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THE EFFECT OF ADRENALECTOMY, CORTISONE AND OTHER STEROID HORMONES ON THE HISTOCHEMICAL REACTION FOR SUCCINIC DEHYDROGENASE

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A technique for the histochemical demonstration of succinic dehydrogenase in tissue sections was described by Seligman & Rutenberg (1951). The technique involves the incubation of sections of fresh frozen tissue in a substrate mixture containing sodium succinate, phosphate buffers and ditetrazolium chloride (blue tetrazolium). The dehydrogenation of the succinate results in the reduction of the tetrazolium (which is a hydrogen acceptor) to a blue insoluble diformazan which precipitates more or less at the site of enzyme activity. Malaty & Bourne (1953) have studied the application of this technique to guinea-pig, rabbit, mouse and rat tissues. Seligman & Rutenberg (1951) carried out their reaction without taking special anaerobic precautions, but Padykula (1952) carried out the incubation in nitrogen and secured a more uniform reaction and she also added calcium and aluminium ions to the incubating medium in order to activate the enzyme. With these refinements of the technique she made a detailed study of the reaction in the organs and tissues of the rat. Shelton & Schneider (1952) tested out the usefulness of a number of tetrazolium salts for the histochemical demonstration of succinic dehydrogenase and found the most satisfactory compound to be neotetrazolium. This compound has been used in the present work and it has been found to give extremely reliable results. Leduc & Wislocki (1952) have also investigated the distribution of succinic dehydrogenase in a number of tissues.

METHODS

Neotetrazolium (*pp'*-diphenylene-bis 2–3 [3, 5 diphenyl] tetrazolium) was used to study the distribution of succinic dehydrogenase activity and the substrate solution used consisted of equal parts of (*a*) neotetrazolium (0·1 % solution), (*b*) sodium succinate (0·2 M solution), (*c*) M/10-phosphate buffer pH 7·5, (*d*) distilled water.

Rats were used throughout; they were killed by a blow on the head and tissues were removed immediately. The organs used were liver, heart, kidney and cerebellum, and were excised in this order in both normal and experimental groups. A small piece of each organ was removed, frozen at once without fixation, and sectioned at 30 μ. The sections were dropped straight into substrate solution. This process took approximately 5 min for each organ, making a total of 20 min in all.

It was found necessary to adhere to a strict order in taking the tissues since it was found that there was some loss of activity over this period. The sections were incubated in the substrate at 37° C for 45 min. A positive result was obtained after 5 min, but incubation for longer than 45 min resulted in no further intensification.

After incubation the sections were fixed in neutral 10% formol-saline for 30 min, washed in distilled water, straightened on a slide, dried for a short time on a hot plate and mounted in Apathy's medium. Drying the sections on the slide reduced curling when the mounting medium was applied.

It is difficult to obtain reasonable sections of fresh tissue at a thickness of much less than 30μ on the ordinary freezing-microtome. Accordingly, in some cases 2 mm slices of fresh frozen tissue were incubated in the above substrate mixture, fixed in formalin and, since the diformazan pigment is soluble in alcohol, embedded in water-soluble wax (Carbowax). Sections 10μ in thickness were cut by a rotary microtome in the usual manner and mounted in glycerine jelly. With some of the tissues this thinness permitted more accurate microscopical observation at a cytological level.

The following experimental groups of male rats were used: three were controls; four were injected daily for 4 days with cortisone (8 mg/kg body weight); 21 were adrenalectomized under pentobarbitone anaesthesia. Of the adrenalectomized rats 3 were given no treatment, but were given normal saline to drink and food *ad lib.*; 3 were given daily intramuscular injections of cortisone (8 mg/kg body weight) for 4 days beginning on the 6th day after the operation; under the same schedule 3 were given deoxycorticosterone acetate (10 mg/kg body weight); 3 cortisone and deoxycorticosterone acetate (same dosage as above); 3 testosterone (50 mg/kg body weight); 3 oestrone (5 mg/kg body weight); and 3 progesterone (5 mg/kg body weight). In addition, 3 animals were given mock adrenalectomy operations, without removal of adrenals, to serve as additional controls and were killed after 4 days.

RESULTS

Tissues from control rats

Liver. Under low power (×60) of the microscope a generalized blue coloration of the hepatic cells could be seen, which was more intense in the peripheral parts of the lobules and which became progressively paler towards the central vein. This has also been recorded by Seligman & Rutenberg (1951) and by Padykula (1952) (see Pl. 3, fig. 21).

High-power observation of the cells showed that the blue reaction was localized in rods and granules. The rods were longer and more filamentous in the cells closer to the central vein. The nuclei appeared to be unstained, although in some cases a faint positive reaction was present.

In general, the cytoplasmic distribution of the reaction suggested that it was localized in the mitochondria. In some cells rather larger masses of blue-coloured material were seen, these may have been due to a condensation of an unusually large number of diformazan crystals on the surface of the mitochondria.

