Brit. J. Nutr. (1964), **18**, 55, *with* 1 *plate*

Adrenal hypertrophy in rabbits fed with cholesterol

By W. FORBES, MARIAN H. STEELE AND H. N. MUNRO

Institute of Physiology and Department of Biochemistry, University of Glasgow

(*Received* 25 *April* 1963—*Accepted* 9 *August* 1963)

Cholesterol has been extensively used as an atherogenic agent. Several authors have observed changes in the adrenal cortex of animals fed on diets containing excessive amounts of cholesterol. Thus Krylow (1914), Sternberg (1915) and McMillan, Klatzo & Duff (1954) fed rabbits with cholesterol dissolved in a vehicle of fat and observed an increase in the breadth of the adrenal cortex with an increase in its lipid content. The cells of the cortex were enlarged and tended to become fused with disintegration of cell membranes. Similar changes in the adrenal cortex of the rabbit were reported by Reineck (1928) and by Kay & Whitehead (1935) who gave cholesterol without the vehicle of fat. Finally, Bernick & Patek (1961) noted deposition of fat in all zones of the adrenal cortex of rats given cholesterol with cottonseed oil, but they make no comment on the size of the adrenal glands.

There is thus evidence that administration of cholesterol leads to changes in the adrenal cortex, but it will be noted that several of these authors gave fats along with cholesterol, and that in all these experiments intake of food was uncontrolled; in consequence it is possible that total food intake may have differed in the control and cholesterol-fed groups. It therefore seemed desirable to repeat these studies, using cholesterol as the only dietary additive and controlling food intake so that it would be similar in the group given cholesterol and in the control group. Further, it is now possible, with the advent of more precise chemical and histochemical procedures, to explore the type of adrenal change. A study has therefore been made of the effect of cholesterol administration on a number of adrenal constituents. The effect obtained with cholesterol has been compared with the effect produced by giving adrenocorticotrophic hormone (ACTH), which also causes adrenal hypertrophy. A preliminary account of these experiments has already appeared (Forbes, Munro & Steele, 1962).

EXPERIMENTAL

Animals. Mature rabbits of both sexes were selected within the weight range 1·8–2·2 kg. Equal numbers of males and females were included in the control and cholesterol-fed groups. The rabbits were kept in individual cages and were housed under thermostatically controlled conditions. Each experiment lasted from 10 to 16 weeks.

Diets. By trial and error, it was found that 100 g daily of stock diet (diet 18 of Bruce & Parkes, 1947) were adequate to prevent loss of weight by rabbits of this size. The animals in the control groups were given this amount daily; those on the

https://doi.org/10.1079/BJN19640006 Published online by Cambridge University Press

cholesterol diet received 99 g stock diet with 1 g cholesterol. Diet 18 was ground, cholesterol was added, and the mixture moistened and repelleted.

Administration of ACTH. Twenty i.u./kg body-weight of corticotrophin zinc hydroxide (Cortrophin Zn; Organon Laboratories, London) were given intramuscularly daily for 9 days before killing.

Removal of organs. At the end of the experimental period each rabbit was killed by a blow on the head. The adrenal glands, liver, thyroid, pituitary gland, heart and aorta were rapidly removed. Fat and connective tissue were carefully removed from the adrenal glands, which were then weighed on a torsion balance. Portions of the adrenal glands were taken for chemical and for histological examination. Each gland was halved lengthwise; half was used for chemical analysis and the remainder was cut into several portions for histological examination. The remaining organs were examined histologically, as part of another study to be reported separately.

