THE EFFECTS OF VITAMINS A AND E ON THE
BIOSYNTHESIS OF 17-OXOSTEROIDS

by

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INTRODUCTION

It has been well established that vitamins and steroid hormones are involved in the intermediary metabolism and various other essential vital processes in life. It is known that some of the vitamins are correlated with the biosynthesis of many other biologically active substances including steroids. The definite role played by the vitamins in this respect is still to be determined. It has been shown that changes in the concentration of the steroids are associated with many diseases and disorders. This abnormality can be verified by the isolation and identification of the steroids from the biological fluids through which they are excreted.

Thus, the assay of urinary excretion of the 17-oxosteroids has been extensively used in an endeavor to study diseases of the adrenal gland, and to differentiate tumors of the adrenals from hyperplasia of the organ. In many of these instances the 17-oxosteroid assay has proved to be of considerable value. In Addison's disease there was a decrease in the output of the 17-oxosteroids (32). Elevated 17-oxosteroid levels were seen in Cushing's disease (4).

Since about one-third of the 17-oxosteroids were derived from the testicular hormones, the assay for the 17-oxosteroid had been of some aid in diagnosing hypogonadism in the male (11). When there was an interstitial tumor of the testes the 17-oxosteroid output was usually markedly elevated (12).

The 17-oxosteroids coupled with the assay of the corticoste-

roids as performed by the 17-ketogenic steroid assay of urine,
furnished valuable information to the clinician in cases of adre-
nal gland imbalance (15).

However, during the past few years interest has grown in the
possibility that vitamin A has a function in the adrenal gland.
In 1955, Clark and Colburn (10) found that when cortisone was in-
jected into rats, the level of vitamin A decreased in the liver
and kidneys. There was also a decrease in vitamin A when Reich-
stein's compound was injected (7).

These experiments demonstrated some connection between the
function of an adrenal steroid and the storage or metabolism of
vitamin A. Hays and Kendall (22) injected progesterone into
vitamin A deficient rats, and found that it did not insure normal
growth, but restored the estrous cycle to normal. By the injec-
tion of pregnenolone it was found that it had no effect on the
estrous cycle, although it is known to be the precursor of pro-
gesterone (46). It was concluded that vitamin A acted in that
step. The foregoing considerations indicated a diversity of ap-
proach to the problem of the function of vitamin A. In spite of
this diversity, a few main areas are emerging. The most promising
one is in the biosynthesis of the adrenal steroids. This is main-
ly because of the basic importance for the maintenance of life, and
also because of the early involvement of the adrenals in vitamin A
deficiency. It may also be noted that both the adrenal steroid
biosynthesis and a vitamin A requirement are found in mammals only.

The adrenal glands have also been reported to be affected by
vitamin E deficiency. It was found that the action of vitamin E

was to some extent, comparable to that of ACTH from the anterior
pituitary in stimulating secretion of the corticosteroids (1).

Ingelman-Sundberg (27) studied genital function of guinea
pigs, fed a vitamin E deficient diet with or without supplementa-
tion of daily doses of vitamin E. Estrogens could not be demon-
strated in the urine of guinea pigs, but the excretion of the
17-oxosteroids and corticosteroids was similar in both cases.

From various sources of information it is also evident that
vitamins A and E are related in metabolism in several ways. The
liver vitamin A reserves were reported to be lowered in avitamino-
sis E (34). It was also shown that the depletion, or absorption
and storage, of vitamin A in the liver was somewhat dependent on
vitamin E. Optimum utilization of vitamin A or β-carotene is
dependent on an adequate amount of vitamin E. This effect has
often been referred to as "physiological synergism" and is in part
due to the action of vitamin E as a physiological antioxidant.
Vitamin E can affect the rate of growth in animals on low levels
of vitamin A, and the extent of liver deposition of vitamin A.
The beta carotene could produce two molecules of vitamin A more
often with adequate vitamin E. This was probably due to the anti-
oxidant properties of vitamin E (36). This antioxidant property
of vitamin E in the conversion of beta carotene was demonstrated
in chickens and turkeys by Kramke, et al. (29). Hence, it is
quite likely that vitamin E plays some role in the biosynthesis of
steroids.

The main purpose of the experiment was to determine the ef-
fects of avitaminosis A and E on the biosynthesis of the 17-oxo-

steroids. The effects on general health and growth were also
studied.

REVIEW OF LITERATURE

Among the many by-products of the process of metabolism which
are excreted in the urine are a group of steroid hormones which
possess a ketone group at carbon 17. These are designated as 17-
oxosteroids. The first 17-oxosteroid isolated was androsterone,
crystallized from male urine in 1931 by Butenandt (33). At pres-
ent there are more than 48 known compounds which can be grouped
under 17-oxosteroid classification (55). In the male, the 17-
oxosteroids arise from two organs, approximately one-third from
the testes and two-thirds from the adrenal cortex (33). The por-
tion arising from the testes are metabolites of the androgen,
testosterone. The portion arising from the adrenal cortex are
metabolites of the corticosteroids, and are secretory products of
the zona reticularis of the adrenal gland. In the female, the
17-oxosteroids arise from the adrenal cortex and the ovaries (33).

From the clinical point of view, the major 17-oxosteroids of
diagnostic importance are: androsterone, etiocholanolone, dehydro-
epiandrosterone, 11-hydroxyandrosterone, 11-hydroxyetiocholano-
lone, 11-oxoetiocholanolone and 11-oxoandrosterone (9). Besides
these naturally occurring compounds, artifacts of hydrolysis appear
quite often. These compounds are: 3 β-chlorodehydroepiandroste-
rone, and Δ 2(or 3)-androstene-17-one. TheΔ9, 11-dehydration prod-
ucts of the 11-oxygenated steroids are found in insignificant
amounts (31).