The intensity of the reaction was approximately equal in two of the controls. In the third it was somewhat less and it was observed that this decreased reaction appeared to be due to the presence of fewer of the blue granules in the cells. Also some diformazan had dissolved in the fat droplets of the liver cells colouring them reddish purple.

Heart. An intense reaction was given by heart muscle. With neotetrazolium the reaction in this tissue was at least as strong as that shown by the kidney (in which it was very intense). This was not the case using blue tetrazolium (Malaty & Bourne, 1953). Also with neotetrazolium the reaction was more uniform. High-power observation showed that the deposit was granular. The granules were large and in many cases were obviously arranged in a linear fashion. This arrangement suggests that the enzymic activity is located in the sarcosomes lying between the muscle fibrils and confirms previous work (Watanabe & Williams, 1951) which showed that sarcosomes do in fact, at least in insect wing muscle, contain a number of Krebs cycle enzymes and that in this respect they are similar to mitochondria. In fact, it is now generally recognized that they are mitochondrial in origin. In addition to the granular deposit the fibres presented a light pink background (Pl. 2, fig. 11).

Kidney. In all controls the glomeruli were negative, all the rest of the cortex was positive and so was the outer portion of the medulla. (A similar result has been obtained by Padykula, 1952.) The proximal convoluted tubules gave the most intense reaction, but a strong positive reaction was also given by Henle's loop and the distal convoluted tubules. A fainter reaction was given by the collecting tubules and in some cases these were negative. Like Padykula we found that the positive reaction stopped sharply at about the middle of the medulla, the inner half of which was completely negative. An examination of the cytological distribution of the reaction showed that the nuclei gave either a negative or a very faint reaction. In the cytoplasm the result of the reaction was a deposit of blue granules some of which, in some cells, were arranged in a peri-nuclear fashion. In most cells, however, the granules were distributed thickly throughout the cytoplasm and, as has been pointed out by Malaty & Bourne, resemble mitochondria in form and distribution. In some cells there was a deposit of larger masses of diformazan pigment which appeared to be due to excessive accumulation of pigment crystals around granular elements in the cytoplasm. Pink-stained globules were also found in the tubule cells of some rats (Pl. 1, fig. 1).

Cerebellum. In the cerebellum the white matter was negative and the grey matter (both granular and molecular layers) positive (see also Padykula, 1952; Leduc & Wislocki, 1952; Malaty & Bourne, 1953). The reaction of the grey matter was a diffuse pink, but in addition the cells contained blue granules and in some cells there were aggregations of granules around or near the nucleus which simulated the Golgi apparatus in form. In the molecular layer some cells contained pink-staining globules surrounded by small blue granules and rodlets (according to Seligman & Rutenberg, 1951). The pink staining, shown by some regions, is said to be due to low enzyme activity and the blue deposit to indicate regions of high activity (Seligman & Rutenberg, 1951). It is possible that some of the colour in this case may have been due to the solution of some of

the diformazan pigments in the lipids of the brain; a phenomenon which has been recorded by the authors quoted above. The Purkinje cells contained a number of dark blue cytoplasmic granules scattered through the cytoplasm (see also Leduc & Wislocki, 1952). See Pl. 4, fig. 31.

Cortisone-injected animals

Liver. A great increase in the positive reaction was given by the liver cells of all experimental animals, particularly at the periphery of the liver lobules. Some of the more centrally placed cells seemed to contain a number of extremely fine positive blue granules (? microsomes), which were just visible under oil-immersion magnification. Some central cells also contained positive 'blobs' which under higher magnification could be seen to be made up of pink-stained fat globules surrounded by blue granules and rods.

The increase of blue colour in the cells appeared to be due partly to increase in intensity of positively reacting bodies (? mitochondria) already there and partly to an increase in their number. In part the increased colour was due to the replacement of a number of the positive rods by positive granules (see Pl. 3, fig. 22).

Heart. There was a decreased pink staining of the background but there was a great increase in the number and intensity of blue-staining granules. The intercalated disks had become completely negative and the linear arrangement of the granules in the other parts of the fibres was more obvious. In this respect they differed from the controls in which some blue granules were present in these regions (see Pl. 2, fig. 12).

Kidney. There was a great increase in the number and intensity of the granules in the tubule cells. In many cells they appeared finer. The reaction seemed to be particularly intensified in the loops of Henle, but some increase was also found in the blue granular deposit in the proximal and distal convoluted tubules (Pl. 1, fig. 2).

Cerebellum. The reaction was considerably increased both in the generalized pink staining in the grey matter and in the number of diformazan granules in the cells. There was a great increase in this granular reaction in the Purkinje cells. The granules themselves appeared to be of similar size but their number was increased by three or four times. There were also more granules in the cells of the granular layer (see Pl. 4, fig. 32).

