VITAMIN A DEFICIENCY IN THE GERM-FREE RAT

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Deficiency of vitamin A in experimental animals is so regularly associated with infection[1,2] that the term "anti-infective vitamin" has often been applied.[3] It has been pointed out, however, that secondary bacterial invasion may actually be due to alterations in mucous membranes rather than to lowered resistance *per se*.[4] These mucosal changes were first emphasized by Wolbach and Howe,[5] who found cornification of the epithelium of various organs. Since that time "keratinizing metaplasia" has been accepted as a specific pathologic manifestation of avitaminosis A and the *sine qua non* of A deficiency in experimental animals.

In A-deficient conventional animals, the almost constant occurrence of infection obviates the necessity of postulating a specific cause of death. In the germ-free animal such is not the case. In fact, if it is true that simple epithelial alterations in the mucosa and ducts of nonvital organs are the only significant changes which occur, then the peculiar environment of the A-deficient germ-free rat should be conducive to long life and relatively good health. It was felt, therefore, that a study of vitamin A deficiency in the germ-free animal would not only provide an unclouded pathologic picture of tissue changes which have heretofore been obscured, but would also clarify the role of secondary infection in the pathogenesis of the disorder.

MATERIAL AND METHODS

Ten germ-free Lobund albino weanling rats, 7 females and 3 males, were transferred from a Reyniers holding tank to a Trexler type plastic isolator,[6] where they were housed in pairs in wire-bottom cages and maintained under accepted germ-free conditions—i.e., by common cultural methods they were free from bacteria and fungi.[7–10] Excreta, food, water, and tank debris were routinely cultured, but only when the tank was entered for other purposes and at the end of the experiment. From the time of transfer at age 21 days until the experiment was terminated, the rats were kept on the following diet:

Casein (G. B. I.)	20 %	Corn starch	71 %
Salt mixture (Wesson)	4.0 %	Vitamin D_3 (oil soluble)	0.5 %
Corn oil	4.5 %	Thiamine hydrochloride	1 mg. %

Accepted for publication, September 19, 1960.

Pyridoxine hydrochloride	1 mg. %	B_{12}	10 γ %
Riboflavin	2 mg. %	Para-aminobenzoic acid	3 mg. %
Calcium pantothenate	2 mg. %	Folic acid	1.25 mg. %
Niacinamide	10 mg. %	Ascorbic acid	10 mg. %
Biotin	5 γ %	Menadione	0.2 mg. %
Choline chloride	200 mg. %	α-tocopherol acetate	5 mg. %

After the diet ingredients were mixed, 250 cc. of water per kg. of diet were added. The diet was then made into rolls and autoclaved at 250° to 255° F. for 30 minutes in the steam lock of a Reyniers tank, from whence it was passed into the plastic tank via a chemical lock (aerosol fog of 2 per cent peracetic acid with detergent). Food and water were supplied *ad libitum*.

In addition, 10 conventional non-germ-free Lobund rats of the same age and sex were maintained on the same diet, but outside the tank, being housed separately in wire-bottom cages. Both the germ-free and control groups were kept on the experimental regimen until 80 per cent of the animals in each group had died; the remaining rats were then sacrificed.

Tissues were fixed as soon as possible after death in 10 per cent buffered neutral formalin. For histologic examination the tissues were embedded in paraffin and stained with azure A-eosin B. Selected sections were also stained by the periodic acid-Schiff (PAS), method, Perls's method for iron, the Dunn and Thompson technique and the Wilder reticulum stain.[11] Fat stains were done with oil red O on frozen sections.[12]

Results

Excellent descriptions of both the clinical course and the pathologic features of vitamin A deficiency in conventional, non-germ-free rats are readily available not only in individual articles,[2,5,13–16] but also in books.[17–19] Observations, therefore, unless specifically indicated, will be confined to *gnotobiotes*.* Germ-free conditions were maintained throughout the experiment and all cultures were negative.

Clinical Observations

The germ-free animals continued to grow and to appear outwardly healthy until the 47th day on the diet, when one of the rats began to exhibit a slight squint and minimal periorbital crusting. By the end of another week, the squint and crust had become slightly more pronounced and the same change had begun to appear in several of the other animals. There were no grossly detectable corneal lesions. However, by this time all of the animals had begun to show slight weight loss, a rough, dull coat, and humped posture. Epilation, which was patchy, partial, and reminiscent of a "moth-eaten" type of alopecia, appeared and increased in severity. In the final stages of the disease, the rats developed a watery diarrhea which at times became profuse. Ac-

* A term coined by Reyniers to include the germ-free animals—the word being derived from the Greek stem *gnos*, known; and *bios*, life.[20]

tivity decreased and a fine tremor could frequently be seen while the animal was at rest. Although there was possibly slight ataxia and incoordination of movement, actual paralysis did not develop.

In spite of the emaciation and diarrhea, food and water consumption remained surprisingly constant, after having shown a decrease at the onset of symptoms. Toward the end of the experiment, spontaneous activity was for the most part confined to feeding. The rats had no apparent difficulty, however, in finding food, in mastication, or in deglutition. Anosmia did not develop; nor did priapism in the males.

The first death in the germ-free group occurred on the 82nd day of the experiment; and in the control group, on the 38th. Nevertheless, 4 control animals lived for 108 days, while 3 germ-free animals survived for 109 days. Average survival time for the 8 animals allowed to die from the disease was 82 days in the controls and 95 days in the germ-free group.

Gross Pathologic Observations

The germ-free group exhibited relatively few significant lesions. There was a decrease in but not complete absence of body fat. Lymphoid tissue was hypoplastic. Pituitary glands were quite small, about one half normal size. The livers appeared smaller than normal and varied in color from yellow-brown to rust. There was a slight increase in friability but no other abnormalities. Spleens also were small and somewhat browner than usual. Three rats exhibited renal calcification and hydronephrosis, but there was no accompanying ureteral or bladder dilatation. Another 2 animals had cystic dilation of the bladder which contained cloudy urine and flaky material. The preputial glands, though not enlarged, were multicystic. The stomach contained undigested food and tended to be dilated, as did the remainder of the intestinal tract. There was enormous dilatation of the cecum.

Microscopic Features

Skin. There was cystic atrophy of the sebaceous glands and associated hair follicles in those areas where the former are more concentrated—viz., the skin of the buccal pouch, the eyelid and the peri-anal region (Figs. 1 and 10). Skin changes in other areas were negligible. In no case was there hyperkeratinization of the skin, the hair follicles or sebaceous ducts.

Breast. Although sections of the mammary glands were not taken regularly, a definite proliferative reaction accompanied by cellular vacuolation suggestive of secretory activity was noted in the 4 females in which breasts were examined (Fig. 2).

Preputial Glands. There was marked cystic atrophy and stasis of secretion without apparent obstruction or hyperkeratinization of the excretory duct (Fig. 3). The acinar cells showed a partial to complete loss of perinuclear granules [21] so that many of the acinar cells resembled ordinary sebaceous cells (Fig. 4). The granules which did remain were weakly PAS-positive.

Salivary, Lacrimal and Orbital Glands. Squamous metaplasia was focal and primarily confined to the ducts, which occasionally were slightly dilated and contained keratinized debris along with a few leukocytes. Otherwise, the almost total lack of inflammation was striking, and accentuated even more the degenerative and necrotic alterations in the parenchymal cells. The submaxillary and parotid glands were the most severely affected and frequently were microscopically indistinguishable one from the other. Involvement of the major salivary glands tended to be uniform (Fig. 5), whereas the appearance of the glossal and tracheal mucosal glands (Fig. 6) varied from area to area. The lacrimal glands were less affected but did occasionally show focal areas of hyaline droplet degeneration (Fig. 7) and sometimes necrosis (Fig. 8). The harderian glands, except for a rare metaplastic duct, exhibited only absence of the usual pigmented secretion.