In early days, while working with adrenal extracts, it was
assumed by Hartman, et al. (20) that there was only one cortico-
steroid, "Cortin." Then investigations were being carried out by
numerous chemists in order to determine the active principle of
the corticosteroids. At present, at least 46 steroids have been
isolated from the adrenal cortex by Dorfman (14), and more recently
by Wettstein and Anner (50).

Owing to similarity in structure, it was assumed that choles-
terol was the precursor for all the steroids. First experimental
evidence for this was shown by Bloch (6). He isolated C^{14}-proges-
terone and pregnanediol after administering C^{14}-cholesterol to a
human subject. This was further proved by Kurath, et al. (30)
and by Werbin, et al. (49). Both groups isolated C^{14}-cortisol by
using C^{14}-cholesterol. It was demonstrated by Zaffaroni, et al.
(54) that cortisol and corticosterone might be produced quite
efficiently by using acetate as precursor. Probably this occurred
in two pathways, one involving cholesterol and the other going
around this step, as found by Bligh, et al. (5).

It was demonstrated by Gloor and Weiss (17) that the biosyn-
thesis of cholesterol and squalene was influenced by vitamin A
deficiency in the rat. They fed rats $2\text{-}C^{14}$-mevalonic acid during
the deficiency period, and detected 2 percent of the total radio-
activity of unsaponifiable material in squalene and 95 percent in
cholesterol.

As shown by Saba, et al. (37), the first step of conversion
of cholesterol was to Δ^5-pregnenolone. This was also proved by
Staple, et al. (42). The intermediates formed during this conver-

sion were probably 20β-OH-cholesterol and 20, 22-dihydroxycholes-
terol. The next step was the conversion of Δ^5-pregnenolone to
progesterone. There was also some evidence that synthesis of the
corticosteroids might proceed from acetate without going through
cholesterol (24). However, progesterone may undergo 11, 17, 18 or
21 hydroxylations. Of these, 11 β-hydroxylation is not to be con-
sidered as a major pathway. It is due to the fact that by using 11
β-OH-progesterone as substrate, corticosterone and cortisol were
produced by Eichhorn and Hechter (16) only in insignificant amounts.
Usually, the 11 and 18 hydroxylating enzymes are found in the mito-
chondria, and the 17 and 21 ones are found in the microsomes. The
hydroxylation reactions were studied by Stachenko and Giroud (41).
The 18 hydroxylation was found to occur in the zona glomerulosa,
the 17 hydroxylations in the zona fasciculata and reticularis, and
the 11 and 21 hydroxylations in the various zones of the adrenal
cortex.

Ungar and Dorfman (45) showed that androgens were produced in
significant amounts by the adrenal cortex. They incubated the
adrenal tissue with C^{14}-carboxyl-labeled acetate and cholesterol,
and isolated: dehydroepiandrosterone, 3β-chloro-Δ^5-androstene-
17-one, Δ^5-androstene-3β, 17β-diol, etiocholane-3α-ol-17-one,
androsterone, and Δ^5-pregnene-3β, 20α-diol.

Another main aspect in the corticoid production is the role
of ACTH, secreted by the anterior pituitary. Its action was found
to be somewhere in between cholesterol and progesterone, and more
specifically in the scission of the side chain (43). Haynes and
Berthet (21) showed that ACTH stimulated the phosphorylase activity

which in turn produced more TPNH, and the latter produced more
steroids because of the many steps which required it. Another
hypothesis put forward by Hechter (23) was that ACTH might act by
increasing the entry of cholesterol or some other necessary sub-
stances into the mitochondria.

The biosynthesis of the corticosteroids from progesterone by
microorganisms is also of considerable importance. Thus Ophiobo-
lus herpotrichus, Cunninghamella blakesleeana or Currvularia
lunata, Cephalothecium roseum were used for hydroxylation of pro-
gesterone at 21, 11β , and 17α positions respectively (24).

However, complete oxidative removal of the side chain in cho-
lesterol and progesterone will lead to the formation of the C_{19}
steroids, androgens. The principal naturally occurring androgens
are: androsterone, dehydroepiandrosterone and testosterone (28).
Testosterone was first isolated from the testes; the other two to-
gether with a large number of related substances have been obtained
from urine. It was first thought that the testes produced only
one hormone, testosterone (35). But the concept was further ad-
vanced by Vidgoff, et al. (47) that the testes secreted more than
one hormone. Dominguez (13) incubated a testicular tumor from a boy
with progesterone-4-C^{14} /n~-hydroxyprogesterone-4-C^{14}, and isolated:
4-pregnene-17 α , 20 α-diol-3-one, 4-pregnen-17α , 20 β -diol-3-one,
6β -hydroxyprogesterone, 11-deoxycorticosterone, testosterone, 4-
androstene-3, 17 dione, progesterone, and 4-pregnene-20 α -ol-3-
one. Chromic acid oxidation of dibromocholesteryl acetate yielded
after several steps and among other products, Δ^5-androsten-3β -ol-
17-one (dehydroepiandrosterone). Previously, this reaction was an

important avenue of approach to the partial synthesis of steroids.

Certain microorganisms might cause removal of the steroid side chains (24). Thus Penicillinum and Aspergillus species were used for microbiological oxidation of progesterone and of various 17-hydroxysteroids to Δ^4-androstene-3, 17-dione, testosterone, and testolactone. Pseudomonas testosteroni was used for reversible reduction of androstenedione to testosterone and its reduction to dehydroepiandrosterone. The reduction of androstenedione to etiocholan-17β -ol-3-one was possible by Bacillus putrificus.

