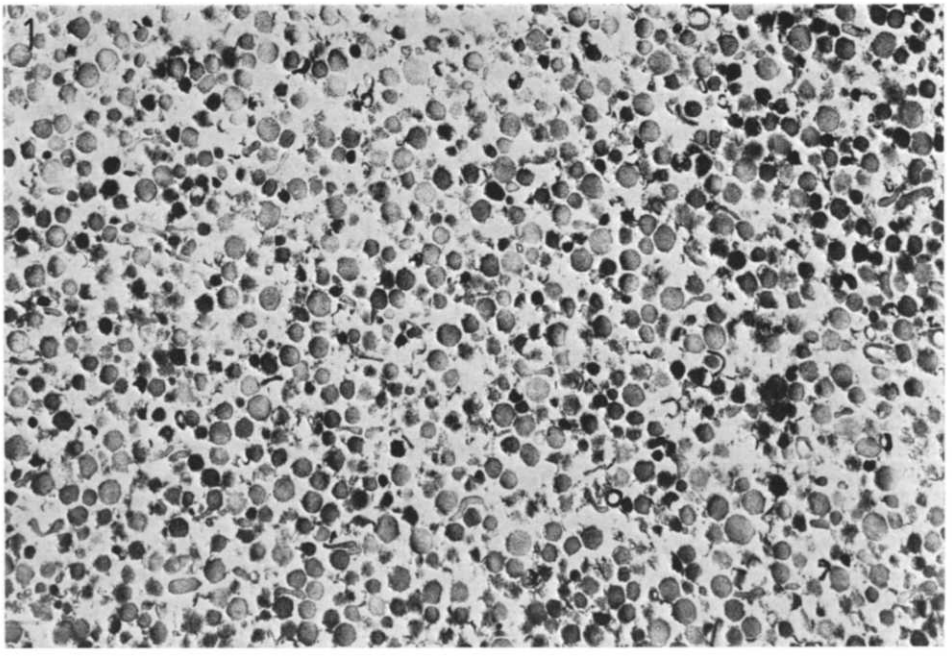
Spectrophotometry

All the spectrophotometric determinations were carried out on a Shimadzu MPS-50 L recording spectrophotometer.

RESULTS AND DISCUSSION

Electron microscopic studies

Electron micrographic examination of chromaffin granules isolated by the standard procedure (the pellet P_c) revealed well-preserved structures (Figs. 1 and 2) characteristic of chromaffin granules as observed in intact cells of bovine adrenal medulla[13]. As in the tissue sections two populations of particles were seen which differ in characteristic ways with respect to size and electron density. Both populations consisted of intact, well-filled and mostly spherical particles with electron-dense matrices (condensed forms). The major population (75 % of the counted granules) was represented by light, predominantly large (approx. 200–250 nm in diameter) and the other (25 %) by dark, predominantly small granules (approx. 150–180 nm in diameter), in close agreement with the size and percentage distribution of granules reported by COUPLAND[13]. In addition to these granules, amorphous material of varying electron density could be seen randomly distributed between the granules, often partly associated with the granule membrane. These 'structures' probably represent lipoprotein material precipitated by the fixation procedure and were significantly reduced in the trailing fractions (S_{WI}–S_{WIII}) obtained upon resedimentation of pellet P_c in 1.6 M



Figs. 1–5. Electron micrographs of chromaffin granules isolated from bovine adrenal medulla: 1, × 9000; 2–4, × 42000; 5, × 120000.

Figs. 1 and 2. A representative section from a pellet (P_c) of granules isolated by the standard procedure. Note that this pellet is composed of two populations of electron-dense particles, *i.e.* light, predominantly large granules and dark, predominantly small granules. Essentially no contamination of this fraction with either mitochondria, lysosomes or smooth-surfaced/rough-surfaced fragments of endoplasmic reticulum is found.