Adrenalectomized animals

Liver. Of the three animals used for this experiment one showed a reaction more or less equal to the weakest control liver, one was completely negative and one gave a slight reaction. The distribution of the positive granules in those specimens in which they were present was similar to the controls (see Pl. 3, fig. 23).

Heart. The reaction in all three animals was greatly reduced. The reaction

was patchy and the granules in the positive patches seemed to be clustered in a band around the nuclei. The granules appeared also to be disorientated, that is, they were no longer arranged in chains or lines as in the controls (see Pl. 2, fig. 13).

Kidney. The reaction was greatly reduced in the proximal convoluted tubules. The distal convoluted tubules, collecting tubules and ascending limbs of Henle's loop were almost completely negative. The descending limbs of Henle's loop still showed a fairly high degree of activity, although the intensity of the reaction was reduced here also (see Pl. 1, fig. 3).

Cerebellum. In two animals the reaction was almost completely negative and in the third it was greatly reduced, but certain, deeply lying, large unidentified cells were strongly positive. The Purkinje cells were almost completely negative (see Pl. 4, fig. 33).

Mock adrenalectomy

The kidneys and the hearts of these rats gave a normal intensity of reaction, but there seemed to be some reduction of intensity in the liver and cerebellum. This reduction was in no way comparable with that seen in the adrenalectomized animals (see Pl. 3, fig. 24; Pl. 2, fig. 14; Pl. 1, fig. 4).

Injection of adrenalectomized rats with steroid hormones

The results are summarized in Table 1 and illustrated in Pl. 1, figs. 5–10; Pl. 2, figs. 15–20; Pl. 3, figs. 25 and 26; and Pl. 4, figs. 27–30 and 34.

TABLE 1. Results of injection of adrenalectomized rats with steroid hormones
(Note: 'Normal' refers to intact, untreated rats)

	Intensity of reaction			
	Heart	Liver	Cerebellum	Kidney
Cortisone	Returned to normal, except that intercalated disks negative	Returned to normal in two animals, partly so in third	Increased but did not reach normal level	Similar to cerebellum but still almost negative in distal convoluted tubules
Deoxycorticosterone acetate. (DOCA)	Only partial restoration to normal level	Similar reaction to heart muscle	Similar to heart muscle	Almost back to normal. Greatest increase was in that of convoluted tubules
Cortisone and DOCA	Appeared normal. Intercalated disks showed slight positive reaction	Almost normal	Purkinje cells positive. Just below normal over whole of section	Appeared normal
Testosterone	Intensity of reaction in all four tissues showed no increase over that of untreated adrenalectomized animals			
Oestrone	Intensity of reaction in heart muscle, liver and cerebellum showed no increase over that of untreated adrenalectomized animals. Kidney reaction was depressed below this level			
Progesterone	In all four tissues there was considerable increase of reaction above that of untreated adrenalectomized animals In kidney, cerebellum and liver it was about three-quarters normal, in heart about half normal, and in kidney proximal convoluted tubule cells the reaction was of normal intensity			

DISCUSSION

Various workers (Seligman & Rutenberg, 1951; Padykula, 1952; Malaty & Bourne, 1953), studying histochemically the distribution of succinic dehydrogenase, appear in general to agree concerning the histological and cytological distribution of this enzyme activity. Malaty & Bourne are also in agreement with Leduc & Wislocki that the Purkinje cells of the cerebellum show a concentration of succinic dehydrogenase activity.

When changes in enzyme activity of the various tissues (liver, heart, kidney and cerebellum) are demonstrated histochemically it is obvious that there are many possibilities of error in making an assessment as to whether a particular experimental treatment of the animal has or has not had an effect. In the first instance the assessment of enzyme activity is by observation not by measurement, and is therefore largely subjective. However, this was partly overcome by assessing the intensity of reaction of the sections without knowing whether they were from controls or treated animals and in any case most of the variations were so gross that there was little or no doubt of the result. Experimental variations, however, still affect the results. It is difficult to cut sections of identical thickness on a freezing microtome and the thickness of the sections affects the intensity of the colour. However, by studying large numbers of sections of each tissue from both experimental and control animals it was possible to overcome errors of judgement due to this variation. Precise standardization of all other steps in the technique was carried out.

After assessment of our results, therefore, we concluded that the injection of cortisone into intact rats caused an increase of enzyme activity in all four tissues examined, even in the intact animal. In the normal heart muscle the intercalated disks did not have as much enzyme activity as the other parts of the muscle fibres but injection of cortisone into the intact or into the adrenalectomized animals caused these disks to become completely negative. In the liver, cortisone appeared to increase the fineness of the positive reaction within the cells. The most noteworthy effect of cortisone in the cerebellum was the increase it caused in the reaction of the Purkinje cells.