Chemical analysis of the adrenal glands. The adrenal tissue was weighed and then homogenized (Potter & Elvehjem, 1936) in chilled water and the volume was made up to 10 ml. Half the homogenate was taken for estimation of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), protein and phospholipid, by procedures slightly modified from those described by Munro, Hutchison, Ramaiah & Neilson (1962). Trichloroacetic acid (TCA) was added to the homogenate to give a concentration of 10% (w/v) with respect to TCA. The precipitate was separated by centrifugation at 0° and washed twice by suspension in 10% (w/v) TCA. The residue was then extracted successively with ethanol, ethanol–chloroform (3:1), ethanol–diethyl ether (3:1) twice, and finally diethyl ether. The determination of phosphorus (Griswold, Humoller & McIntyre, 1951) in the combined extracts provided a measure of the phospholipid content of the tissue. The tissue residue was incubated in 1 ml N-NaOH for 18 h at 37° to hydrolyse RNA, from which the DNA was separated by acidification; the RNA was estimated by the orcinol procedure of Kerr & Seraidarian (1945), the period of heating in the reagent being extended to 30 min. The colour formed was compared with that from ribose, and was expressed as RNA phosphorus on the assumption that 10 μg ribose give the same colour intensity as 4·13 μg RNA phosphorus. The precipitate containing the DNA was dissolved in N-NaOH and the DNA estimated by Ceriotti's (1952) deoxypentose reaction. A calf thymus DNA preparation was used as standard and the results were expressed as DNA phosphorus. Protein nitrogen was determined on a portion of the alkaline digest by the method of Paul (1958). Cholesterol measurements in the adrenal gland homogenate were carried out by the method of Zlatkis, Zak & Boyle (1953) as described by Knobil, Hagney, Wilder & Briggs (1954). Portions of 0·5 ml homogenate were dissolved in 10 ml glacial acetic acid, and to 3·5 ml of this solution 2·5 ml of the chromogenic reagent were added.

The chemical analyses were evaluated by statistical analysis using the *t* test after logarithmic transformations of the results in order to equalize the variances.

Histological and histochemical procedures. The adrenal gland was divided into three parts. One was placed in a solution containing 10% (w/v) silver nitrate and 10% (w/v) acetic acid for the demonstration of ascorbic acid (Cater, 1951). A second portion was fixed in neutralized 12% (v/v) formaldehyde and frozen sections (15 μ thick)

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were cut and treated to demonstrate the presence of various lipid substances: (*a*) total fat by the standard Sudan Black technique; (*b*) neutral fat by means of Sudan IV; (*c*) neutral fat by the Fettrot method as described by Pearse (1960), but modified by floating the sections out in the stain; (*d*) phospholipids by Menschik's (1953) method, modified by using cold acetone for 10 sec in place of hot acetone for 30 min; (*e*) fatty acids by the Fischler method (Pearse, 1960); (*f*) total cholesterol by the modification of Schultz's method described by Weber, Phillips & Bell (1956); (*g*) free cholesterol was differentiated from its esters by the method of Feigin (1956). Glycogen (Best's carmine method) and RNA (Kurnick, 1955) were demonstrated on cryostat sections cut from the third portion of tissue.

The amounts of these substances observed histochemically were evaluated quantitatively as far as possible and shown in Table 3 by using plus and minus signs to indicate the direction and degree of change from the pattern observed in the untreated control animals.

RESULTS

Adrenal size, composition and histological appearance after prolonged administration of cholesterol

In the first series of experiments the rabbits were fed on the diet with cholesterol or on the control diet for periods of 14 or 15 weeks. During this time, the control rabbits gained an average of 165 ± 62 g in weight and the group given cholesterol gained 345 ± 91 g; these small weight gains do not differ significantly. The size of the adrenal glands and the amounts of their constituents are expressed per kg final body-weight (Table 1).

Table 1. *Effect of giving rabbits cholesterol on adrenal gland composition*

(Combined results of two experiments lasting for 14 weeks and for 15 weeks; total number was ten animals/group; the results are mean values with their standard errors)

	Value/kg final body-weight		
Measurement	Control group	Cholesterol-fed group	Difference
Weight (mg)	78 ± 5	192 ± 19	+146 %**
Cholesterol content (mg)	$6{\cdot}7 \pm 0{\cdot}7$	$33{\cdot}8 \pm 7{\cdot}0$	+403 %**
Protein content (mg N)	$1{\cdot}00 \pm 0{\cdot}09$	$1{\cdot}52 \pm 0{\cdot}23$	+52 %*
RNA content (μg P)	42 ± 3	61 ± 6	+47 %**
Phospholipid content (μg P)	144 ± 11	176 ± 21	+22 %†
DNA content (μg P)	25 ± 2	28 ± 3	+11 %†