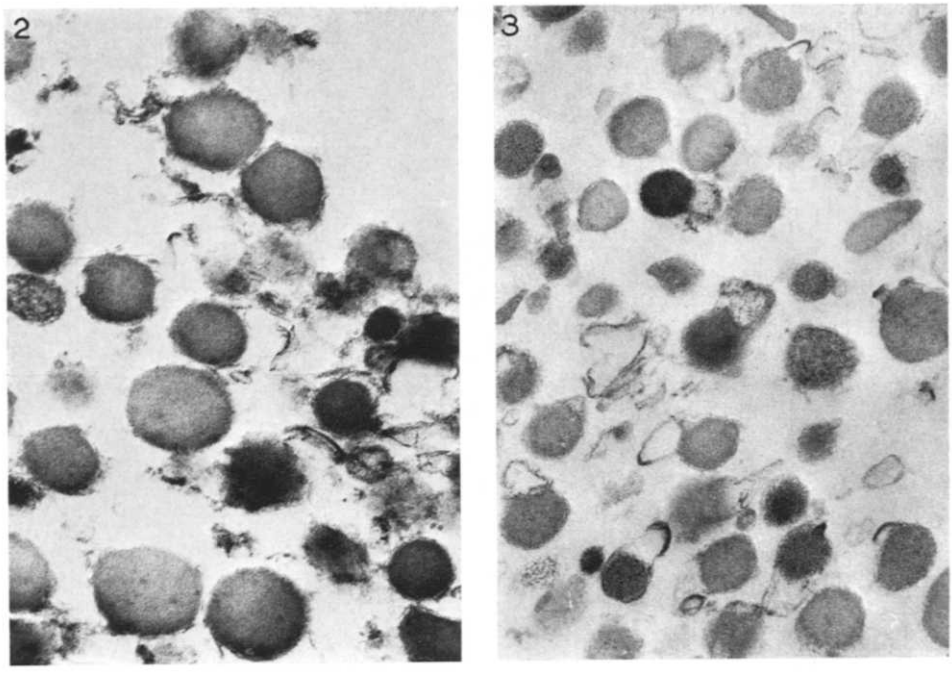


Fig. 3. Section through the pellet of 'washed' granules, fraction S_{WI}.

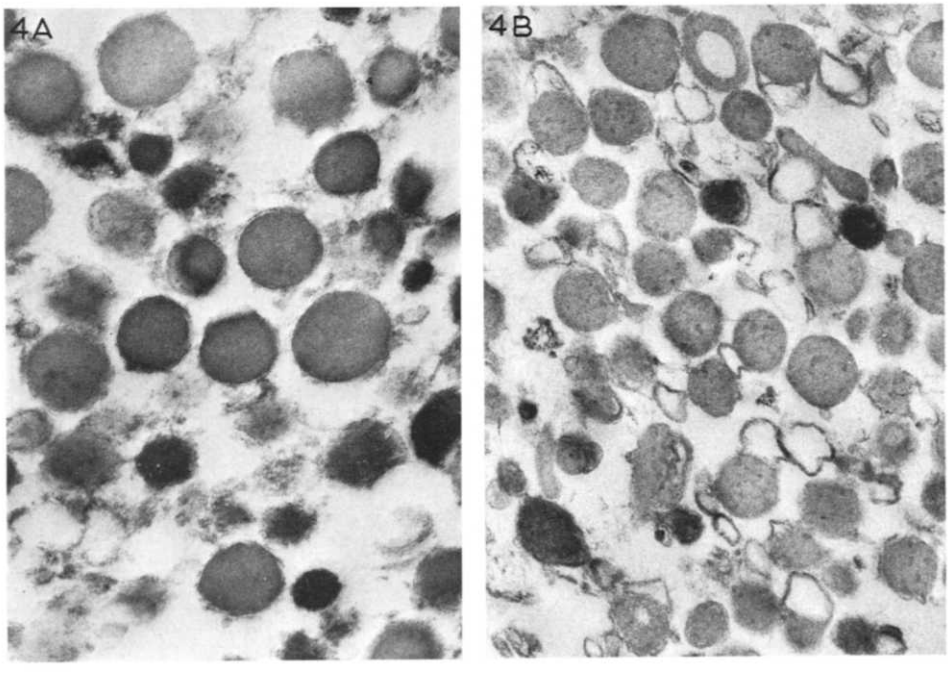


Fig. 4. Section through the pellet of 'washed' granules, fraction P_{WIII} (A) and fraction S_{WIII} (B).

sucrose (see below). The preparation was essentially free of contamination with either mitochondria, lysosomes or smooth- and rough-surfaced microsomal vesicles.