However, in mammals, androgens are produced mainly by the adrenal cortex, the testes and the ovaries. While the production of androgens by the adrenals is controlled by ACTH of the anterior pituitary, the gonadal biosynthesis of androgens is regulated by the gonadotropic hormones of the pituitary and placenta. Probably, as in the case of adrenocortical hormones, cholesterol is not an obligatory precursor of testicular androgens.

There was ample evidence that the ovary became capable of liberating effective amounts of androgen during unusual or abnormal circumstances (44). It was reported and confirmed that ovarian homotransplants persisting in the ears of orchidectomized mice and rats frequently secreted sufficient androgens to maintain the vesicular glands and prostates in a normal condition. Although it was known that progesterone evoked certain androgenic effects, there was strong evidence by Hill and Strong (25) that the action of ovarian grafts was due to another androgen similar to that produced by the testes.

The 17α -hydroxylation and the side chain splitting were not

the rate-limiting steps in the biosynthesis of androgens in the normal animal, as both were reduced in the cryptorchid animal (26). Yet the seminal vesicles were slightly larger than normal, indicating a greater output of androgenically active substances.

The necessary steps for the biosynthesis of the corticosteroids were demonstrated by Van Dyke (46), as shown in figure 1. Similarly, the steps for the biosynthesis of the androgens were demonstrated by Heftman and Mosettig (24), as shown in figure 2. Important neutral 17-oxosteroids were determined and isolated from human urines by Mason and Engstrom (33), as shown in table 1.

However, the present experiment was concerned with the 17-oxosteroids, which are excreted in the urine as metabolites of the corticosteroids and the androgens, and the effects produced by avitaminosis A and E in this regard.

EXPERIMENTAL PROCEDURES

Basal Diet

A modification of a semi-purified vitamins A and E deficient diet used by Wooley (52), Wolf, et al. (51), Greenberg, et al. (18 and 19), Brown and Sturtevant (8), and Sherman and Trupp (40) was employed, and consisted of the following:

Ingredients	gms./1000 gms. diet
Casein (vitamin free)	350.00
Lard (molecularly distilled)	250.00
Corn starch	80.00
Dextrose	80.00

Acetate

↓

Mevalonic acid

↓

Squalene

Estrogens

↑

Androstene- ← Dehydro- ← Cholesterol
dione   epiandrosterone

↓   ↓

11-OH-Androstenedione   Δ^5-Pregnenonolone

↓

Progesterone

17α-OH-Progesterone   Desoxycorti-
                            costerone

↓   11β-OH-Progesterone   ↓

17α-OH-Desoxycortico-   Cortico-
sterone   sterone

↓   ↓

17α-OH-Corticosterone   Aldosterone

Fig. 1

Biosynthesis of Corticosteroids

Δ^5-Pregnenolone

17α-OH-Pregnenolone   Progesterone

Dehydroepiandrosterone   17α-OH-Progesterone

     Androstenedione
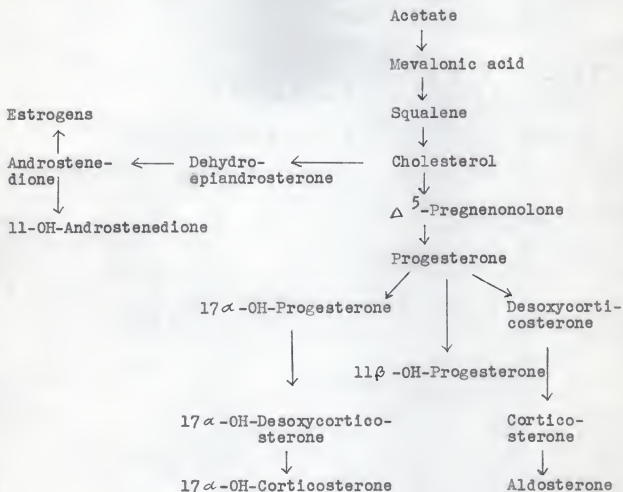
 Testosterone   11β-OH-Androstenedione
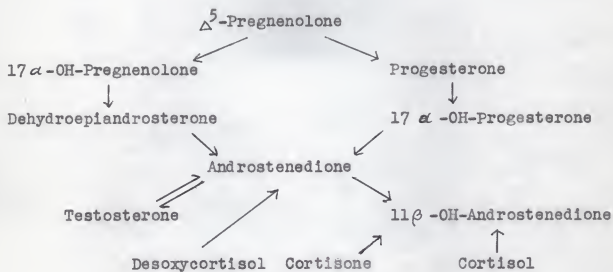
  Desoxycortisol Cortisone   Cortisol

Fig. 2

Biosynthesis of Androgens

Table 1. Important neutral 17-oxosteroids isolated from human
       urines.

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1. Androsterone (Androstan - 3 α-ol-17-one).

2. Etiocholanolone (Etiocholan-3 α-ol-17-one).

3. Dehydroepiandrosterone (Δ^5-Androsten-3 β-ol-17-one).

4. Isoandrosterone (Androstan-3 β-ol-17-one).

5. Androstanedione (Androstane-3, 17-dione).

6. Etiocholanedione (Etiocholane-3, 17-dione).

7. Androstenedione (Δ^4-Androstene-3, 17-dione).

8. 11-Hydroxyandrosterone (Androstane-3 α, 11 β-diol-17-one).

9. 11-Hydroxyetiocholanolone (Etiocholane-3 α, 11 β-diol-
       17-one).

10. 11-Ketoandrosterone (Androstane-3 α-ol-11, 17-dione).

11. 11-Ketoetiocholanolone (Etiocholan-3 α-ol-11, 17-dione).

12. $\Delta^{2(or\ 3)}$-Androsten-17-one.

13. $\Delta^{3,5}$-Androstadien-17-one.

14. 3-Chloro-Δ^5-Androsten-17-one.