Eyes. The corneas without exception exhibited a thin superficial layer of keratinized cells (Fig. 9), but there was no evidence of inflammation or of vascularization. The conjunctivas showed foci of cornification (Fig. 10) although interspersed mucous cells were abundant. Among the internal ocular structures, degeneration of the retinal bacillary layer and underlying "pigmented" epithelium was noted (Fig. 11). Several of the retinas were detached in the sections, but this may have been artifactual.

Pituitary Glands. No characteristic cellular changes were noted, and castration cells [22,23] were absent.

Thyroid and Parathyroid Glands. There was questionable slight atrophy of the thyroid acini. This was associated with decreased colloid and occasional sloughing of the lining cells. The parathyroid glands were unremarkable.

Heart. In half of the germ-free animals, the ventricular myocardium exhibited focal areas of necrosis (Fig. 12) predominantly associated with an infiltration of mononuclear cells and, rarely, calcification. Four of the animals had died, but the lesion was also present in one of the rats killed at the termination of the experiment.

Respiratory Tract. There was patchy squamous metaplasia and keratinization of the larynx and trachea (Fig. 13); the bronchi and bronchioles were uninvolved. The lungs frequently showed vascular con-

gestion and, occasionally, perivascular edema. In only one animal was there alveolar edema, although two rats exhibited focal pulmonary hemorrhage.

Gastrointestinal Tract. An apparent increase in intraluminal mucus was noted throughout but was unaccompanied by discernible structural alteration in the mucosa, except for an occasional retention cyst with a few leukocytes at the base of a crypt. No gastric ulcers were seen.[24]

Liver. The most remarkable changes occurred in the liver. These varied from the presence of cytoplasmic hyaline droplets in occasional hepatic cells of the two sacrificed animals (Fig. 14) to actual liver necrosis in animals dying of the disorder (Fig. 15). The necrosis was coagulative in type and tended to involve individual cells, converting them into eosinophilic hyaline masses. Nuclear fragments were occasionally present, although frequently no nuclear remnants remained. Even with widespread involvement of the liver, the focal cellular nature of the injury was maintained, in that apparently viable cells were interspersed throughout (Fig. 16). There was total lack of any cellular inflammatory response to the necrotic tissue, no significant stromal collapse, and a negligible increase in reticulin. A moderate amount of fatty metamorphosis was noted in several livers and tended to be periportal in distribution. The hepatic parenchymal cells were pleomorphic and had vesicular but hyperchromatic nuclei with prominent nucleoli. One half of the animals also exhibited a peculiar type of tubular "degeneration" or transformation, manifest by ductlike arrangements of hepatic parenchymal cells (Figs. 15 and 17). In addition, proliferation of the bile duct epithelium was marked in most instances. This process extended from the portal areas and penetrated the lobule, the small ductular structures serpiginously intertwining among the hepatic cells (Figs. 18 and 19).

The common bile duct exhibited superficial squamous metaplasia and slight keratinization, but with associated parakeratosis (Fig. 20). The duct system was apparently unobstructed, and no evidence of bile stasis was noted.

The portal areas contained considerable deposits of an extracellular granular material consisting of pigments which stained green to brown with azure A-eosin B, gave a positive Prussian blue reaction for iron, stained with the PAS method, and to some extent with oil red O. This apparent combination of hemosiderins[11] and lipofuscins[11] was also present in small amounts within the Kupffer cells throughout the liver. There was no associated increase in fibrous connective tissue.

Spleen and Lymph Nodes. Although the lymphoid tissue proper, with the exception of Peyer's patches, was hypoplastic, hemosiderosis of the

spleen and abdominal lymph nodes was marked, but was unaccompanied by fibrosis.

Pancreas. The pancreas showed only minimal squamous metaplasia of occasional interlobular ducts without keratinization and without evidence of obstruction. In a few animals the major pancreatic duct exhibited changes similar to those noted in the common bile duct. No lesions were manifest in the islets and acini.

Adrenals. The adrenal glands were atrophic, had a thin zona glomerulosa, and exhibited a reduced amount of lipid on fat stain. In two animals the glands showed vacuolar degeneration of the zona glomerulosa; in two other rats there was extensive but focal bilateral cortical hemorrhagic necrosis (Fig. 21).

Urinary Tract. The kidneys of all of the animals exhibited mild degenerative changes such as cloudy swelling and fatty degeneration. In 4 rats, however, focal tubular necrosis (Fig. 22) was extensive, bilateral, and associated in 2 instances with pigmented casts in the convoluted and collecting tubules (Fig. 23). These casts gave a positive reaction with the Dunn and Thompson method for hemoglobin. In the more severely affected kidneys, occasional hyaline thrombi were seen in several glomeruli.

In addition to the above, 3 rats exhibited rather extensive tubular calcification and hydronephrosis (Fig. 24), the calcium being deposited in a PAS-positive matrix. In one rat the calcinosis and hydronephrosis were unilateral; in two, bilateral.

In no instance did the renal pelves show squamous metaplasia; nor did the ureters in the 3 animals examined. The bladder and particularly the urethra, however, did, in all cases, show considerable squamous metaplasia and keratinization.

Sex Organs. In males the testes were the site of marked tubular degeneration of both the Sertoli cells and the germinal epithelium. No spermatozoa were present. The tubular epithelium and basement membrane were separated from the interstitial tissue by protein-rich fluid (Fig. 25), and in one case by recent hemorrhage. The prostate glands exhibited atrophy, degeneration and focal squamous metaplasia. The epithelium of the seminal vesicles showed degranulation[25] and atrophy, along with some necrosis, but no metaplastic epithelial changes were noted. Mild cystic degeneration and focal metaplasia were found in the epididymis, and in one rat there was squamous metaplasia of the ductus deferens as well.

In females the vaginas were without exception heavily keratinized, but no leukocytic infiltration was present (Fig. 26). In the uterus the most extensive squamous metaplasia occurred in the glands near the

cervix but was also present focally within the endometrial glands of the cornua. Although "keratin pearls" were the rule, if plugging of a gland neck occurred, an exudation of leukocytes into the obstructed gland was not uncommon (Fig. 27). The superficial endometrium showed atrophy and vacuolar degeneration, but was not metaplastic. There was a striking absence of eosinophils in the endometrial stroma. In no case was there squamous metaplasia of the fallopian tubes. The ovaries of all 7 females had multiple large and prominent corpora lutea; the presence of spindle cells interspersed among the lutein cells suggested that none of these bodies were of recent origin. In addition there was an apparent absence of developing follicles as well as actual atresia of those already present. In all instances characteristic cart-wheel "deficiency" nuclei[26] were present in the surrounding thecal cells (Fig. 28).

Nervous System. Sections of the brain were essentially normal, except for some acute neuronal degeneration and cell shrinkage. Peripheral nerve ganglia and branches of peripheral nerves present in other sections were also not remarkable with conventional histologic techniques. An extensive histologic examination of the nervous system, however, was not carried out. (The teeth were given to the National Institute of Dental Research.)

Control Animals

In general, the control rats exhibited the usual accepted stigmas of A deficiency—i.e., widespread keratinizing metaplasia. Infection and inflammation, particularly of the tongue, salivary glands, respiratory and urinary tracts, were invariably present. Except for slight hemosiderosis, the livers appeared histologically normal, as did the kidneys. The adrenal glands were somewhat atrophic, depleted of lipid, and in two instances contained occasional colloid droplets in the cells of the zona glomerulosa; there was no hemorrhage or necrosis. The ovaries in the conventional animals tended to be more atrophic than in the germ-free, but there was less arrested follicular development, although luteal remains appeared more involuted. Deficiency cells, however, were present in all cases. Changes in the preputial glands and skin resembled those in the germ-free animals; sections of the mammary glands were not obtained.

