It is well known that vitamin A influences the differentiation of epithelial cells. Deficiency of vitamin A causes squamous metaplasia of columnar epithelium (11, 13). Excess vitamin A causes conversion of some normally keratinizing epithelia to a columnar, secretory type (1, 9). In addition, vitamin A has been shown to influence the morphology of myxoviruses (4). In view of these effects, it was of interest to determine whether high doses of vitamin A would influence the development of the Shope rabbit papilloma, a virus-induced, epithelial neoplasm.

MATERIALS AND METHODS

1. GENERAL

Vitamin A was administered by i.p. injection 3 times per week to 28 young adult New Zealand white rabbits of both sexes and to 6 wild rabbits from Kansas. Control solutions were administered to corresponding numbers of New Zealand and wild rabbits. Observations of the following were made on the New Zealand rabbits: (a) the time to macroscopic appearance of papillomas (incubation period); (b) the height of the fleshy portions of the papillomas (consisting principally of viable, nonkeratinized epithelium with a small amount of vascularized connective tissue stroma); and (c) the presence or absence of permanent papilloma regression. Observations of the effect of vitamin A on virus titer were made on the wild rabbits.

The dosage of vitamin A used was high enough to cause toxicity, as indicated by substantial weight loss and mortality. The vitamin-treated rabbits lost an average of 27% of their initial weight in an average interval of 48 days, compared to a 1% weight gain for the control rabbits during the same period. The mortality was 55% for the vitamin-treated rabbits, with death occurring 7—96 days following start of treatment, compared with a 13% mortality for the control rabbits.

Treatment schedules were as follows:

Group I.—Two New Zealand rabbits, initially 2.0 and 2.7 kg each, received vitamin A palmitate (Aquasol A), 250,000 IU per injection, starting 15 and 23 days after virus inoculation, for 26 and 22 days respectively.

Group II.—Five New Zealand rabbits, initially 2.0 to 2.7 kg each, received, starting 26 days after virus inoculation, vitamin A palmitate (Aquasol A), 250,000 IU per injection for 27 days, followed by 150,000 IU/kg/injection for 16 days, followed by vitamin A alcohol, 144,000 IU/kg/injection for 27 days. (Total treatment duration was 39—70 days, depending on survival.)

Group III.—Five New Zealand rabbits, initially 3.1 to 3.6 kg each, received, starting 63—71 days after virus inoculation, vitamin A palmitate (Aquasol A), 250,000 IU per injection for 27 days, followed by vitamin A alcohol, 144,000 IU/kg/injection for 27 days. (Total treatment duration was 39—70 days, depending on survival.)

2 Vitamin A palmitate, made water soluble with 12% soyaethan oilate, manufactured by U. S. Vitamin and Pharmaceutical Corporation, New York, N. Y.

3 Vitamin A alcohol, crystalline, kindly supplied by Hoffmann-LaRoche, Inc., Nutley, N. J., was dissolved in 16.6% (w/w) aqueous polyoxyethylene sorbitan monoleate (Tween 80) to give a concentration of 14.1 mg of vitamin A alcohol/gm of solvent. The solution was then filtered with a Seitz filter and stored frozen in sealed ampules until used.

Inhibition of Growth of Shope Rabbit Papilloma by Hypervitaminosis A

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SUMMARY

Vitamin A retards the growth and delays the initial appearance of the Shope rabbit papilloma when administered systemically in high dosage. Such treatment, when continued long enough, causes atrophy of the fleshy portion of the papilloma. In some rabbits this may be followed by shedding of the keratinized portion of the papilloma and macroscopic disappearance of the tumor. When treatment is stopped, such tumors regress.

Microscopic examination of the tumors suggests that these effects are brought about either by acceleration of differentiation and keratinization of the epithelial papilloma cells or by inhibition of their mitotic rate.

Observations were made suggesting that excess vitamin A may cause a moderate increase in concentration of infective virus in papillomas grown on wild rabbits. It is thought that this effect could be due to the higher proportion of keratinized material in vitamin A-treated papillomas.

1 Supported in part by Grant 5-TI-CA-5022-08 and General Research Support Grant, Subcontract No. 1, both from the National Institutes of Health.