15. $\Delta^{9,11}$-Androsten-3 α-ol-17-one.

16. Δ^{11}-Androsten-3 αol-17-one.

17. $\Delta^{9,11}$-Etiocholen-3 α-ol-17-one.

18. 1-Androsten-6-ol-17-one.

Sucrose	90.00
Briggs' salt mixture[a]	60.00
Linolenic acid	10.00
Potassium acetate	25.00
Magnesium oxide	5.00
Vitamin mix[b]	50.00
	1000.00

[a]Briggs' salt mix	%
Calcium carbonate	25.00
Dipotassium hydrogen phosphate	15.00
Disodium hydrogen phosphate	12.17
Tricalcium phosphate	23.33
Sodium chloride	14.67
Magnesium sulfate (hydrated 7$H_2$0)	8.33
Manganese sulfate (hydrated 7$H_2$0)	0.53
Potassium iodide	0.07
Zinc carbonate	0.03
Cupric sulfate	0.03
Ferrous sulfate	0.84

	mgs./100 gms. of vitamin mixture
[b]Thiamine hydrochloride	250
Riboflavin	150
Calcium pantothenate	400

Nicotinic acid	1000
Pyridoxine hydrochloride	60
Folic acid	40
2-methyl-1, 4-naphthoquinone	40
Cobalamin	0.5
Choline chloride	3000
Inositol	4000
p-Aminobenzoic acid	2400
Ascorbic acid	500
Vitamin D_3 (15000 units/gm.)	100
Biotin	6
Glucose (make the mix 100 gms.)	88093.5

Preparation of the Animals for Collection of Urine

Thirty-two weanling, albino male, 3 weeks old rats, purchased from Sprague-Dawley, Inc., Madison, Wisconsin, were used for the experiment. They were divided into 4 groups, comprising 8 in each group, and were subjected to a semipurified regimen for a period of three months as follows:

> Group I--Complete with all nutrients
> Group II--Deficient in vitamin A
> Group III--Deficient in vitamin E
> Group IV--Deficient in vitamins A and E

At the beginning of the experiment, all rats weighed 40-50 gms. Group I was fed the basal diet, supplemented with 120 I.U. vitamin A acetate and 0.3 mg. alpha tocopherol acetate per rat daily. Group II was fed the basal diet, supplemented with alpha tocopherol

acetate, 0.3 mg. per rat per day. Group III was fed the basal
diet, supplemented with vitamin A acetate, 120 I.U. per rat per
day. Group IV was fed the basal diet only. The diet was freshly
prepared every 7 days, and kept under refrigeration. The rats
were weighed every 3 days until the end of the feeding period for
30 days. The weights were recorded as shown in tables 2, 3, 4 and
5, and Plate I.

At the end of 30 days feeding, the animals were transferred
to metabolism cages for the collection of urine from each group.
A considerable amount of urine was collected also from a normal
bull for a comparative study. Several 48-hour specimens of urine
were collected from each rat, then combined for their respective
groups. All the specimens were preserved by the addition of a few
ml. of chloroform frozen and stored.

Hydrolysis and Extraction of Urine

The majority of the procedures followed in this investigation
were taken from the work of Watson and Besch (48).

Fifty ml. of urine were taken separately from each specimen.
To each aliquot was added 300 U./ml. of β-glucuronidase (from beef
liver). The pH was carefully adjusted to 4.5, and 1/10th volume
of acetate buffer (1.5 parts of 1 M aqueous acetic acid added to
3 parts of 1 M sodium acetate soln.) was added. All the urine
specimens were incubated at 37° C for 24 hours, permitting hydroly-
sis of the glucuronide-conjugated steroids. The incubated urines
were extracted with 2 x 1 volumes of ethyl acetate. The organic
phases were washed with 1/10th volume of 10 percent aqueous NaOH,

1/5th volume of NaHCO$_3$ soln. and 1/10th volume of water. They were dried over a small amount of anhydrous Na$_2$SO$_4$, the same being washed with ethyl acetate, and the washings added to the organic phases, which were evaporated to dry residues. The residues, containing the free and glucuronide-conjugated steroids, were transferred to test tubes for chromatography. The aqueous phases of the urines were acidified to pH 1 with HCL (about 15 percent) and boiled for 1 hour under a fume hood. They were cooled, and extracted with 1 x 1 and 2 x 1/2 volumes of ether. The combined ether phases were washed similarly as organic phases of urine, evaporated, and transferred to test tubes for chromatography.

Chromatographic Separation of Steroids

Chromatography was carried out as done by Zaffaroni, et al. (53), with some modification (2). The solvent system used for the purpose was ligroin/propylene glycol as shown by Savard (38 and 39). All the chromatograms were 35 cm. long and 2 cm. wide.

About 200 ml. of mobile solvent (ligroin, B. P. 65°-90°C) were placed in cylindrical chromatography chambers, allowing them to equilibrate over night. Standards were made by mixing 50γ each of androsterone, etiocholanolone and dehydroepiandrosterone in a test tube, and evaporating them to a dry residue. Chromatography papers were impregnated with propylene glycol: methanol (1:1), removing the excess solvent by blotting the papers between two sheets of filter paper. Both samples from the extractions were streaked on impregnated papers, dissolving the residues in acetone. While streaking, the spots were dried with the aid of a current

of dry nitrogen. Standards were spotted on a side limb for loca-
tion of the unknown steroids from the urines of the different
groups of rats. The papers were placed in the chambers for chro-
matographic separation of the steroids by the ligroin/propylene
glycol system for 12 hours. At the end of that period, the papers
were removed, dried, and the steroids identified by the modified
Zimmermann's reagent (3). For that, the paper strips were im-
mersed in a mixture of 1 percent of aqueous m-dinitrobenzene and
15 percent of aqueous KOH (2:1), removing the excess reagent by
thorough blotting. The colors were quickly developed by gentle
warming of the strips. The urine extracts were also chromato-
graphed in toluene/propylene glycol system for 4 hours.