Discussion

It has long been accepted that tissues such as the liver, which ordinarily contain a high concentration of vitamin A,[27,28] exhibit the least morphologic alteration in A deficiency.[18] Of particular interest, there-

fore, are the striking hepatic lesions in the germ-free rats. In several instances the necrosis alone was of sufficient magnitude to have been at least contributory to, if not the cause of, death. The bizarre focal necrosis and hyalinization of liver cells bear no resemblance to the diffuse cell death of "dietary hepatic necrosis,"[29] and, in fact, are not duplicated in any known deficiency state. On the other hand, the cellular pleomorphism, the duct formation from hepatic cells, and the proliferation of bile duct epithelium are indicative of injury or a metabolic defect which has been operative over a prolonged period of time. Similar changes are seen occasionally in chronic choline deficiency[30] and as an effect of certain carcinogens.[31] Why such a lesion should occur in the A-deficient germ-free rat and be completely absent in control animals on exactly the same diet remains an enigma. It may be that an A deficiency in the absence of normal bacterial flora precipitates a deficiency of another substance, or it may be that the presence of bacteria is necessary for the degradation of certain toxic metabolites produced by the deficiency, and that in the germ-free state these are re-absorbed. The possibilities are infinite and any attempted explanation would be no more than speculative, for even a viral etiology could not be excluded.

Such sporadically occurring lesions as the hemoglobinuric-like nephrosis and renal tubular degeneration may or may not be due to the A deficiency *per se;* more likely they are related to liver necrosis and shock. Although focal hemorrhages can certainly occur with shock and anoxia, hemorrhagic necrosis of the adrenal gland is not usually seen. Adrenal hemorrhage and necrosis do occur in pantothenic acid deficiency,[32] but calcium pantothenate was given in sufficient amount to protect the ordinary germ-free rat from this condition. Menadione (vitamin K) was also supplied in ample quantity. Ascorbic acid, which is present in the adrenal gland[33] and which could conceivably be involved in a hemorrhagic diathesis, was added to the diet but may not have survived autoclaving. Although conventional and germ-free rats on normal diets have not as yet demonstrated a need for an exogenous source of this vitamin, it has been reported that in the conventional A-deficient rat there is a co-existent depletion of tissue ascorbic acid.[34] Other investigators[35] have claimed that the C depletion is related to the decreased food intake of the A-deficient animal. If the latter statement is true, then the A-deficient germ-free rat should not be affected, as food intake was apparently ample. Utilization, of course, is another matter, but this phase has not been investigated as yet. In this same regard, the close topographic association between vitamins A and C should also be remembered—that is, those sites having a high tissue concentration of vitamin A are frequently also high in C; e.g., the adrenal gland and the corpus luteum.[33,36]

In the germ-free rat as in the conventional animal, a deficiency of vitamin A results in widespread squamous metaplasia and keratinization. In the absence of secondary infection, however, such changes lose much of their pathogenic significance. "Xerophthalmia," long regarded as the pathognomonic sign of avitaminosis A, was represented only by a thin superficial layer of keratin. Interestingly enough, keratinization of the cornea and conjunctiva, as well as dilatation of the meibomian glands, has also been reported in sodium deficiency.[37] Even the heavily keratinized vagina, which is generally accepted as one of the earliest manifestations of the disease,[38] presented no distinctive characteristics and was completely devoid of the leukocytic infiltration which has been said to distinguish the A-deficient from the estrogen-stimulated vagina.[39,40]

There were no tongue abscesses to interfere with swallowing.[2,41] There was no purulent rhinitis or nasopharyngitis to produce anosmia[5]; no ulcerative urethritis, common in conventional animals, to account for priapism[5]; and inflammatory lung lesions were nonexistent. The occurrence of "bronchiectasis" secondary to the desquamation of keratinized epithelium[5] was not observed. In fact, even in those organs such as the salivary glands, which were severely involved, degeneration and necrosis of the parenchyma—not simply atrophy—far overshadowed ductular keratinization. The interstitial infiltration by lymphoid and plasma cells observed by Wolbach and Howe[5] in the reported absence of infection was not seen. Except for a few leukocytes in an occasional apparently plugged duct, inflammatory reactions of any kind were rare.

In those tissues and organs not ordinarily susceptible to "keratinizing metaplasia," infection in conventional animals is extremely unusual. Other alterations do occur, however, which reflect the generalized nature of the underlying disturbance, but which, because of the more striking epithelial changes, have in the past been minimized. Less specific, to be sure, but still significant, are the focal lesions in the heart, which Wolbach and Howe[5] observed in 16 of 19 rats. In spite of the occurrence of somewhat similar lesions in other conditions—e.g., potassium deficiency,[42] thiamine deficiency[43] and biotin deficiency[44]—the foci of myocardial necrosis are nonetheless a part of A deficiency. In the germ-free animals, lesions were present in half of those dying and in one of the killed rats. The cardiac lesion, therefore, is probably neither necessarily lethal in effect nor agonal in origin. In fact, the presence of calcium in some of the foci suggests but is not proof for some degree of chronicity.

In regard to retinal degeneration, our findings are in general agreement with the observations reported by Johnson.[45] They are not as severe, since only the bacillary layer and the "pigmented" epithelium

were involved, and no "rosettes" were formed. Johnson found that lesions involving the rod and cone layer were reversible, but that if there was involvement of the greater part of the outer nuclear layer or more, the damage was irreparable.[46] By fluorescence microscopy, vitamin A is localized in the bacillary and pigmented epithelial layers of the retina.[47]

Cystic atrophy of sebaceous glands and hair follicles was striking; whether these adnexal changes bear any relationship to the acquired alopecia, except perhaps in certain localized areas, is doubtful. Deficient rats, regardless of the specific deficiency, often exhibit a pica-like trichophagy[48] in an apparent attempt at diet supplementation. In any event, the skin changes bear no histologic resemblance whatsoever to such human conditions as Darier's disease, keratosis pilaris or keratosis follicularis, for which a relationship to vitamin A deficiency has been postulated.[49] This association between vitamin A and human hyperkeratosis was first suggested by applying the experimental findings of Wolbach and Howe[5] to cutaneous lesions in man.[50] However, not only did Wolbach and Howe confine their experimental observations in rats to the skin of the eyelid and base of the ear, but they also reported "no striking changes."[5] Hyperkeratinization of the epidermis does not occur in A-deficient rats.[51] The idea has, nevertheless, persisted in regard to human A deficiency, although hyperkeratotic skin lesions have not been produced in experimental A deficiency in man,[52] nor have they been reported in necropsied cases.[53] It is true that the latter, for the most part, consist of infants and children with fibrocystic disease of the pancreas. In these, because of squamous metaplasia at various sites, A deficiency was assumed to be present; therefore, they may not be valid examples. In fact, the microscopic description of the first necropsied human case of vitamin A deficiency[54] would lead one to believe that the patient not only had cystic fibrosis of the pancreas, but salivary gland inclusion disease as well. Interestingly enough, it is this complicated case which has been cited as being comparable to the disease in rats.[5]