** $P < 0{\cdot}01$. * $0{\cdot}05 < P < 0{\cdot}10$. † $P > 0{\cdot}05$.

Chemical analysis. Table 1 shows that there was a gross increase (+146 %) in the weight of the adrenal glands of the group given cholesterol. By analysis of variance, this change was shown to be statistically significant ($P < 0{\cdot}01$). The cholesterol content of the gland was even more extensively raised (+403 %, $P < 0{\cdot}01$), and there was a statistically significant increment in RNA content (+47 %, $P < 0{\cdot}01$) and an almost significant increase in protein content (+52 %, $0{\cdot}05 < P < 0{\cdot}10$). Changes in DNA and in phospholipid content were not statistically significant ($P > 0{\cdot}05$). These observations imply that the gland enlargement after giving cholesterol was

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essentially due to hypertrophy of existing cells without a significant increase in cell number.

Since the total DNA content of an organ is a measure of cell number (Thomson, Heagy, Hutchison & Davidson, 1953), the effect of cholesterol administration on the composition of the average cell can be computed by expressing the values given in Table 1 in relation to the amount of DNA in the gland (Table 2). The mean cell weight and its content of cholesterol, protein and RNA were all significantly increased ($P < 0.01$), whereas the phospholipid content per cell was unaffected ($P > 0.05$).

Table 2. *Effect of giving rabbits cholesterol on the composition of the average adrenal cell*

(Values computed from the data summarized in Table 1; the results are mean values with their standard errors)

Measurement	Value/μg DNA‡ P: Control group	Cholesterol-fed group	Difference
Weight (mg)	3·7 ± 0·2	7·2 ± 0·8	+94 %**
Cholesterol content (μg)	276 ± 27	1277 ± 281	+356 %**
Protein content (μg N)	40 + 2	53 ± 3	+33 %**
RNA content (μg P)	1·68 ± 0·08	2·23 ± 0·09	+33 %**
Phospholipid content (μg P)	6·0 ± 0·5	6·4 ± 0·5	+7 %†

** $P < 0.01$. † $P > 0.05$. ‡ As a measure of cell number (see above).

The main source of the enlargement in cell size was not identified chemically; the change in adrenal size was three times greater than the increase in protein and in RNA content of the gland, and the amount of cholesterol deposited could not account for more than a fraction of the excess weight. A few measurements of dry weight made on adrenal glands obtained from other animals receiving various cholesterol-enriched diets showed that the enlargement is not accompanied by an increase in cell water content; the increased fat content seen histochemically is a more likely cause of the gland enlargement in the groups given cholesterol.

Histological examination. The animals given cholesterol had adrenal cortices about twice as broad as those of the control group, the increase being confined to the zona fasciculata. The zona glomerulosa was not notably increased in breadth, and the zona reticularis had shrunk to a thin layer of cells incompletely surrounding the medulla.

Considerable changes in the vascular pattern were observed in the cortices of the rabbits given cholesterol. A plexus of widely dilated venules and capillaries was apparent in the zona glomerulosa and outer fasciculata, forming a ring round the cortex at this level, and this change was accompanied by a widening of the blood sinusoids between the cords of the outer fasciculata cells. The sinusoids of the inner fasciculata, on the other hand, were no longer visible, probably owing to the fact that, in this region, the cells were no longer arranged in columns.

The mean size of the cells of the zona fasciculata was enlarged to about four times the area of the fasciculata cells in the control group, as seen in cross section (Pl. 1). These enlarged cells stained weakly with haematoxylin and eosin, contained many

https://doi.org/10.1079/BJN19640006 Published online by Cambridge University Press

vacuoles and were often binucleate. Some very large cells showed, by the presence of broken cell membranes, that they had arisen by confluence of adjacent cells. There were also large areas of the zone in which the cells had very clear cytoplasm. By contrast, the only change noted in the cells of the zona glomerulosa was an increase in the number of cytoplasmic vacuoles. The cells of the zona reticularis of the cholesterol-fed animals were moderately enlarged and showed a number of vacuoles that were rarely observed in this zone in the control animals.