Preparation of Histological Sections

After collection of the urine specimens, the rats were trans-
ferred to ordinary cages, while restricted to the same diets. On
the 15th day after this transfer, 2 rats died from Group IV; 4
died previously. Next day, the other 2 from that group, and 2 each
from other groups were sacrificed, autopsied, and the adrenals,
the testes, the gastrochnemius muscle and the sciatic nerve were
collected and preserved in 10·/. buffered formalin. Later, histo-
logical sections were made from the specimens, using hematoxylin
eosin stain. All the sections were examined under the microscope.

Recording Electroretinograms (ERG)

The vitamin A deficient group of rats was tested for night
blindness at the end of three months feeding. For this, electro-

retinograms were differentially amplified by a Tektronix Type 122 preamplifier, displayed on a Tektronix Type 502 oscilloscope, and photographed with a Tektronix C-12 polaroid camera. One each from the control and vitamin A deficient animals was prepared for recording sessions. The brain stem of the ether-anesthetized rat was severed at the mid-collicular level, and anesthesia was then discontinued. The rim of the bony orbit, and the eyelids were removed, and the eye muscles severed. After immobilization of the animals, 30 minutes were allowed for dark accommodation of the eye. The indifferent electrode was placed near the origin of the optic nerve, and the recording electrode* was placed on the center of the cornea in such a manner as to minimize interference of the light beam used to evoke the ERG. The duration of light flash was one second.

RESULTS AND DISCUSSION

Weights and Symptoms

The individual weights of each rat of Groups I to IV inclusive are shown in tables 2 to 5 respectively. The average weights of each group per unit of time are depicted in Plate I.

Table 2 shows the weights of the control (Group I) rats. The group grew normally, manifesting no abnormal symptoms. Except for the first 3 days, the group showed maximum rate of gain in weights. No animal died from this group.

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*The electrode consisted of a glass tube with a cotton wick in one end, and a chlorided silver wire in the other. The tube was filled with mammalian Ringer's solution. The wire was attached to the input of the preamplifier.

Table 3 shows the weights of the vitamin A deficient (Group II) rats. The group showed only a little less rate of gain in weights. There were indications of blindness and loss of appetite after two months of feeding. One animal died on the 70th day of feeding.

Table 4 shows the weights of the vitamin E deficient (Group III) rats. The group was apparently normal, manifesting no abnormal symptoms throughout the experiment. No animal died from the group.

Table 5 shows the weights of the vitamins A and E deficient (Group IV) rats. For the first 3 days, the group showed maximum rate of gain in weights, then minimum. The group was actually losing weight during the last 3 days. The animals started developing paresis and blindness from the third week. The syndrome was quite pronounced at the end of the fourth week. At first, they manifested a disturbance in the gait, which became slower and slightly incoordinated. Later, thetfleet of the hind legs were placed a little out to the side, and the gait became dragging. The muscular power of the hind legs was still good, and the group had a normal appetite. The function of the adductors of the thigh became impaired, and most conspicuous when the animals were taken out of the cage. In walking, the knees were extended abnormally. In moving about, the hind quarters were kept close to the floor. There was distinct atrophy of the musculature of the hind quarters and lower extremities. The hair coat became scanty. The fore legs were normal. The animals commenced to move slowly. While moving, they kept the hind legs greatly abducted, without

Table 2. Weights (gms.) of Group I (control rats) taken every three days, mentioned as wt. no. 1, wt. no. 2 etc.

Rats	Wt. no. 1	Wt. no. 2	Wt. no. 3	Wt. no. 4	Wt. no. 5	Wt. no. 6
No. 1	36	46	62	83	105	125
" 2	43	53	68	85	105	124
" 3	50	59	72	89	108	126
" 4	40	50	66	84	105	125
" 5	41	51	68	85	106	126
" 6	42	52	69	87	106	124
" 7	45	54	70	88	107	127
" 8	50	54	7_	90	110	129
Total	347	424	546	691	852	1006

Rats	Wt. no. 7	Wt. no. 8	Wt. no. 9	Wt. no. 10	Wt. no. 11
No. 1	141	160	180	199	218
" 2	140	161	177	194	214
" 3	141	160	177	198	215
" 4	140	160	178	196	216
" 5	143	160	177	198	215
" 6	140	161	178	197	214
" 7	142	160	177	197	217
" 8	143	160	182	201	219
Total	1130	1282	1426	1580	1728

Table 3. Weights (gms.) of Group II (vitamin A deficient rats)
taken every three days, mentioned as wt. no. 1, wt. no. 2
etc.

Rats	Wt. no. 1	Wt. no. 2	Wt. no. 3	Wt. no. 4	Wt. no. 5	Wt. no. 6
No. 1	43	57	71	87	107	124
" 2	40	54	70	86	106	123
" 3	43	57	72	87	106	124
" 4	46	60	74	90	108	124
" 5	43	57	71	87	108	125
" 6	44	58	72	88	110	125
" 7	50	64	80	94	112	125
" 8	42	54	72	88	107	122
Total	351	464	582	707	864	992

Rats	Wt. no. 7	Wt. no. 8	Wt. no. 9	Wt. no. 10	Wt. no. 11
No. 1	136	153	170	185	201
" 2	135	152	172	189	205
" 3	137	154	171	186	202
" 4	134	152	170	189	203
" 5	136	154	170	186	201
" 6	137	155	171	185	202
" 7	135	153	171	185	201
" 8	134	153	170	189	203
Total	1084	1226	1364	1494	1618

Table 4. Weights (gms.) of Group III (vitamin E deficient rats) taken every three days, mentioned as wt. no. 1, wt. no. 2 etc.