Adrenalectomy caused a marked reduction in the succinic dehydrogenase activity in all four tissues examined. This confirms the work of Tipton, Leath, Tipton & Nixon (1946), who found a decrease of both succinic dehydrogenase and cytochrome oxidase activity in rat liver. On the other hand, Stucki, Shipley & Meyer (1952) found an increased O_2 uptake in adrenalectomized animals, 8–12 days after adrenalectomy, together with a relative increase of succinic dehydrogenase activity. However, there was no evidence in the paper that any attempt had been made to see if the adrenalectomies were complete and, in any case, our results were obtained 4 days after the operation, compared with their 8 days. In our results in the kidney it was noteworthy that

the greatest reduction of activity was in the cells of the proximal convoluted tubules, which in the intact animal are probably the sites of the greatest activity. In the cerebellum the almost complete lack of reaction of the Purkinje cells in adrenalectomized animals is of interest. The possibility that these decreases in enzyme activity in various tissues may have been due to the operation and not due to the removal of the adrenals *per se* was checked by mock adrenalectomies. The tendency to reduced intensity of reaction in some tissues after such an operation did not appear to be significant.

Ingle, Nezamis & Morley (1951) have shown that cortisone and 17-hydroxy-corticosterone result in increased work performance by adrenalectomized rats, and various other workers (including Chiu & Needham, 1950; Leupin & Verzár, 1950) have shown an increase of carbohydrate metabolism by cortical hormones. In this work it has been found that the succinic dehydrogenase activity of liver and heart was almost normal, that of cerebellum partly restored to normal and kidney not much improved in adrenalectomized rats treated by cortisone. Deoxycorticosterone, on the other hand, brought back a good positive reaction in the convoluted tubules of the kidney but only partly restored the reaction in the other three tissues. The two hormones together brought the intensity of the reaction of the four tissues nearly back to normal, but not quite in cerebellum and liver.

Oestrone depressed the enzyme activity in some cases (particularly in kidney). Testosterone had no effect and progesterone brought the reaction intensity at least half-way back to normal in all four tissues. The effect of oestrone in decreasing the reaction intensity is of interest in view of the fact that synthetic and natural oestrogens are known as *in vitro* inhibitors of succinic dehydrogenase, but were not found to have this effect *in vivo* (Meyer & McShan, 1950). Kalman (1952) also found that testosterone had an inhibitory effect on the succinic dehydrogenase system of homogenates of rat liver.

It is appreciated that cortisone and deoxycorticosterone are not necessarily the forms in which the gluco-corticoids and mineralo-corticoids are secreted by the adrenal cortex, in fact the failure of the two hormones together to bring back a completely normal succinic dehydrogenase reaction in all of the four tissues examined either supports this conception or suggests that some other factor or factors are missing.

The effect of progesterone in partially restoring the succinic dehydrogenase reaction in the tissues examined is of interest in view of the finding (Bourne, 1939) that progesterone prolonged the lives of adrenalectomized animals, that testosterone had no effect and that oestrone shortened their lives.

SUMMARY

1. Intramuscular injection of cortisone causes an increase of intensity of the histochemical reaction for succinic dehydrogenase in liver, heart, kidney and cerebellum of intact rats.

2. Adrenalectomy causes a considerable reduction of this reaction in all four tissues.

3. Cortisone, injected into adrenalectomized rats restores the intensity of the reaction almost to that found in intact, untreated animals, in heart muscle, liver, cerebellum and partly restores the reaction intensity in the kidney.

4. Deoxycorticosterone acetate injected into adrenalectomized rats produces a partial restoration of intensity of the reaction in all four tissues, that of the kidney being almost equal to that of the intact, untreated animals.

5. Cortisone and deoxycorticosterone acetate injected together into adrenalectomized rats produce a nearly normal reaction-intensity in all four tissues.

6. In adrenalectomized rats oestrone decreases the reaction; testosterone has no effect and progesterone brings the reaction in all four tissues partly back to normal.

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EXPLANATION OF PLATES

PLATE 1

Succinic dehydrogenase reaction in kidney.

Fig. 1. Normal control (intact, untreated rat). Note negative glomeruli (*G.*) and granular deposit in surrounding tubules.

Fig. 2. Intact animal. Effect of cortisone. Note increased intensity of reaction in tubules. Individual granules difficult to see. Glomeruli still negative.

Fig. 3. Effect of adrenalectomy. Note great reduction in reaction.

Fig. 4. Effect of mock adrenalectomy. Note reaction intensity virtually same as normal.

Fig. 5. Effect of cortisone on adrenalectomized animal. Note reaction intensity only about half that of normal.

Fig. 6. Effect of deoxycorticosterone on adrenalectomized animal. Reaction-intensity about two-thirds that of normal.