Table 3. *Changes demonstrated by histochemical tests in the adrenal glands of rabbits given cholesterol or injected with adrenocorticotrophic hormone (ACTH)*

(Rabbits studied after cholesterol feeding, rabbits injected with ACTH while on the stock diet and rabbits injected with ACTH while on a cholesterol-rich diet were those reported in Tables 1, 4 and 5, respectively. The signs indicate the degree of change in the gland constituent by comparison with the control group of untreated animals in each experiment: + = more than control group; o = no change from control group; − = less than found in control group. The results are reported for three areas of the adrenal cortex, the zona glomerulosa (Glom.), the zona fasciculata (Fasc.) and the zona reticularis (Retic.))

	Cholesterol group			ACTH group			Cholesterol and ACTH group		
	Glom.	Fasc.	Retic.	Glom.	Fasc.	Retic.	Glom.	Fasc.	Retic.
Total lipids	+	++	++	++	−	−	++	−	+
Neutral lipids	+	+	+	++	−	o	++	−	−
Fatty acids	++	−	o	−	−	−	−	−	−
Phospholipids	+	+	+	+	+	+	+	+	+
Total cholesterol	++	++	++	+	−	−	++	−	−
Free cholesterol	+	+	+	o	o	o	o	o	o
RNA	+	+	o	++	++	++	++	+++	++
Ascorbic acid	−	−	−	−	−	−	−	−	−
Glycogen	o	+	+	+	o	o	+	+	+

The results of the histochemical tests are summarized in Table 3. In consequence of giving cholesterol, changes were observed in all zones, but were most pronounced in the zona fasciculata. Total fat, neutral fat and phospholipid content were increased in all parts of the cortex. The cholesterol content was also increased, almost completely owing to deposition in the esterified form, although some free cholesterol was noted in every zone in many sections taken from the animals given cholesterol. A marked increase was observed in the fatty acid content of the zona glomerulosa, but a fall in the content of the zona fasciculata. There was an increase in glycogen content in the cells of all zones except those of the glomerulosa, and RNA content increased in both the glomerulosa and fasciculata. All zones showed a decrease in ascorbic acid content.

Effect of duration of cholesterol administration and of sex of the animal

The experiments described above demonstrated the action of cholesterol administered for from 14 to 15 weeks, in ten control and ten cholesterol-fed rabbits. In subsequent experiments the period of feeding with cholesterol was varied from 10 to 16 weeks. Though the changes caused by cholesterol administration varied considerably in degree in successive experiments, there was no progressive increase in the intensity of the adrenal effects as the time of administration was prolonged.

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Consequently, the changes in adrenal size and structure were well established in less than 10 weeks of administration of cholesterol.

The sex of the rabbits made no significant difference in the response to cholesterol administration. The mean values obtained from all the experiments from a total of nineteen male and eleven female rabbits given the cholesterol diet show that the adrenal glands underwent increases in weight of 94% in the bucks and 68% in the does when compared with those of control animals of the same sex. This sex difference in degree of adrenal enlargement was not statistically significant ($P > 0.05$).

Table 4. *Effect of injecting rabbits with adrenocorticotrophic hormone (ACTH) on the weight and individual chemical components of the adrenal gland*

(Rabbits injected intramuscularly daily with 20 i.u. ACTH/kg body-weight for 9 days; four animals/group; the results are mean values with their standard errors)

	Value/kg final body-weight		
Measurement	Control group	ACTH-treated group	Difference
Weight (mg)	65 ± 4	173 ± 32	+165%**
Cholesterol content (mg)	3·6 ± 0·5	1·9 ± 0·13	−49%*
Protein content (μg N)	584 ± 13	1854 ± 346	+218%**
RNA content (μg P)	28 ± 1	81 ± 17	+191%**
Phospholipid content (μg P)	84 ± 8	306 ± 82	+261%**
DNA content (μg P)	15 ± 1	23 ± 3	+52%†