Since cystic atrophy occurs in the skin adnexal structures, it is not surprising that the preputial gland, which embryologically is derived from the skin[55] and which normally contains vitamin A,[56] should take part. The peculiar evanescence of the perinuclear granules, however, is an interesting phenomenon, since the granules themselves ordinarily share some of the histochemical reactions of keratin[55] and might be expected to increase in prominence. Why the few remaining granules become PAS positive when ordinarily they are not stained by this method is also unexplainable. Except for the normal glandular size and the acinar

cell degranulation, the cystic atrophy resembles that seen following prolonged testosterone stimulation.[55]

It has long been recognized that vitamin A is in some way intimately concerned with reproduction.[57] The exact mechanism is unknown. The large corpora lutea, the lack of follicular development, and the stimulation of breast tissue which were seen in the germ-free animals may not be significant but are interesting in that they fall into an endocrinologic pattern, i.e., all of the changes could be brought about by any substance capable of producing a functioning corpus luteum. The reported occurrence of spontaneous deciduomas in A deficiency [58] is interesting in this same regard. On the other hand, although the appearance of ovarian deficiency cells is generally accepted as being due to a decreased output of pituitary gonadotropin,[59] the A-deficient pituitary has been said to contain an increased number of basophils [60] and to exert above normal gonadotropic activity.[61] If this is true, and if uterine squamous metaplasia follows avitaminosis A only when the epithelial cells are under the influence of estrogen,[62] what was its source and why was there no accompanying infiltration of the endometrial stroma with leukocytes? [63] At the present time it is impossible to account for all of these endocrinologic aberrations, since some are mutually exclusive.

The failure of either the germ-free or control animals to develop paralysis cannot be explained. According to Wolbach and Bessey,[64] if weanling rats were started on an A-deficient diet, paralysis almost invariably appeared between 6 and 9 weeks of age, generally beginning about the time weight gain fell below normal. It was thought that this paralysis was due to a mechanical phenomenon caused by disproportionate growth between the central nervous system and the skeletal system although no discernible changes could be demonstrated in the bone itself. Though probably not significant, it should, nevertheless, be mentioned in passing that during the early days of investigation, several dietary factors, including B_{12}, were unknown and that brewers' yeast frequently served as the only source of vitamin supplementation.

In still another category are lesions which have been reported in A deficiency, but which also occur in the nondeficient germ-free state—e.g., hemosiderosis of the liver and spleen, and renal calcinosis. It is impossible to tell whether the final effect in the A-deficient germ-free rat has been additive or synergistic. To be sure, the hemosiderosis is much more marked than that seen in A deficiency alone or in the occasional, generally older, germ-free animal killed at random. Renal calcinosis with hydronephrosis, on the other hand, was no more severe than that seen in germ-free rats in general, but did occur at a much younger age. In fact, renal calcification has even been reported in hyper-

vitaminosis A.[65] Lymphoid hypoplasia and dilatation of the cecum, of course, are products solely of the germ-free environment, and bear no relationship to A deficiency at all.

One of the more surprising aspects of the experiment was in regard to survival time. In the germ-free group 3 animals lived for 109 days and in the control group 4 rats lived for 108 days. In spite of the severe liver, kidney and adrenal lesions in the former, and the constant presence of suppurative infection in the latter, survival time was not appreciably altered, and, in fact, compared favorably with the 61 to 109 day survival originally reported by Wolbach and Howe for a similar group of young rats.[5]

Summary

In addition to the usual stigmas of vitamin A deficiency, the germ-free rats exhibited severe hepatic, renal, and adrenal lesions, which were not present in conventional rats on an identical diet. In other organs, the lesions in the germ-free group resembled those in non-germ-free animals, but were modified by the absence of infection and inflammation, so that atrophy, degeneration, and necrosis became the predominant features of the disease, although focal keratinizing metaplasia did occur. Death supervened independently of the germ-free state, and survival time was not increased.

References

1. Boynton, L. C., and Bradford, W. L. Effect of vitamins A and D on resistance to infection. *J.Nutrition*, 1931, **4**, 323–329.
2. Tyson, M. D., and Smith, A. H. Tissue changes associated with vitamin A deficiency in the rat. *Am. J. Path.*, 1929, **5**, 57–70.
3. Green, H. N., and Mellanby, E. Vitamin A as an anti-infective agent. *Brit. M.J.*, 1928, **2**, 691–696. Cited by Thatcher and Sure.[13]
4. Clausen, S. W. The pharmacology and therapeutics of vitamin A. *J.A.M.A.*, 1938, **111**, 144–154.
5. Wolbach, S. B., and Howe, P. R. Tissue changes following deprivation of fat soluble A vitamin. *J. Exper. Med.*, 1925, **42**, 753–777.
6. Trexler, P. C. The use of plastics in the design of isolator systems. *Ann. New York Acad. Sc.*, 1959, **78**, 29–36.
7. Reyniers, J. A.; Trexler, P. C., and Ervin, R. F. Germ-free Life Studies. Lobund Reports No. 1. University of Notre Dame Press, Notre Dame, Ind., 1946, pp. 62–63.
8. Gustafsson, B. Germ-free Rearing of Rats, General Technique. Berlingska Boktryckeriet, Lund, Sweden, 1948. *Acta path. et microbiol. scandinav.*, 1948, Suppl. 73, 130 pp.
9. Wagner, M. Determination of germ-free status. *Ann. New York Acad. Sc.*, 1959, **78**, 89–101.

10. LEVENSON, S. M.; MASON, R. P.; HUBER, T. E.; MALM, O. J.; HOROWITZ, R. E., and EINHEBER, A. Germfree animals in surgical research. *Ann. Surg.*, 1959, **150**, 713–730.

11. LILLIE, R. D. Histopathologic Technic and Practical Histochemistry. The Blakiston Co., Inc., New York, 1954, ed. 2, 501 pp.

12. LILLIE, R. D., and ASHBURN, L. L. Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degenerations not shown by Herxheimer technic. *Arch. Path.*, 1943, **36**, 432–435.

13. THATCHER, H. S., and SURE, B. Avitaminosis. III. Pathologic changes in tissues of the albino rat during early stages of vitamin A deficiency. *Arch. Path.*, 1932, **13**, 756–765.

14. WOLBACH, S. B., and BESSEY, O. A. Tissue changes in vitamin deficiencies. *Physiol. Rev.*, 1942, **22**, 233–289.

15. ZIMMERMAN, H. M. Lesions of the nervous system in vitamin deficiency. I. Rats on a diet low in vitamin A. *J. Exper. Med.*, 1933, **57**, 215–228.

16. WOLBACH, S. B., and HOWE, P. R. The incisor teeth of albino rats and guinea pigs in vitamin A deficiency and repair. *Am. J. Path.*, 1933, **9**, 275–294.

17. FOLLIS, R. H., JR. The Pathology of Nutritional Disease. Physiological and Morphological Changes Which Result from Deficiencies of the Essential Elements. Amino Acids, Vitamins and Fatty Acids. Charles C Thomas, Springfield, Ill., 1948, 291 pp.

18. WOLBACH, S. B. Effects of Vitamin A Deficiency in Human Beings. In: The Vitamins, Chemistry, Physiology and Pathology. SEBRELL, W. H., and HARRIS, R. S. (eds.). Academic Press, Inc., New York, 1954, Vol. 1, pp. 137–163.

19. FOLLIS, R. H. Deficiency Disease. Charles C Thomas, Springfield, Ill., 1958, 600 pp.

20. REYNIERS, J. A. The pure-culture concept and gnotobiotics. *Ann. New York Acad. Sc.*, 1959, **78**, 3–16.

21. BEAVER, D. L. Der Einfluss verschiedener Fixierungsmittel auf das Strukturbild der Präputialdrüsen der Ratte. *Ztschr. Zellforsch.*, 1959, **51**, 88–96.