As a further control of the purity of this preparation, the pellet P_c was resuspended in sucrose of slightly lower osmolarity (1.5 M) as described in MATERIALS AND METHODS. After resedimentation in 1.6 M sucrose a certain degree of trailing could be observed, and the material that did not sediment (S_{WI}) was studied in the electron microscope. The morphological appearance of S_{WI} (Fig. 3) indicates that the trailing material consisted of granules which mostly diverge from the forms given in Figs. 1 and 2. The membrane of many granules was seen to stretch out in loops from the inner core of the electron-dense matrix to form a clear 'pocket' (open forms). The preparation did not exhibit structures of either mitochondrial or microsomal origin. The patterns obtained for the trailing material, S_{WIII}, obtained after the third resedimentation in 1.6 M sucrose are given in Figs. 4B and 5B–5D, and the corresponding pellet of chromaffin granules, P_{WIII}, is presented in Figs. 4A and 5A. It may be seen that many of the granules (75 % of those counted) that did not sediment in 1.6 M sucrose contained the open forms already described for fraction S_{WI}, and empty vesicles could also be detected. The complete absence of open forms and empty vesicles in the pellets P_c and P_{WI}–P_{WIII} eliminates the possibility of histological artifacts and probably reflects a structural change in some of the granules caused by the slightly hypo-osmotic washing procedure.

In the condensed forms the surrounding membrane appeared to be in close contact with the electron-dense matrix in light (Figs. 5A and 5B) as well as in dark granules (Fig. 5C). When subjected to the slight hypo-osmotic conditions employed in the

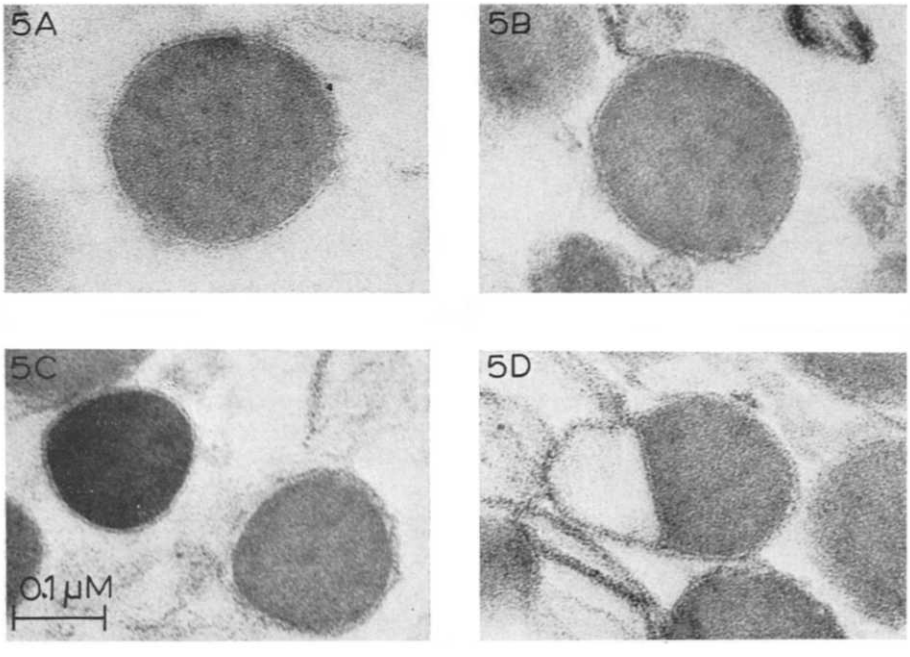


Fig. 5. Details in the structure of intact chromaffin granules taken from the following fractions: P_{WIII} (A) and S_{WIII} (B, C and D).

resedimentation experiments, granules surrounded by 'wrinkled' membranes (Fig. 5C) could be seen together with the open forms already described. In some of the latter structures the clear space of the 'pocket' could be seen to be separated from the matrix by a membrane-like structure (Fig. 5D), suggesting that the chromaffin granule is surrounded by a double-layered membrane.