** $P < 0.01$. * $P < 0.05$. † $0.05 < P < 0.10$.

Response of the adrenal gland to administration of ACTH

The giving of cholesterol and the administration of ACTH each causes adrenal enlargement. A comparison was therefore made between the changes in adrenal structure caused by cholesterol administration and those induced by injecting ACTH. After intramuscular injection of ACTH for 9 days into rabbits on the stock diet, there was adrenal enlargement of magnitude similar to that observed in rabbits given cholesterol for 15 weeks (Table 4), the effect being statistically significant ($P < 0.01$). There was also a significant increase in the gland content of protein, RNA and phospholipid ($P < 0.01$), the change in the amounts of these constituents being proportional to the change in gland weight. In this respect, the effect of ACTH differed from that of cholesterol, which caused an increase in gland size accompanied by increments in protein and RNA which were proportionately much smaller and without significant change in phospholipid content of the gland (Table 1). Further, the administration of ACTH caused an increment in the DNA content of the gland which just failed to attain significance ($0.05 < P < 0.10$); this finding suggests that some multiplication of cells may have occurred, a feature absent from the enlarged glands of animals given cholesterol (Table 1). In agreement with much published data on other species, the cholesterol content of the glands was sharply reduced by administration of ACTH.

Histological examination showed that the adrenal cortex of the ACTH-treated animals was broader than normal, owing to enlargement both of the zona fasciculata

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and of the zona reticularis; both zones now consisted of cells of the same type, namely 'compact' cells with highly eosinophilic cytoplasm and few vacuoles (Pl. 1). There were no changes in the cells of the zona glomerulosa, but a considerable increase in the number of patent blood vessels in the zona glomerulosa and subglomerulosa regions; the sinusoids between the cords of the zona fasciculata and zona reticularis were more distinct than is usual.

On histochemical examination, the zona glomerulosa showed a marked increase in all lipids, including esterified cholesterol, whereas the fasciculata and reticularis exhibited a decrease in these constituents, except that the neutral fat content of the zona reticularis was unaltered by ACTH administration. All zones showed increments in free fatty acids and in phospholipid content. The RNA content of all zones was increased, but glycogen deposition was limited to the zona glomerulosa. In all parts of the cortex there was a reduction in ascorbic acid content.

It is thus obvious that giving cholesterol in the diet to rabbits induced a pattern of chemical, histological and histochemical change in the adrenal cortex quite different from that caused by stimulation with ACTH.

Effect of administering ACTH to animals given cholesterol

In view of the marked differences in the response of the rabbit adrenal gland to cholesterol and to ACTH, the next obvious step was to find if the action of ACTH on the gland could be modified by previous administration of cholesterol. Rabbits were given the cholesterol diet for 10–12 weeks and during the last 9 days of the period they received each day an intramuscular injection of ACTH. In spite of the increase in adrenal weight caused by cholesterol feeding, the administration of ACTH induced a further enlargement of the gland (Table 5). Further, the change in individual chemical constituents caused by ACTH administration to the cholesterol-fed rabbits was similar to that induced by ACTH injection in control animals on the stock diet without cholesterol. Thus prolonged administration of cholesterol did not alter the specific pattern of response to injection of ACTH.

Histological examination of the glands confirmed that the response of animals to ACTH was not grossly altered by previous feeding on a diet rich in cholesterol. There were, however, minor differences from the changes induced by ACTH in animals on the stock diet alone. Giving cholesterol and then injecting ACTH exaggerated the vascular response and produced areas of very clear cells in the zona fasciculata, similar to the areas seen in the adrenals of animals given cholesterol alone. The lipid, phospholipid, fatty acids and ascorbic acid content and distribution underwent changes similar to those observed in animals on the stock diet receiving ACTH injections. Compared with the picture seen after ACTH alone had been given, rather more esterified cholesterol was deposited in the zona glomerulosa. More RNA was found in the outer part of the zona fasciculata, and glycogen deposition was observed in all the zones, whereas animals on the stock diet responded to ACTH with glycogen deposition confined to the zona glomerulosa.