22. ADDISON, W. H. F. The cell-changes in the hypophysis of the albino rat, after castration. *J. Comp. Neurol.*, 1917, **28**, 441–462.

23. ELLISON, E. T., and WOLFE, J. M. The effect of castration on the anterior hypophysis of the female rat. *Endocrinology*, 1934, **18**, 555–575.

24. COX, A. Magenveränderungen bei Ratten bei A-Avitaminose. *Beitr. path. Anat.*, 1937, **98**, 362–374.

25. MOORE, C. R.; HUGHES, W., and GALLAGHER, T. F. Rat seminal-vesicle cytology as a testis-hormone indicator and the prevention of castration changes by testis-extract injection. *Am. J. Anat.*, 1930, **45**, 109–135.

26. SELYE, H.; COLLIP, J. B., and THOMSON, D. L. On the effect of the anterior pituitary-like hormone on the ovary of the hypophysectomized rat. *Endocrinology*, 1933, **17**, 494–500.

27. POPPER, H., and GREENBERG, R. Visualization of vitamin A in rat organs by fluorescence microscopy. *Arch. Path.*, 1941, **32**, 11–32.

28. POPPER, H. Distribution of vitamin A in tissue as visualized by fluorescence microscopy. *Physiol. Rev.*, 1944, **24**, 205–224.

29. FITE, G. L. The pathology of dietary liver necrosis—a preliminary report. *Ann. New York Acad. Sc.*, 1954, **57**, 831–838.

30. SALMON, W. D., and COPELAND, D. H. Liver carcinoma and related lesions in chronic choline deficiency. *Ann. New York Acad. Sc.*, 1954, **57**, 664–677.

31. WILSON, J. W. Hepatomas produced in mice by feeding bentonite in the diet. *Ann. New York Acad. Sc.*, 1954, **57**, 678–687.

32. ASHBURN, L. L. The effect of administration of pantothenic acid on the histopathology of the filtrate factor deficiency state in rats. *Pub. Health Rep.*, 1940, **55**, 1337–1346.

33. BOURNE, G. The role of vitamin C in the organism as suggested by its cytology. *Physiol. Rev.*, 1936, **16**, 442–449.

34. SURE, B.; THEIS, R. M., and HARRELSON, R. T. Vitamin interrelationships. I. Influence of avitaminosis on ascorbic acid content of various tissues and endocrines. *J. Biol. Chem.*, 1939, **129**, 245–253.

35. MAPSON, L. W., and WALKER, S. E. The synthesis of ascorbic acid in the rat deprived of vitamin A with and without addition of chloretone. *Brit. J. Nutrition*, 1948, **2**, 1–14.

36. LOCKWOOD, J. E., and HARTMAN, F. A. Relation of the adrenal cortex to vitamins A, B, and C. *Endocrinology*, 1933, **17**, 501–521.

37. FOLLIS, R. H., JR. Pathologic effects produced by deficiency of single metallic and nonmetallic elements. *Arch. Path.*, 1942, **34**, 451–468.

38. EVANS, H. M. The effects of inadequate vitamin A on the sexual physiology of the female. *J. Biol. Chem.*, 1928, **77**, 651–654.

39. BERN, H. A.; ALFERT, M., and BLAIR, S. M. Cytochemical studies of keratin formation and of epithelial metaplasia in the rodent vagina and prostate. *J. Histochem.*, 1957, **5**, 105–119.

40. BURRILL, M. W., and GREENE, R. R. Vitamin A and the vaginal response to sex hormones in the rat; estrogens. *Endocrinology*, 1941, **28**, 765–766.

41. GOLDBLATT, H., and BENISCHEK, M. Vitamin A deficiency and metaplasia. *J. Exper. Med.*, 1927, **46**, 699–707.

42. FOLLIS, R. H., JR.; ORENT-KEILES, E., and MCCOLLUM, E. V. The production of cardiac and renal lesions in rats by a diet extremely deficient in potassium. *Am. J. Path.*, 1942, **18**, 29–39.

43. ASHBURN, L. L., and LOWRY, J. V. Development of cardiac lesions in thiamine-deficient rats. *Arch. Path.*, 1944, **37**, 27–33.

44. DAFT, F. S.; ASHBURN, L. L., and SEBRELL, W. H. Biotin deficiency and other changes in rats given sulfanilylguanidine or succinyl sulfathiazole in purified diets. *Science*, 1942, **96**, 321–322.

45. JOHNSON, M. L. The effect of vitamin A deficiency upon the retina of the rat. *J. Exper. Zool.*, 1939, **81**, 67–89.

46. JOHNSON, M. L. Degeneration and repair of the rat retina in avitaminosis A. *Arch. Ophth.*, 1943, **29**, 793–810.

47. GREENBERG, R., and POPPER, H. Demonstration of vitamin A in the retina by fluorescence microscopy. *Am. J. Physiol.*, 1941, **134**, 114–118.

48. PAPPENHEIMER, A. M., and LARIMORE, L. D. The occurrence of gastric lesions in rats; their relation to dietary deficiency and hair ingestion. *J. Exper. Med.*, 1924, **40**, 719–732.

49. ALLEN, A. C. The Skin, a Clinicopathologic Treatise. C. V. Mosby Co., St. Louis, 1954, 1048 pp.

50. FRAZIER, C. N., and HU, C. K. Cutaneous lesions associated with a deficiency in vitamin A in man. *Arch. Int. Med.*, 1931, **48**, 507–514.

51. SULLIVAN, M., and EVANS, V. J. Nutritional dermatoses in the rat. XI. Vitamin A deficiency superimposed on vitamin B complex deficiency. *Arch. Dermat. & Syph.*, 1945, **51**, 17–25.

52. HUME, E. M., and KREBS, H. A. Vitamin A Requirement of Human Adults. An Experimental Study of Vitamin A Deprivation in Man. Medical Research Council, Great Britain. Special Report Series No. 264. His Majesty's Stationery Office, London, 1949, 145 pp.

53. BLACKFAN, K. D., and WOLBACH, S. B. Vitamin A deficiency in infants; clinical and pathological study. *J. Pediat.*, 1933, **3**, 679–706.

54. WILSON, J. R., and DUBOIS, R. O. Report of a fatal case of keratomalacia in an infant, with postmortem examination. *Am. J. Dis. Child.*, 1923, **26**, 431–446.

55. BEAVER, D. L. A re-evaluation of the rat preputial gland as a "dicrine" organ from the standpoint of its morphology, histochemistry and physiology. *J. Exper. Zool.*, 1960, **143**, 153–173.

56. WARD, R. J., and MOORE, T. 7-Dehydrosterol in the sexual organs of the rat. *Biochem. J.*, 1953, **55**, 295–298.

57. POPPER, H. Histologic distribution of vitamin A in human organs under normal and under pathologic conditions. *Arch. Path.*, 1941, **31**, 766–802.

58. BISHOP, K. S., and MORGAN, A. F. Occurrence of deciduomata in rats low in vitamins A and E. *Proc. Soc. Exper. Biol. & Med.*, 1928, **25**, 438.

59 SELYE, H. The effect of adaptation to various damaging agents on the female sex organs in the rat. *Endocrinology*, 1939, **25**, 615–624.

60. SUTTON, T. S., and BRIEF, B. J. Cellular changes in the anterior hypophyses of vitamin-A deficient rats. *Endocrinology*, 1938, **23**, 211–215.

61. SUTTON, T. S., and BRIEF, B. J. Physiological changes in the anterior hypophysis of vitamin A-deficient rats. *Endocrinology*, 1939, **25**, 302–307.

62. BO, W. J. Relationship of vitamin A deficiency and estrogen to induction of keratinizing metaplasia in the uterus of the rat. *Am. J. Clin. Nutr.*, 1957, **5**, 666–673.