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inoculation, vitamin A alcohol, 233,000 IU per injection for 51 days, followed by 144,000 IU/kg/injection for an additional 43 days. (Total treatment duration was 24–94 days, depending on survival.)

**Group IV.**—Ten New Zealand rabbits, initially 2.4 to 3.2 kg each, received, starting 20 days after virus inoculation, vitamin A alcohol, 144,000 IU/kg/injection, for 31 days. (Treatment duration was 21–31 days, depending on survival.)

**Group V.**—Six New Zealand rabbits, initially 2.3 to 2.6 kg each, received, starting 1 day after virus inoculation, vitamin A alcohol, 144,000 IU/kg/injection, for 68 days. (Treatment duration was 43–68 days, depending on survival, except for 1 rabbit with immunologic regression of papillomas, where treatment was stopped after 38 days.)

**Group VI.**—Six wild cottontail rabbits obtained from Kansas, initially 1.0 to 1.2 kg each, received, starting 4 days before virus inoculation, vitamin A alcohol, 144,000 IU/kg/injection, for 46 days after virus inoculation. (Treatment duration was 27–46 days after virus inoculation, depending on survival.)

Control rabbits of corresponding number for each group received the following solutions instead of vitamin A.

**Group I.**—Corresponding volumes of physiologic saline solution.

**Groups II–VI.**—Where vitamin A palmitate (Aquasol A) was administered to the experimental rabbits, Aquasol A vehicle (kindly supplied by U. S. Vitamin and Pharmaceutical Corp.) was administered in corresponding volume. Where vitamin A alcohol solution was administered, a solution of identical composition, but lacking vitamin A, was administered in corresponding volume.

Virus was inoculated by rubbing aqueous extracts of pooled wild (Kansas) rabbit papillomas onto scarified skin sites measuring about 12 x 15 mm each. Four sites were inoculated on each rabbit of Groups I, II, IV, and VI; 6 sites were inoculated on each rabbit of Groups III and V. Each site in Groups I–IV and VI received an inoculum equivalent in incubation period to approximately 10^{9.0–10^{11}} virus particles by electron microscope count (C. Breedis, personal communication). On each rabbit of Group V, 2 sites received this dose, 2 sites received 0.1 as much virus, and 2 sites received 0.01 as much virus.

In the case of Groups I, II, and IV, treatment was begun when the papillomas were fleshy and had either no keratinization or scanty keratinization. The maximum diameters of the papillomas in these groups at the start of treatment ranged from a few mm to 27 mm, most being about 15 mm; the heights ranged from 1 to 8 mm, most being about 3 mm. In Group III, maximum diameters of the papillomas at the start of treatment ranged from 22 to 41 mm, averaging 28 mm. Neglecting papillomas which had been chewed, the heights of the papillomas in this group at the start of treatment ranged from 5 to 18 mm, averaging 10 mm. For Group III, these initial height measurements included variable amounts of keratin.

For each group, rabbits were assigned to receive either vitamin A or control solutions so that the distribution of sex, weight, and papilloma size was similar. An attempt was made to minimize chewing of papillomas by locating the papillomas on the backs of the rabbits and by bandaging. Some chewing nevertheless occurred, but to about equal degrees among experimental and control animals. Where a papilloma was significantly chewed, height measurements were thereafter omitted in the compilation of data for Chart 1.

### 2. Rabbit Diet

Rabbits were fed Rockland Rabbit Ration (distributed by Teklad, Inc., Monmouth, Ill.) and water ad libitum and were given a small quantity of green leafy vegetable twice a week.

In order to determine whether the papilloma atrophy observed in the rabbits treated with vitamin A might be a nonspecific effect occurring in association with the severe weight loss seen in these rabbits, a control study was made of the effect of weight loss induced by diet restriction.

Five New Zealand white rabbits bearing a total of 20 papillomas were placed on the above diet, restricted in quantity so that gradual weight loss resulted. Over a period of 49 days, the average weight loss was 25% (range, 24–30%). Six control rabbits bearing a total of 18 papillomas were maintained on the same diet, unrestricted in quantity. Over the 49-day period, their average weight gain was 12% (range, 3–19%). The heights of the fleshy portions of the papillomas of both groups were measured every few days.