Fig. 7. Effect of simultaneous cortisone and deoxycorticosterone on adrenalectomized animal. Reaction intensity about equal to that in fig. 6.

Fig. 8. Effect of testosterone on adrenalectomized rat. Note slight increase of reaction over untreated adrenalectomized animal (fig. 3).

Fig. 9. Effect of progesterone on adrenalectomized rat. Reaction near normal.

Fig. 10. Effect of oestrone on adrenalectomized rat. Reaction less than in untreated adrenalectomized rat.

PLATE 2

Succinic dehydrogenase reaction in heart muscle.

Fig. 11. Normal control (intact, untreated rat). Magnification is too low to show longitudinal arrangement of granules.

Fig. 12. Intact rat. Effect of cortisone. Note great increase in intensity of reaction and negative intercalated discs (*I.C.*).

Fig. 13. Effect of adrenalectomy. Note very great reduction of reaction.

Fig. 14. Effect of mock adrenalectomy. Note reaction virtually normal. Longitudinal arrangement of granules more obvious.

Fig. 15. Effect of cortisone on adrenalectomized rat. Note reaction as good or better than normal, and negative intercalated disks (*I.C.*).

Fig. 16. Effect of deoxycorticosterone on adrenalectomized animal. Note reaction much less than with cortisone (fig. 15) and less than normal.

Fig. 17. Effect of cortisone and deoxycorticosterone on an adrenalectomized rat. Note reaction more intense than normal, and negative intercalated disks (*I.C.*).

Fig. 18. Effect of testosterone on adrenalectomized rat. Reaction equal to untreated adrenalectomized animal (fig. 13).

Fig. 19. Effect of progesterone on adrenalectomized rat. Reaction greater than untreated adrenalectomized rat (fig. 13) but much less than normal.

Fig. 20. Effect of oestrone on adrenalectomized rat. Reaction about same as untreated adrenalectomized (fig. 13) rat.