63. SPECTOR, W. G., and STOREY, E. A factor in the oestrogen-treated uterus responsible for leucocyte emigration. *J. Path. & Bact.*, 1958, **75**, 387–398.

64. WOLBACH, S. B., and BESSEY, O. A. Vitamin A deficiency and the nervous system. *Arch. Path.*, 1941, **32**, 689–722.

65. BERDJIS, C. C. Late effects of hypervitaminosis A in the rat. Disturbance and retardation in the normal growth of offspring. *A.M.A. Arch. Path.*, 1958, **66**, 278–281.

I am deeply indebted to Ernest G. McDaniel of the Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, for his valuable assistance.

[*Illustrations follow*]

Legends for Figures

All illustrations are of tissues obtained from vitamin A-deficient germ-free rats. The photomicrographs were prepared from sections stained with azure A-eosin B.

Fig. 1. Skin of the buccal pouch. There is marked cystic atrophy of sebaceous glands and hair follicles. Note the lack of hyperkeratinization. × 18.

Fig. 2. Mammary gland. Acinar proliferation and apparent secretion are evident. × 120.

Fig. 3. Preputial gland. There are cystic atrophy and stasis of secretion. × 18.

Fig. 4. Preputial gland. Degranulation of acinar cells produces a resemblance to the ordinary sebaceous gland. Perinuclear protein granules are still present in some of the cells in the upper portion of the photograph. × 210.

Fig. 5. Salivary glands: parotid, upper left; sublingual, upper right; submaxillary, lower right. Atrophy and degeneration are accompanied by focal squamous metaplasia of the ducts. Note the total absence of inflammation and edema. × 18.

Fig. 6. Trachea. Mucosal glands exhibit cystic atrophy; the superficial epithelium is also atrophic. × 105.

Fig. 7. Lacrimal gland. Hyaline droplet degeneration is evident in the upper left. There is ductular transformation of an acinus (right) and early squamous metaplasia of a duct (lower right). × 335.

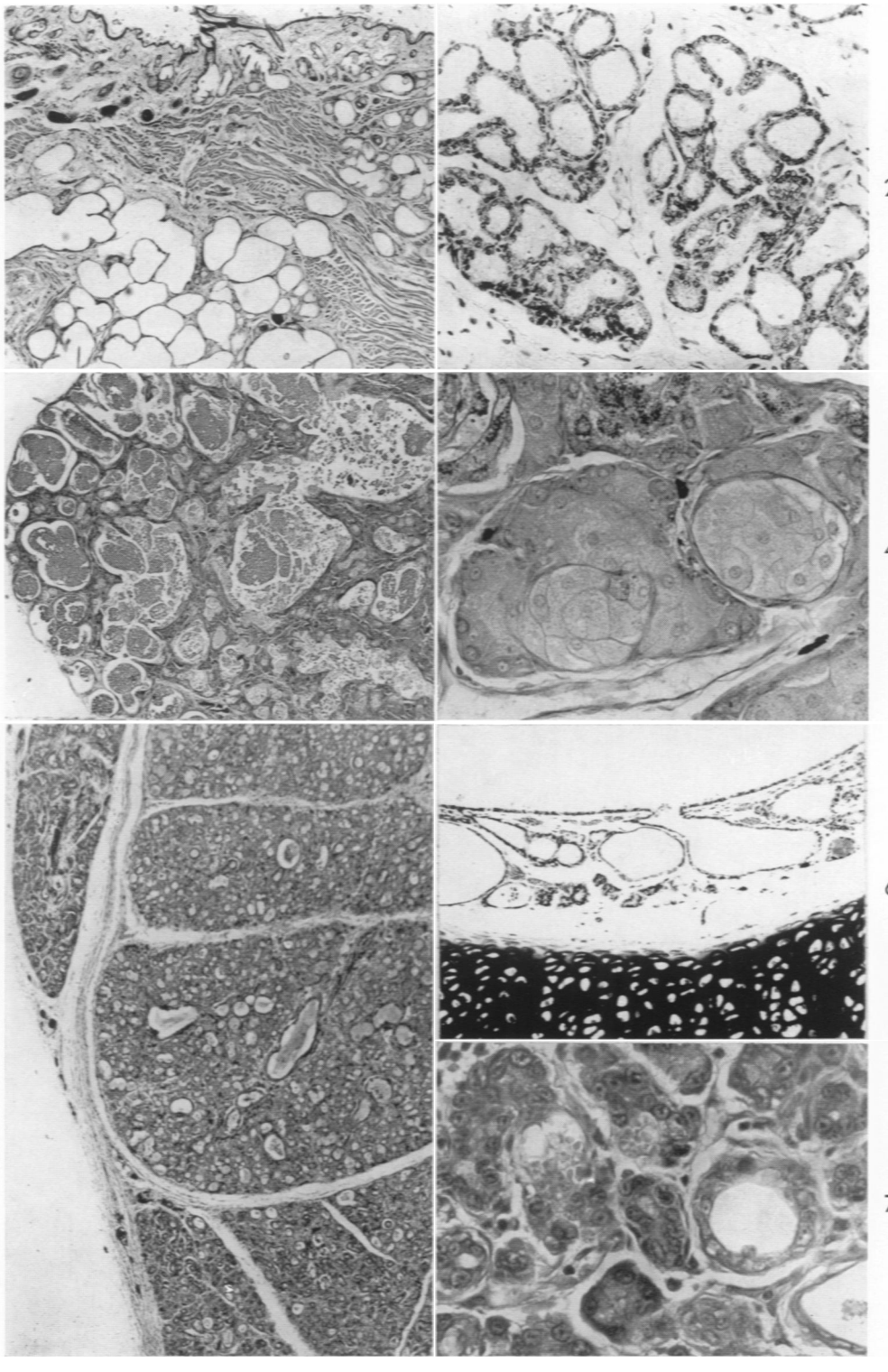
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FIG. 8. Lacrimal gland. Clumping of chromatin and karyorrhexis are manifest. × 435.

FIG. 9. Cornea. Superficial keratinization is associated with a complete lack of inflammation and vascularization. × 125.

FIG. 10. Eyelid. Keratinization of the palpebral conjunctiva appears on the left. There are atrophy and cystic dilatation of the meibomian glands. × 18.

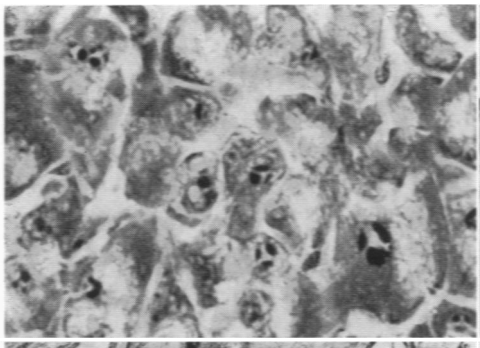
FIG. 11. Retina. Degeneration of bacillary layer and sloughing of "pigmented" epithelium are shown. × 125.

FIG. 12. The ventricular myocardium is the seat of focal necrosis. × 65.

FIG. 13. Trachea. Superficial squamous metaplasia and keratinization are unaccompanied by inflammation. The large dark-staining cells (left) are mast cells. × 250.

FIG. 14. Liver. Cytoplasmic hyaline droplets appear in parenchymal cells. × 775.

FIG. 15. Liver. Isolated cell necrosis is characterized by pyknotic stellate cells (off center) and karyorrhexis (lower right). Duct formation by hepatic parenchymal cells is also evident. × 250.