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Table 5. *Effect of feeding rabbits with cholesterol and injecting them with adrenocorticotrophic hormone (ACTH) on adrenal gland composition*

(Rabbits fed on the stock diet or the stock diet with cholesterol for 10–12 weeks, during the last 9 days of which 20 i.u. ACTH/kg body-weight were injected daily; each entry is the mean value for four animals on the stock diet or for seven animals on the diet with cholesterol)

	Value/kg final body-weight					
	Group given stock diet			Group given stock diet with cholesterol		
Measurement	Without ACTH	With ACTH	Difference	Without ACTH	With ACTH	Difference
Weight (mg)	110	268	+158	150	304	+154
Cholesterol content (mg)	6·6	3·8	− 2·8	8·6	5·7	− 2·9
Protein content (mg N)	1·4	4·1	+ 2·7	1·6	4·5	+ 2·9
RNA content (μg P)	48	162	+114	63	187	+124
Phospholipid content (μg P)	124	459	+335	154	462	+308
DNA content (μg P)	24	46	+ 22	28	45	+ 17

DISCUSSION

These studies substantiate and amplify information about the occurrence of enlargement of the adrenal cortex in rabbits given cholesterol which has been reported in previous investigations (Krylow, 1914; Sternberg, 1915; McMillan *et al.* 1954; Reineck, 1928; Kay & Whitehead, 1935; Bernick & Patek, 1961). The earlier work demonstrated hypertrophy of the cells of the cortex without identifying the cell types involved and without excluding coincident occurrence of an increase in cell number. Our findings showed that the giving of cholesterol caused an increment in cell size which was mainly in the zona fasciculata, and was not accompanied by a significant increase in cell number, as shown by measurement of the total DNA content of the gland (Table 1).

The cell enlargement due to cholesterol was accompanied by a small though significant accumulation of protein and of RNA and of a gross deposition of cholesterol (Table 1). This picture differed markedly from the adrenal hypertrophy induced by administration of ACTH (Table 4). Injection of ACTH caused an increase in cell size of the same order of magnitude as that caused by giving cholesterol, but the chemical constituents were differently affected by the two agents. Unlike cholesterol, ACTH induced an increase in the protein, RNA and phospholipid content of the cortex which was proportional to the increment in gland weight. The hormone also caused formation of new cells in the cortex, as shown by accumulation of DNA. Histochemical examination confirmed that the changes in cell morphology and composition after ACTH administration were quite different from those induced by cholesterol administration.

The different types of adrenal enlargement after giving cholesterol and after injecting ACTH suggest that the mechanism of action of cholesterol does not involve stimulation of the pars distalis of the pituitary gland to secrete more ACTH. It is, nevertheless, possible that the giving of cholesterol may contribute to adrenal enlarge-

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ment by sensitizing it to the action of ACTH secreted at a normal rate. However, the results obtained after administration of ACTH to cholesterol-fed animals show that there is no evidence of any modification of the action of ACTH as the result of previous ingestion of much cholesterol in the diet. Cholesterol absorbed from the gut may well act directly on the adrenal cortex.

SUMMARY

1. A diet containing 1% cholesterol was given to rabbits for periods varying from 10 to 16 weeks and the effects on the adrenal glands were examined. Control rabbits received in similar quantities the same diet without cholesterol.

2. Gland weight was approximately doubled and there were significant increments in the content of protein, of ribonucleic acid and of cholesterol, which did not, however, account for the increased gland weight. As demonstrated by measurement of deoxyribonucleic acid, the number of cells in the gland was not significantly altered by feeding with cholesterol.

3. Histological and histochemical studies showed that the main effect of cholesterol administration occurred in the zona fasciculata, where the cells underwent enlargement and showed gross changes histochemically.

4. Administration of adrenocorticotrophic hormone for 9 days induced enlargement of the adrenal glands similar in magnitude to that seen after feeding with cholesterol, but chemical and histological examination showed that the pattern of changes differed from those caused by feeding with cholesterol.

5. Rabbits that had previously received the cholesterol diet showed no change in their responsiveness to injection of ACTH.

6. Adrenal enlargement caused by feeding with cholesterol is therefore not due to increased secretion of ACTH.

We should like to acknowledge with thanks the technical services of Mr D. McAllister, Miss J. Wilson, Mrs G. Neill, Miss R. McCafferty and Miss E. Douglas. One of us (M.H.S.) took part in this work while employed under a grant received from the Advisory Committee on Medical Research of the Department of Health for Scotland.

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EXPLANATION OF PLATE

Zona fasciculata cells of the rabbit adrenal cortex stained with haematoxylin and eosin.

(*a*) Cells from control animals on stock diet, showing usual size of cells arranged in typical columns.

(*b*) Cells from animals given cholesterol, showing enlarged cells with transparent, foamy cytoplasm and loss of columnar arrangement.