Rats	Wt. no. 1	Wt. no. 2	Wt. no. 3	Wt. no. 4	Wt. no. 5	Wt. no. 6
No. 1	41	56	70	91	109	126
" 2	44	59	72	91	109	127
" 3	46	60	72	89	110	126
" 4	48	62	75	90	112	127
" 5	47	61	73	89	110	128
" 6	46	60	73	87	111	129
" 7	42	57	70	88	109	126
" 8	47	61	74	90	110	127
Total	361	476	579	715	880	1016

Rats	Wt. no. 7	Wt. no. 8	Wt. no. 9	Wt. no. 10	Wt. no. 11
No. 1	143	161	179	195	213
" 2	144	162	180	199	217
" 3	143	162	180	197	214
" 4	144	165	181	200	218
" 5	144	162	180	179	216
" 6	145	161	177	192	212
" 7	144	160	178	194	213
" 8	143	161	179	196	215
Total	1150	1294	1434	1572	1718

Table 5. Weights (gms.) of Group IV (vitamins A and E deficient rats) taken every three days, mentioned as wt. no. 1, wt. no. 2 etc.

Rats	Wt. no. 1	Wt. no. 2	Wt. no. 3	Wt. no. 4	Wt. no. 5	Wt. no. 6
No. 1	48	63	73	90	105	119
" 2	44	61	72	88	104	119
" 3	45	60	72	89	105	120
" 4	46	62	73	89	106	120
" 5	42	59	72	90	107	121
" 6	42	60	71	88	105	121
" 7	47	62	74	91	108	122
" 8	44	60	71	86	104	120
Total	358	487	587	711	844	962

Rats	Wt. no. 7	Wt. no. 8	Wt. no. 9	Wt. no. 10	Wt. no. 11
No. 1	132	141	152	100	152
" 2	130	140	121	100	95
" 3	132	141	148	155	150
" 4	130	140	151	157	151
" 5	132	141	148	154	148
" 6	130	141	151	158	152
" 7	133	140	150	159	153
" 8	131	140	151	157	151
Total	1050	1124	1172	1200	1152

any adduction. The hind quarters were dragging along the floor.
The muscular atrophy increased, involving also the buttocks and
abdominal muscles. Loss of hair increased, forming large bald
spots here and there on the trunk and hind legs. The animals
showed inappetence, were not so lively, sleeping most of the time,
and moving about but little. They were almost unable to walk, and
preferred a reclining position. When they were forced to move,
they dragged the hind quarters along, supporting themselves on the
fore legs. They failed to stand on their feet, and rolled over on
the side. The sensibility appeared lowered, muscular atrophy be-
came extreme. Sometimes, they would step on their tails, or lie
on them for hours. They ate and drank very little. One fourth
(2 out of 8) of the animals died on the 40th, one half (4 out of 8)
on the 42nd, and three fourths (6 out of 8) on the 57th day of the
experiment, about 67 percent (4 out of 6) showing rectal hemorrhages.
On autopsy, hemorrhagic spots were found in the gastrointestinal
tract.

All of the above findings demonstrated the physiological syn-
ergistic effects of vitamins A and E. It also seemed that prob-
ably avitaminosis A and E together had some stimulating effects
initially on growth. The utilization of vitamins A and K appeared
much lowered in the absense of vitamin E, indicating the physio-
logical antioxidant property of the latter. Probably, the actual
depletion of the levels of vitamins A and E from the body took
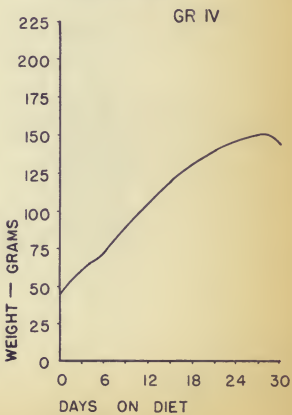about three weeks when Group IV started losing weight.
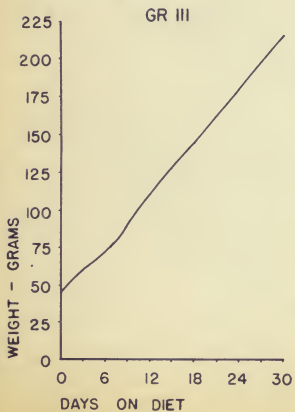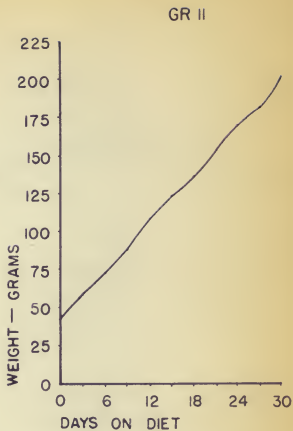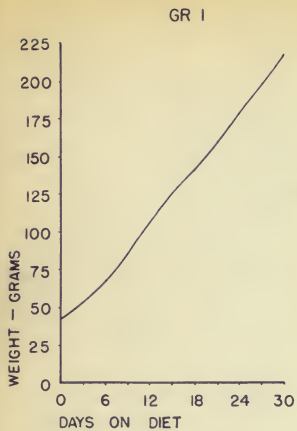
EXPLANATION OF PLATE I

Average weights (gms.) recorded every three days
for different groups of animals during the first 30
days of feeding experiments:

Group I--Diet complete with all nutrients

Group II--Diet deficient in vitamin A

Group III--Diet deficient in vitamin E

Group IV--Diet deficient in vitamins A and E

PLATE I

Isolation and Identification of 17-oxosteroids

Plate II depicts the 17-oxosteroids identified and determined from the different urine extracts. The most common 17-oxosteroids, androsterone, etiocholanolone and dehydroepiandrosterone were used as standards. These known steroids were chromatographed on the paper strip a. The urine extracts from the control, the vitamin E deficient, the vitamin A deficient, and the combined vitamins A and E deficient groups of rats were chromatographed on the paper strips b, c, d and e respectively. The urine extract from the normal bull was chromatographed on the paper strip f. The known steroids were first identified from the paper strip a by noting violet-colored spots for the 17-oxosteroids. The unknown steroids were then identified by a comparison of the violet-colored spots synchronously with those developed on the paper strip a. Thus the control group was found to produce all the three major 17-oxosteroids, androsterone, etiocholanolone and dehydroepiandrosterone. The vitamin E deficient group produced the steroids, androsterone and etiocholanolone. The vitamin A deficient group produced those two steroids in a lesser quantity. The combined vitamins A and E deficient group produced those two steroids in the least quantity. No dehydroepiandrosterone could be identified from the paper strips c, d or e. The bull was found to produce the steroids, androsterone and dehydroepiandrosterone in quite an appreciable quantity, but revealed no presence of etiocholanolone. On rough estimation from the different paper strips, the control group was found to produce a maximum quantity of steroids. The vitamin E deficient group produced less steroids. The vitamin A

deficient group produced still less steroids. The combined vitamins A and E deficient group produced the least quantity of steroids. The urine extracts were further chromatographed in toluene/propylene glycol system for four hours, the results being similar.

From the above findings, it was evident that the control animals did not lack in any of the major 17-oxosteroids produced by the adrenal cortex or the testes. Conversely, all the animals deficient in vitamins A or E separately, or together lacked in the steroids produced exclusively by the adrenal cortex. This demonstrated that vitamins A and E were necessary for the biosynthesis of the 17-oxosteroids. In other words, the deficiencies of these two vitamins affected the production of the 17-oxosteroids. The vitamin E deficiency affected it to the least extent. The vitamin A deficiency affected it to a greater extent. The vitamins A and E deficiencies together affected it to the greatest extent. Thus, the present findings gave evidence to the established fact that vitamins are correlated with the biosynthesis of many other biologically active substances including steroids.

The present findings gave evidences for some relation between the function of an adrenal steroid and the storage or metabolism of vitamin A (7 and 10). Although the actual mechanism of the functions of the adrenal steroids was not clear, the present findings might be correlated with those of Hays and Kendall (22). The amount of the 17-oxosteroids was found lowered more appreciably by the vitamin A than by the vitamin E deficiency, the former situation causing adrenal insufficiency. This gave evidence for the original findings of Lieberman, et al. (32). The quantity of the

Chromatography papers run in ligroin-propylene glycol system
for 12 hours for isolating and separating 17-oxosteroids (from
urine), the latter then identified and determined by the Zimmer-
mann reaction (with m-DNB and KOH), with the development of
violet-colored spots, indicating the presence of 17-oxosteroids:

    a. Paper strip showing the known 17-oxosteroids, androste-
       rone, etiocholanolone and dehydroepiandrosterone, used as
       standards;

    b. Paper strip showing the same steroids as above with dif-
       ferent concentrations, isolated from the urine of control
       rats;

    c. Paper strip showing the steroids, androsterone and etio-
       cholanolone, isolated from the urine of vitamin E defi-
       cient rats;

    d. Paper strip showing the same steroids as above with less
       concentrations, isolated from the urine of vitamin A de-
       ficient rats;

    e. Paper strip showing the same steroids as above with least
       concentration, isolated from the urine of vitamins A dnd E
       deficient rats;

    f. Paper strip showing the steroids, androsterone and dehy-
       droepiandrosterone, isolated from the urine of a normal bull

30

PLATE II

17-oxosteroids was found to be more in the vitamin E than in the vitamin A deficiency. This was due to the fact that the former situation, although causing testicular degeneration, also caused hypertrophy of the zona glomerulosa in the adrenal cortex. It might be correlated with the original findings of Birke, et al. (4). The determination of the urinary steroids was related with the original findings of Drektor, et al. (15). It was evident from the present findings that the action of vitamin E was to some extent similar to that of ACTH from the anterior pituitary (1).

Histological Changes

The control group appeared normal in all cases. The vitamin A deficient group appeared apparently normal, except some atrophy of the adrenal cortex. The vitamin E deficient group indicated moderate hypertrophy of the zona glomerulosa in the adrenal cortex. There was degeneration of the germinal epithelium in the testes along with loss of spermatocytes. In some cases, lysis and fusion of the mature spermatozoa, and spermatids assuming vesicular forms and disintegrations were also observed. The gastrochnemius muscle showed hyalinization and necrosis of the fibers. All these abnormalities were observed in much more pronounced form in the combined vitamins A and E deficient group, demonstrating the synergistic effects of vitamins A and E.

Electroretinograms (ERG)

Figures 5a and 5b depict the electroretinograms of the control and the vitamin A deficient rats respectively. The control

EXPLANATION OF PLATE III

Electroretinograms (ERG) recorded by cl2 Teϕktronic Pola-
roid camera, using silver-silver chloride Ringerswick elec-
trodes, the eyeball stimulated by the luminance of one second
flash, x to y, after allowing darkness for 30 minutes:

      a. Electroretinogram of a rat from the control
         group, showing a measurable ERG, indicating
         normal vision;

      b. Electroretinogram of a rat from the vitamin
         A deficient group, showing very little ERG,
         indicating blindness.
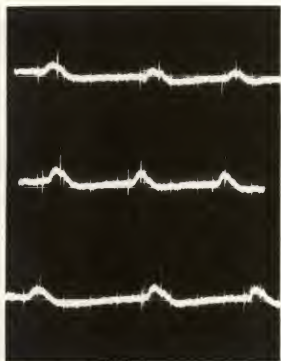
a   b

PLATE III

rat responded to the luminance of one second flash, x to y, indicating a measurable ERG. The vitamin A deficient rat did not so respond, indicating very little ERG. By a comparison of the two figures, it was evident that the vitamin A deficient rat was night blind, the control rat having normal vision.