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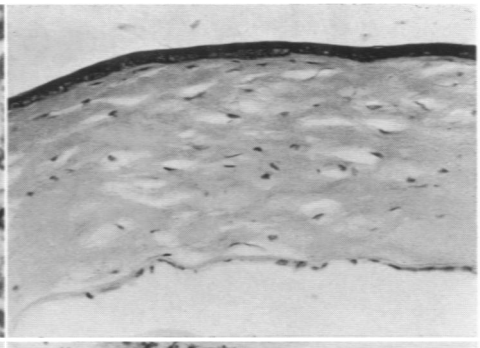

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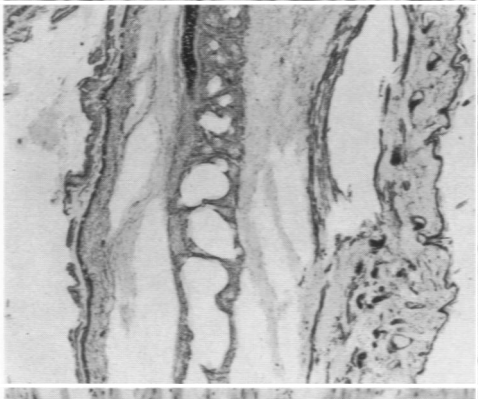

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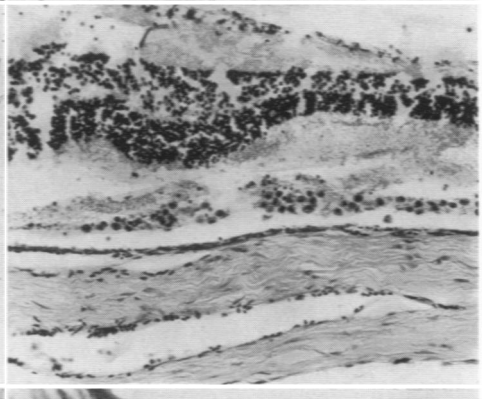

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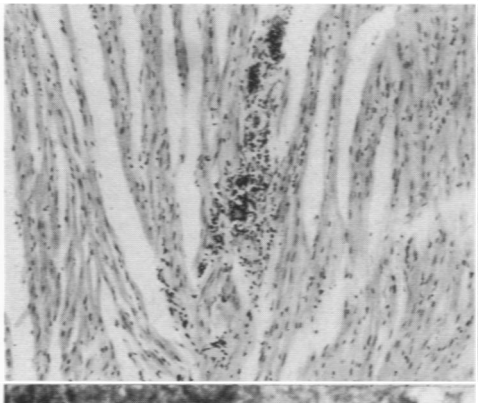

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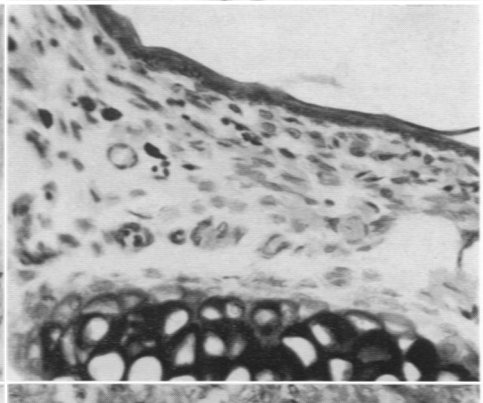

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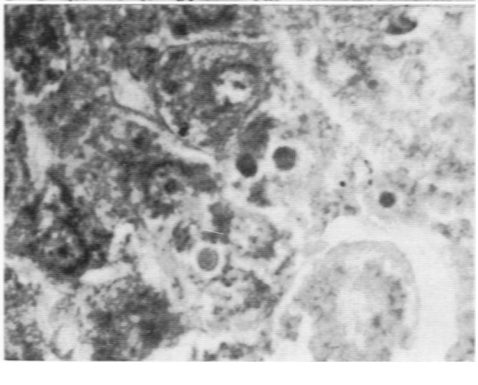

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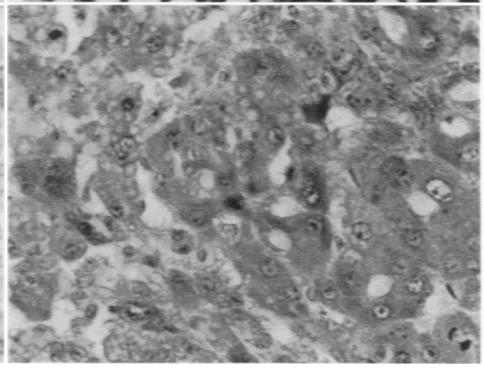

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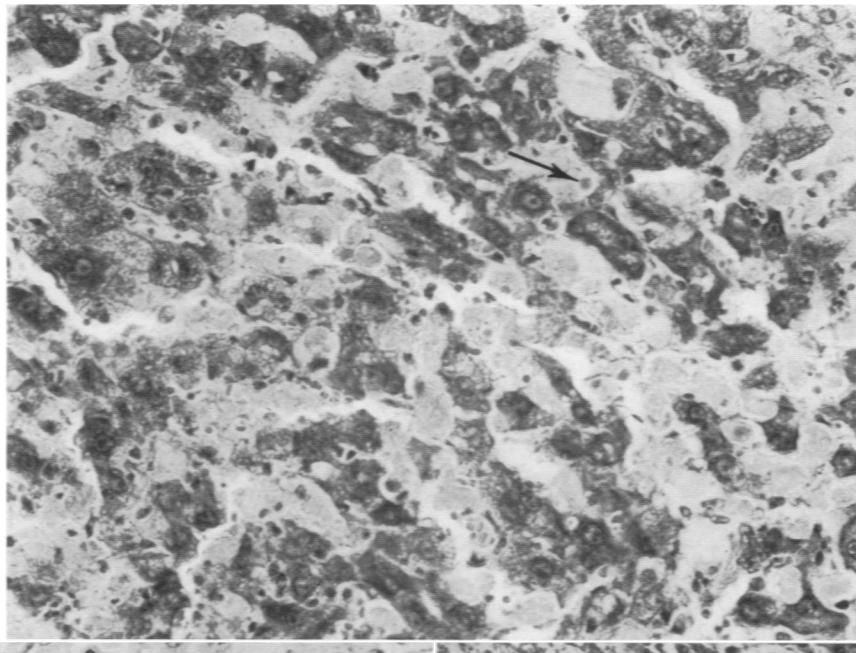
FIG. 16. Liver. There is extensive necrosis with apparently viable hepatic cells interspersed throughout. Occasional inclusion-like cytoplasmic hyaline droplets persist in necrotic cells (arrow). Compare with Figure 14. × 290.

FIG. 17. Liver. Ductlike structure appears to be formed by hepatic parenchymal cells. Note also the nuclear pleomorphism of adjacent cells. × 250.

FIG. 18. Liver, portal area. There is striking proliferation of bile duct epithelium. × 143.

FIG. 19. Liver. A higher magnification of same area shown in Figure 18. × 235.

FIG. 20. Common bile duct. Superficial squamous metaplasia and keratinization with parakeratosis are shown. × 130.