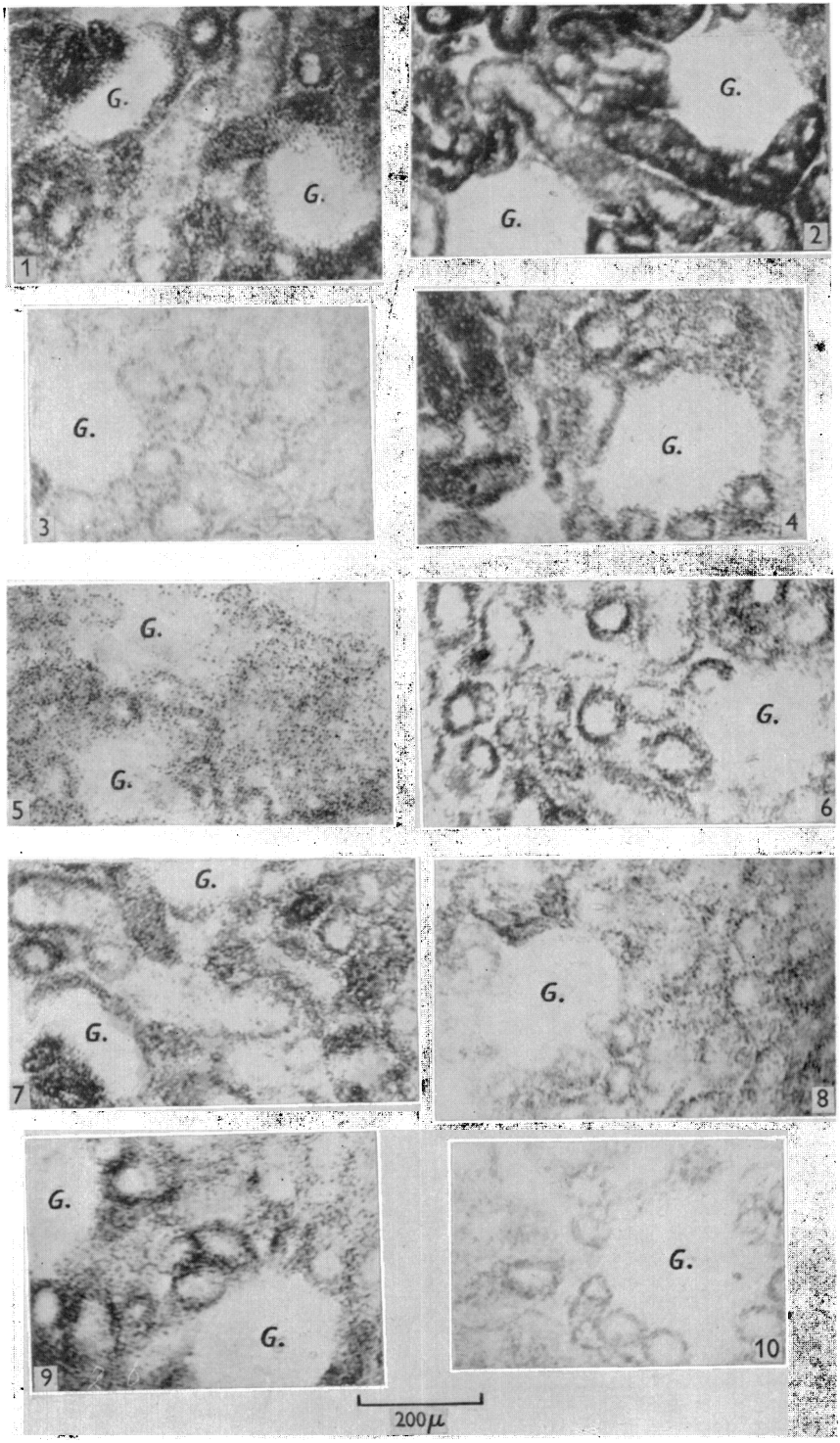
PLATE 3

Succinic dehydrogenase in liver. Each photomicrograph is of a portion of a liver lobule and shows a central vein (*C.V.*).

Fig. 21. Normal control (intact, untreated rat). Note granular reaction in cells. More intense at periphery of lobule.

Fig. 22. Effect of cortisone on intact rat. Note great increase of reaction particularly at periphery of lobule.

Fig. 23. Effect of adrenalectomy. Note almost negative result. Region near central vein completely negative.