(*c*) Cells from animals on the stock diet treated with adrenocorticotrophic hormone, showing cell enlargement and dark-staining cytoplasm, with partial loss of columnar arrangement and prominent blood vessels between the cells.

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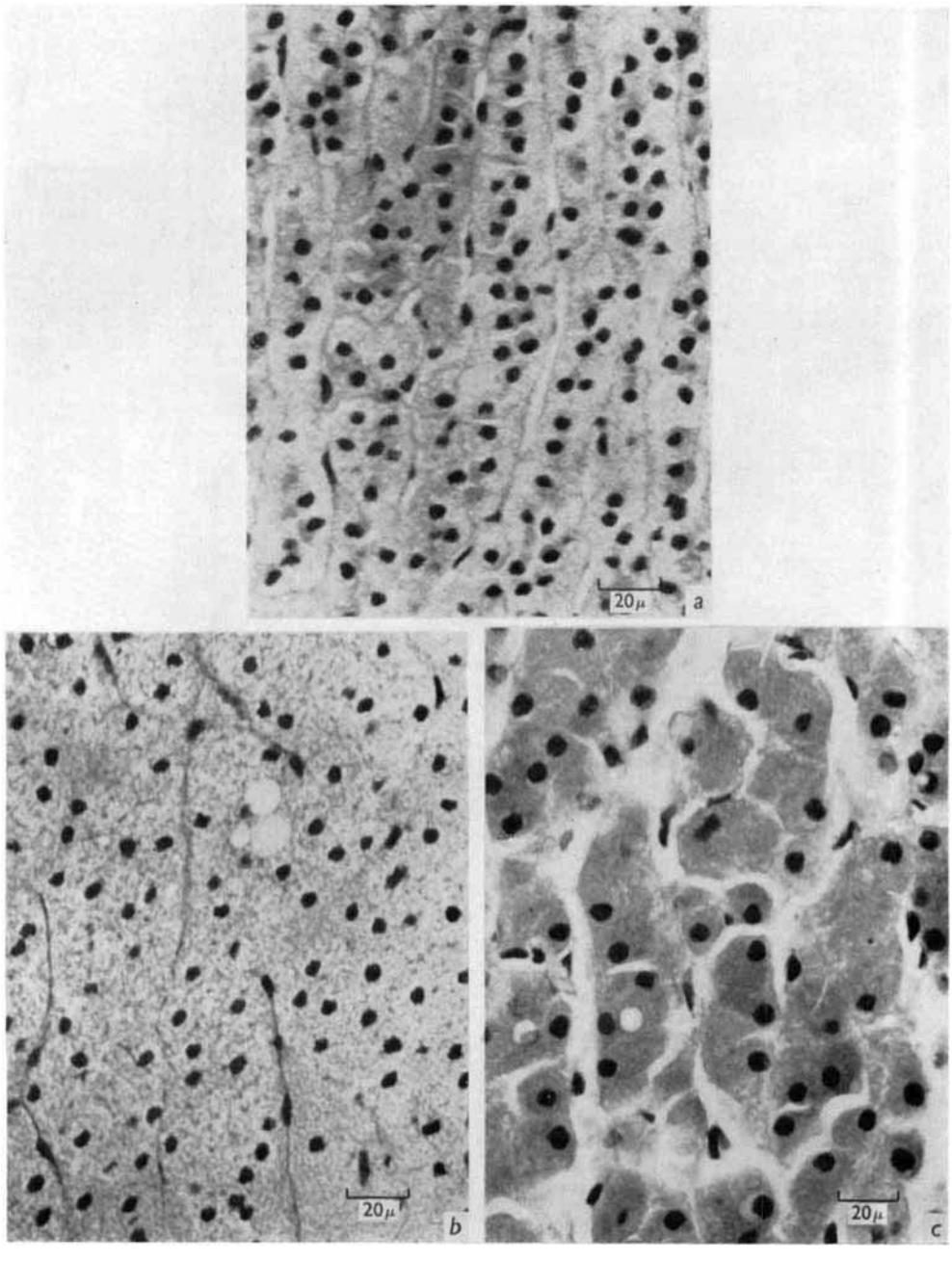

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