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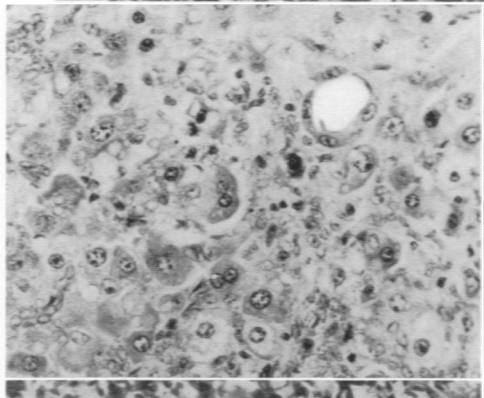

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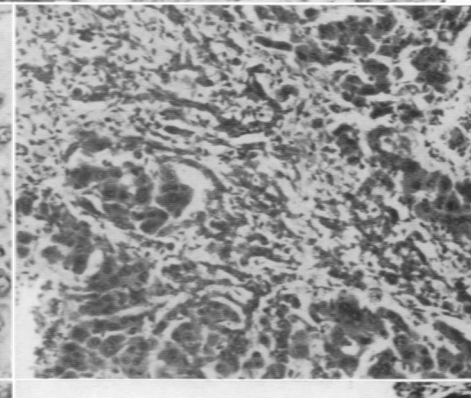

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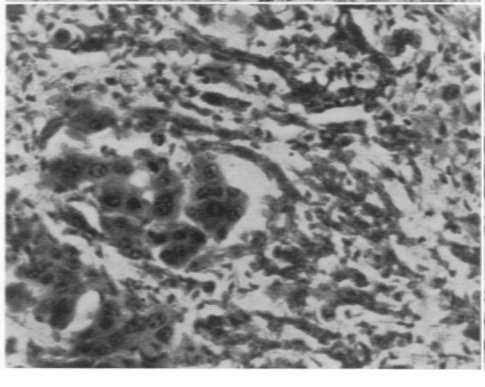

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FIG. 21. The adrenal cortex is the seat of hemorrhagic necrosis (left). × 65.

FIG. 22. The kidney shows focal cortical tubular degeneration and necrosis. × 125.

FIG. 23. Kidney. A collecting tubule contains a pigmented granular cast. × 250.

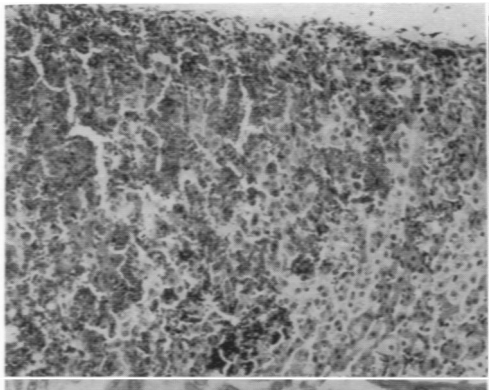
FIG. 24. The kidney exhibits calcinosis and hydronephrosis. There is a dilated calyx on the right. Note the absence of squamous metaplasia. × 18.

FIG. 25. A testis shows severe tubular degeneration and edema. The protein-rich fluid apparently has accumulated between the basement membrane and the interstitial tissue. × 50.

FIG. 26. The vagina is characterized by marked hyperkeratinization; there is no leukocytic infiltration. × 36.

FIG. 27. Uterus. A dilated keratinized endometrial gland exhibits a blocked duct and an intraluminal inflammatory exudate. Vacuolar degeneration has occurred in the superficial endometrium. × 110.

FIG. 28. Ovary. "Deficiency" cells are shown. × 775.



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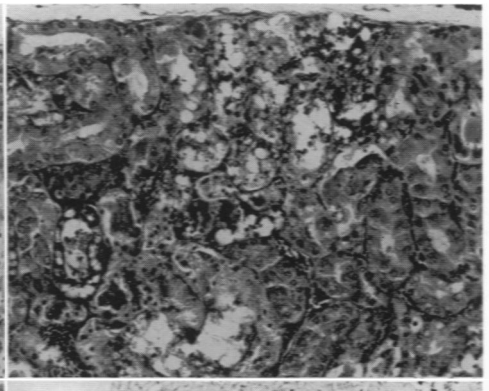

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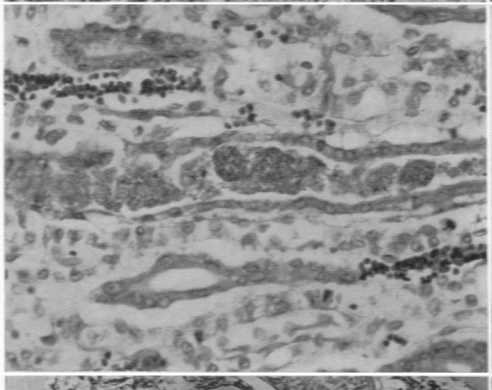

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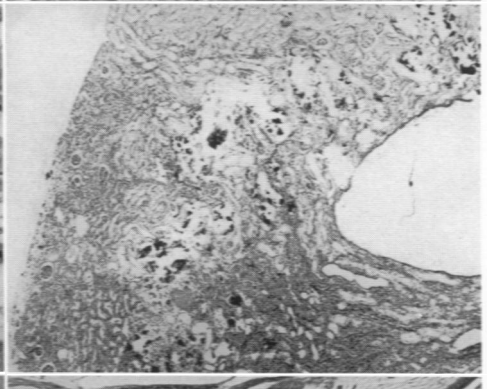

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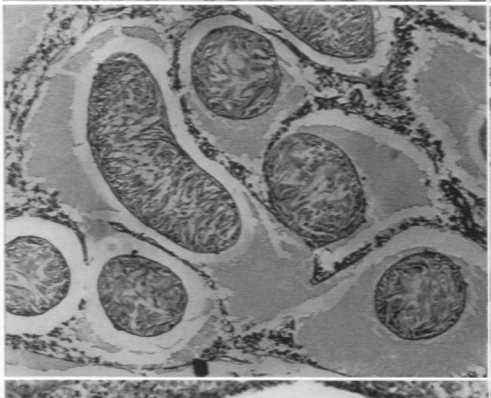

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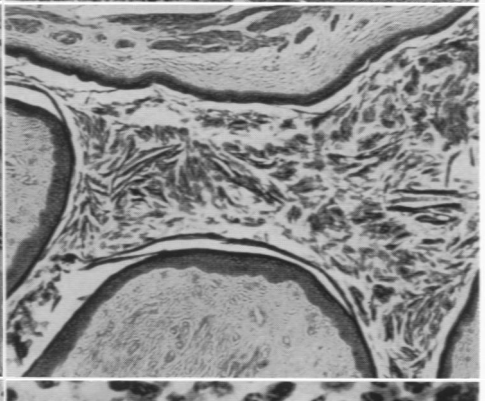

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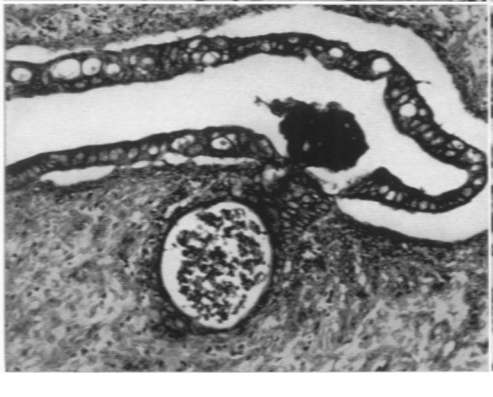

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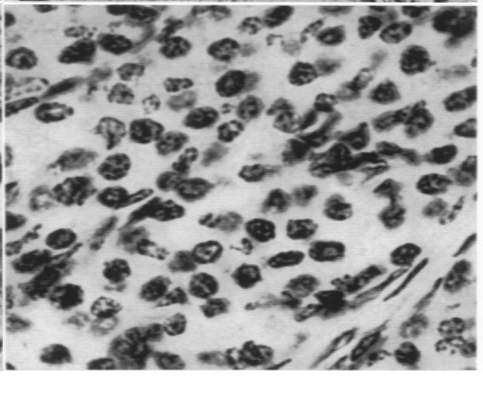

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