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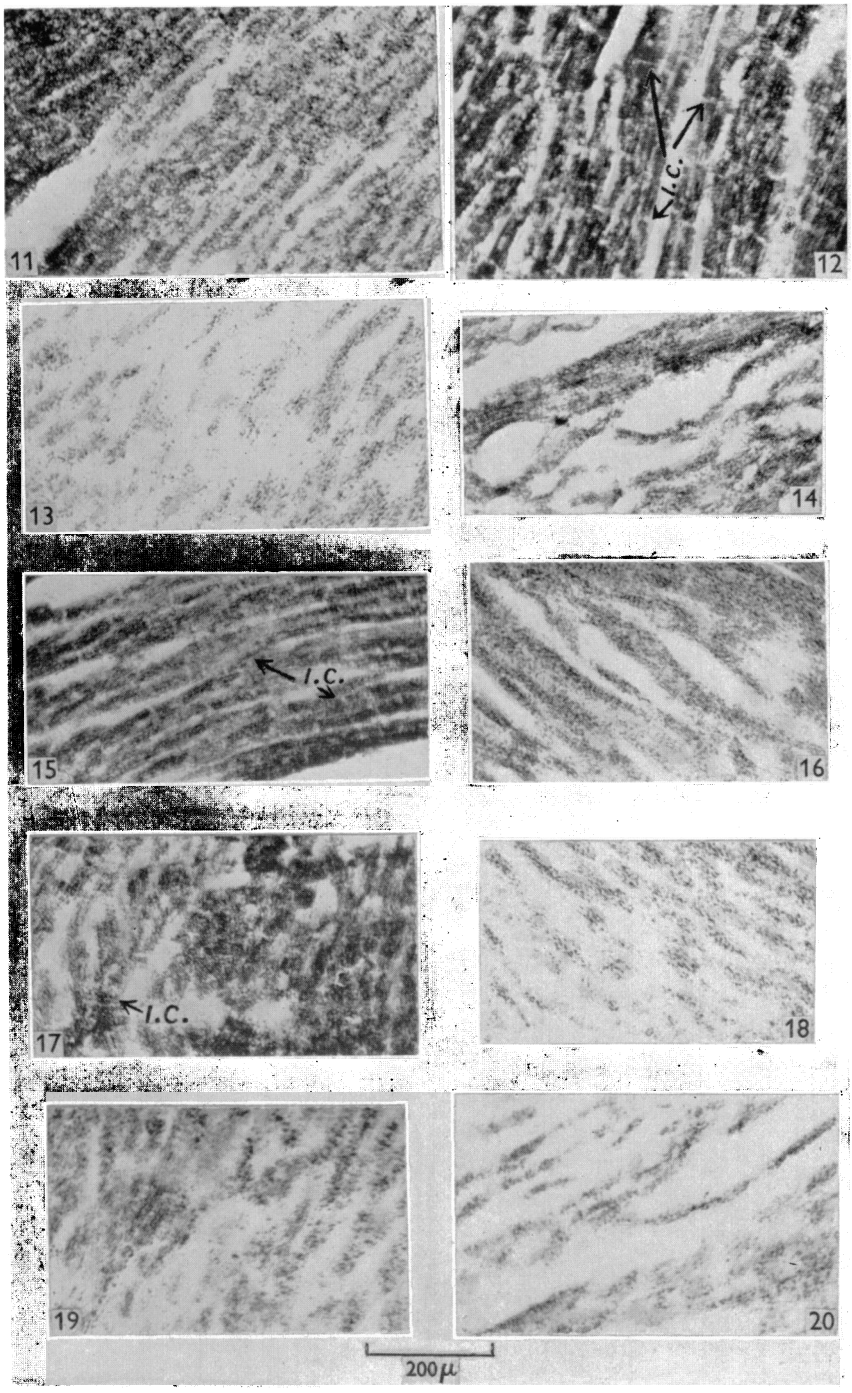
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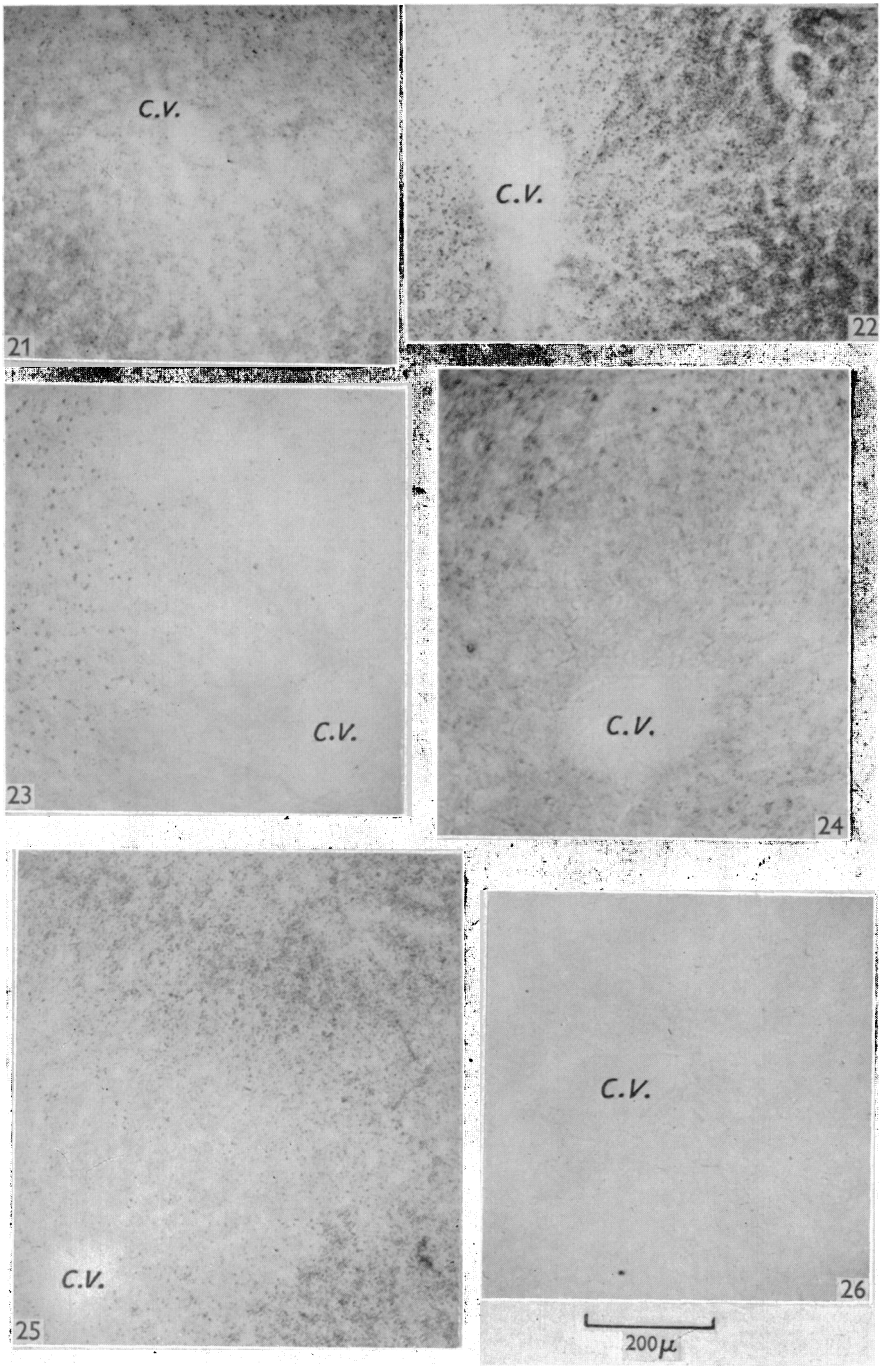
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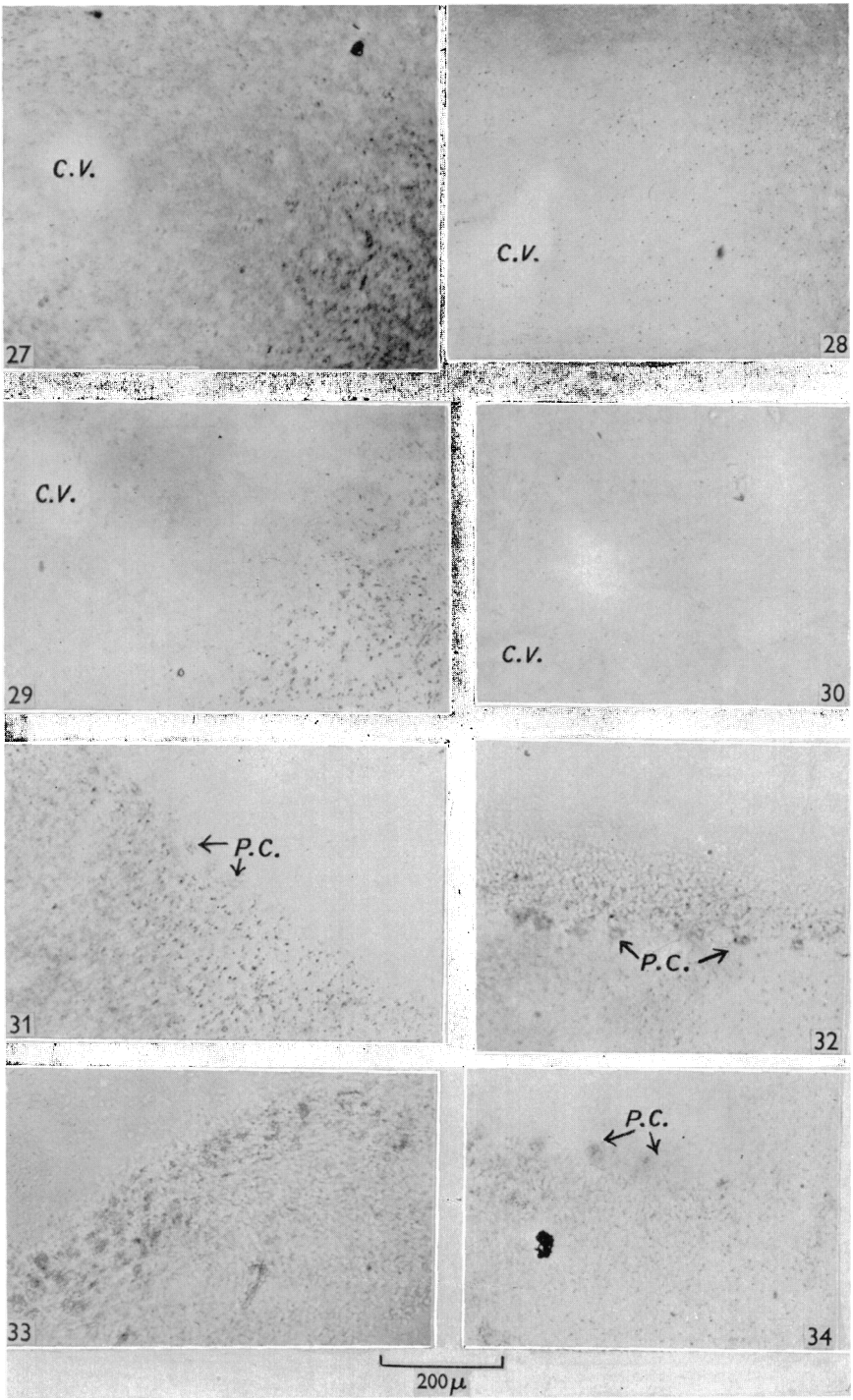
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Fig. 24. Effect of mock adrenalectomy. Reaction intensity approximately normal.
Fig. 25. Effect of cortisone on adrenalectomized rat. Note reaction intensity practically normal.
Fig. 26. Effect of deoxycorticosterone on adrenalectomized rat. Reaction negative.

PLATE 4

Succinic dehydrogenase reaction in liver and cerebellum. (*C.V.* = central vein.)

Fig. 27. Liver. Effect of cortisone and deoxycorticosterone on adrenalectomized rat. Reaction almost normal.
Fig. 28. Liver. Effect of testosterone on adrenalectomized rats. Slight reaction.
Fig. 29. Liver. Effect of progesterone on adrenalectomized rat. Moderate reaction.
Fig. 30. Liver. Effect of oestrone on adrenalectomized rat. Negative reaction.
Fig. 31. Cerebellum. Normal rat. Purkinje cell (*P.C.*) moderately positive.
Fig. 32. Cerebellum. Effect of cortisone on intact rat. Note increased reaction by Purkinje cells (*P.C.*).
Fig. 33. Cerebellum. Adrenalectomized rat. Note Purkinje cells negative.
Fig. 34. Cerebellum. Effect of cortisone on adrenalectomized rat. Note reaction again in Purkinje cells (*P.C.*).