The apparent delay of the inner core of granular material in filling the empty space of the open forms is parallelled by observations made on whole adrenal medullary cells of hamster in which chromaffin granules have been seen to expel their content of electron-dense material at the cell surface by an exocytotic mechanism of secretion[14]. In many instances the electron-dense material expelled had retained its spherical shape in the interstitial space, suggesting that the granular storage complex may not be immediately solubilized in the interstitial fluid.

Assay of some 'marker' enzymes

Confirmation of the absence of mitochondria was obtained by the assay for succinate oxidase activity on the isolated membrane fraction; the reaction was completely negative even when assayed at high protein concentration (Table I). A certain, but low activity of acid phosphatase was always observed in the chromaffin granule membrane fraction, but the activity was partially detached from the membrane by treatment with 0.3 M KCl (Table I). This finding together with the morphological picture (see above) suggest that this enzymic activity is due to redistribution of the enzyme released from lysosomes disrupted before and/or during the homogenization procedure.

The membrane fraction exhibited a low but significant glucose-6-phosphatase activity (Table I) as well as NADH :(acceptor) oxidoreductase activities[5], although the absence of microsomal structures has been documented morphologically. How-

TABLE I

ACTIVITIES OF SOME MARKER ENZYMES IN THE MEMBRANE FRACTION OF CHROMAFFIN GRANULES

Experimental conditions were as described in the text.

	Succinate oxidase * (*nmoles* O_2*/min per mg of protein*)	*Glucose-6-phosphatase* ** (*μmoles* P_i*/60 min per mg of protein*)	*Acid phosphatase* ($\Delta A_{410\,nm}$*/30 min per mg of protein*)
Membrane fraction (chromaffin granules) pellet P_c	0	0.044	2.18 ***
Membrane fraction (chromaffin granules) pellet P_{WIII}	0	0.042	—
Unfractionated microsomes	0	0.272 §	—
Mitochondria	35.8	—	—

* The protein concentration was always in the range 0.5–0.8 mg protein/ml.

** The results represent the mean value of five assays.

*** This activity was significantly reduced (approx. 40%) by resuspension and recentrifugation of the intact granules by using sucrose solutions containing 0.3 M KCl.

§ The corresponding value for unfractionated bovine liver microsomes was 4.54 μmoles P_i/60 min per mg of protein.

ever, neither of these enzymic activities could be removed or reduced even after 3 resedimentations of the chromaffin granules (see MATERIALS AND METHODS). In fact, as can be seen from Table I, the glucose-6-phosphatase activity remained essentially the same following this procedure. This finding indicates a presence of glucose-6-phosphatase activity in the membranes of the chromaffin granules which can not be due to contamination by microsomes as discussed in further detail in the accompanying paper[5].

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REFERENCES

1 K. B. HELLE, *Mol. Pharmacol.*, 2 (1966) 298.
2 K. B. HELLE, *Biochim. Biophys. Acta*, 117 (1966) 107.
3 K. B. HELLE, *Biochem. J.*, 109 (1968) 43P.
4 K. B. HELLE AND G. SERCK-HANSSEN, *Pharmacol. Res. Commun.*, 1 (1969) 25.
5 T. FLATMARK, O. TERLAND AND K. B. HELLE, *Biochim. Biophys. Acta*, 226 (1971) 9.
6 A. D. SMITH AND H. WINKLER, *Biochem. J.*, 103 (1967) 480.
7 R. E. COUPLAND AND D. HOPWOOD, *J. Anat.*, 100 (1966) 227.
8 T. FLATMARK, *J. Biol. Chem.*, 242 (1967) 2454.
9 O. H. LOWRY, N. R. ROBERTS, M-L. WU, W. S. HIXON AND E. J. CRAWFORD, *J. Biol. Chem.*, 207 (1954) 19.
10 C. DE DUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX AND F. APPLEMANS, *Biochem. J.*, 60 (1955) 604.
11 W. B. RATHBUN AND M. V. BETLACH, *Anal. Biochem.*, 28 (1966) 436.
12 T. YONETANI, *J. Biol. Chem.*, 236 (1961) 1680.
13 R. E. COUPLAND, *Nature*, 217 (1968) 384.
14 O. DINER, *Compt. Rend.*, 265D (1967) 610.