The present findings thus gave evidence to the fact that vitamin A functions in certain photochemical changes occurring in the retina. The retinal receptors concerned in dim-light vision contain the pigment, rhodopsin. When exposed to light, rhodopsin breaks into a pigment, retinene, and a protein, opsin. During the reactions in the retina, some of the vitamin A or retinene is lost, and is replaced by vitamin A from the blood. If the blood level of vitamin A is nil or too low, a functional blindness will result. This was evident from the very little ERG, revealed by the vitamin A deficient rat, as compared to the control rat.

Thus, all the above findings demonstrated that vitamins A and E had synergistic effects on general health, growth and the bio-synthesis of the 17-oxosteroids, although not much effect by either alone. However, further study with more animals, and more known steroids, and investigations into the effects of recovery after supplementing the vitamins to the deficient animals in some future experiment would give more evidences for the present findings. The effects of vitamins A and E on the biosynthesis of the 17-oxosteroids would be more clarified thereby.

SUMMARY AND CONCLUSIONS

From the results of the present investigation, it was con-
cluded that vitamins A and E are involved in various essential
vital processes of life. The two vitamins are necessary for gen-
eral health, growth of animals, and the biosynthesis of the 17-
oxosteroids.

It was ascertained from the determination of the 17-oxoster-
oids that the deficiency of either vitamin A or E alone, or
together, affected the production of steroids by the adrenal cor-
tex. This effect was more pronounced when the diet was deficient
in vitamins A and E together rather than either alone. The quan-
tity of steroids production was found maximum by the control ani-
mals. The vitamin E deficient animals produced less steroids.
The vitamin A deficient animals produced still less steroids. The
combined vitamin A and E deficient group produced a minimum quan-
tity of steroids.

In regard to the effects of vitamins A and E on the general
health and growth of the animals, the deficiencies together had
much more pronounced effect than either alone. For instance, the
vitamin A deficient group started to show anorexia and blindness
after two months of feeding; the vitamin E deficient group was
apparently normal throughout the entire investigation. Converse-
ly, the combined vitamins A and E deficient group started to
manifest anorexia, blindness and paresis even from the third week.

Histological abnormalities of the adrenals, the testes and the
gastrocnemius muscle were much more evident in case of deficien-

cies of the vitamins A and E together rather than either alone. The above tissues from the vitamin A deficient group were apparently normal, except some atrophy of the adrenal cortex. The vitamin E deficient group showed moderate hypertrophy of the zona glomerulosa in the adrenal cortex, and degeneration of the testicular and muscular tissues. The combined vitamins A and E deficient group revealed the above abnormalities more prominently.

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THE EFFECTS OF VITAMINS A AND E ON THE
BIOSYNTHESIS OF 17-OXOSTEROIDS

by

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Physiology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1963

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———————————
Major Professor

The research was undertaken, mainly, to investigate the correlation of vitamins A and E with the biosynthesis of other biologically active substances, like the 17-oxosteroids. The general health and development of the animals were also studied.

Thirty-two weanling, albino, male rats of the Sprague-Dawley strain were subjected to a semipurified regimen for a period of three months as follows:

> Group I--Complete with all nutrients
> Group II--Deficient in vitamin A
> Group III--Deficient in vitamin E
> Group IV--Deficient in vitamins A and E

After 30 days on the regimen, the animals were transferred to metabolism cages. Urine specimen was collected separately from each rat, then combined in their respective groups, preserved by the addition of a few ml. of chloroform, frozen, and stored.

Urine specimens collected from different groups were chemically hydrolyzed and extracted. The 17-oxosteroids were separated from the urine extracts by paper chromatography in a ligroin/propylene glycol and a toluene/propylene glycol system. They were identified and determined from the chromatographic paper strips by a modified Zimmermann reaction.

From the results of this investigation, it was concluded that the control group of animals was not lacking in any of the major 17-oxosteroids. Conversely, all the groups deficient in vitamin A or E separately, or together, lacked some of the steroids produced exclusively by the adrenal cortex. The quantity of steroid

production was maximum by Group I. Group III produced less steroids. Group II produced still less steroids. Group IV produced a minimum quantity of steroids. This indicated that the biosynthesis of the 17-oxosteroids was affected by the deficiency of vitamin A or E, more being noted by that of vitamin A. The greatest deficiency of the steroids was noted when vitamins A and E were lacking together. This demonstrated stimulating effects of vitamins A and E on the biosynthesis of the 17-oxosteroids.

The health and body development of the control group were good, manifesting no abnormality. The vitamin E deficient group remained apparently normal. The vitamin A deficient group indicated anorexia and blindness after two months of feeding. The combined vitamins A and E deficient group started to show anorexia, blindness and paresis from the third week. The syndrome was quite pronounced at the end of the fourth week, demonstrating a synergistic relationship between vitamins A and E.

Histological abnormalities of the adrenals, the testes and the gastrochnemius muscle were more evident in vitamins A and E deficiencies together. The above tissues from the vitamin A deficient group were apparently normal, except for some atrophy of the adrenal cortex. A moderate hypertrophy of the zona glomerulosa of the adrenal cortex, and degeneration of the testicular and muscular tissues were observed in the vitamin E deficient group. The combined vitamins A and E deficient group revealed the abnormalities more prominently, demonstrating synergistic effects of these vitamins.