### 3. Virus Extraction and Assay

After excision, the papillomas from the wild rabbits were trimmed of attached skin and subcutaneous tissue. The papillomas from any one animal were then pooled and extracted by grinding with sand in a mortar and pestle with the use of 7 ml of buffered normal saline/gm of papilloma tissue.

The extracts from 4 experimental and 3 control rabbits were assayed for relative virus titer by inoculating a standard dose of extract from each rabbit by scarification onto a test site on each of 6 New Zealand white rabbits. The incubation periods of the papillomas growing on the test sites were determined. As the incubation period is an inverse function of the dose of virus (2), the incubation periods were used as an index of virus concentration.

### 4. Vitamin A Assays

Blood samples were obtained by cardiac puncture from 4 experimental rabbits and 4 control rabbits of Group V. Serum from these samples was analyzed for vitamin A by the method of Bessey et al. (3) modified for the use of larger blood samples.

### 5. Histology

All sections were prepared by routine paraffin embedding, cut at 6 μ, and stained with hematoxylin and eosin.

**RESULTS**

Regardless of the age of the papilloma or the type of vitamin A preparation employed, a reduction in the height of the fleshy portion of the papillomas resulted in the rabbits treated with vitamin A. This decrease in fleshy height is shown in Chart 1. For each duration of treatment indicated in Chart 1, the mean fleshy height of the
keratinized portion was shed. Microscopic examination of such sites showed that viable papilloma tissue remained. This is seen by comparing Fig. 7 (tumor site) and Fig. 8 (skin). The persistence of tumor was further demonstrated by the fact that papillomas regrew on all such sites when vitamin A treatment was stopped.

That this change in fleshy height of the papillomas was not a result of weight loss due to limited food intake by the rabbits receiving vitamin A is shown by the absence of papilloma atrophy in the rabbits placed on the restricted diet without vitamin A treatment. Papilloma atrophy was well established after 6–7 weeks of vitamin treatment (statistically significant papilloma atrophy compared with the untreated controls, \( P < 0.001 \)). By contrast, after a similar time period there was not even a suggestion of papilloma atrophy among the papillomas of the diet-restricted rabbits compared with the papillomas of their unrestricted diet controls (Chart 2).

A distinction must be made between papilloma atrophy induced by excess vitamin A and papilloma regression, which occurs spontaneously in about 25% of New Zealand white rabbits (6). The atrophy induced by vitamin A is reversible and dependent on continued administration of

**Chart 1.**—Effect of vitamin A on height of fleshy portion of papilloma. Data are combined from Groups II–V. For each time interval the mean height of the papillomas on the vitamin A-treated rabbits differs statistically from the mean height of the papillomas on the control rabbits \( (P < 0.001) \).

The change in appearance of the papillomas is seen by comparing Fig. 1 (vitamin-treated rabbits) with Fig. 2 (control rabbits). In the case of 2 rabbits of Group II, 2 rabbits of Group III, and 1 rabbit of Group IV, vitamin A treatment resulted in macroscopic disappearance of all of the fleshy portions of the papillomas before the animals died or treatment was stopped. This degree of change is illustrated in Figs. 3, 4, which show a shed keratinized portion of papilloma and the tumor site from which the papillomas on rabbits treated with vitamin A differs statistically from the mean fleshy height of the papillomas on the control rabbits \( (P < 0.001) \).

**Chart 2.**—Failure of restricted diet to affect height of fleshy portion of papilloma. Average weight loss of rabbits on restricted diet during test period was 26%. Average weight gain of rabbits on unrestricted diet was 12%.
vitamin A. Spontaneously occurring regression almost always results in permanent disappearance of the papilloma and is generally conceded to be brought about by an immunologic mechanism (6, 8). Measurements of papillomas showing permanent regression have been omitted from the data shown in Chart 1. Except for rabbits showing such permanent immunologic regression of papillomas, an increase in fleshy height of papillomas occurred after vitamin A treatment was stopped. This increase was observed within a few days of the last dose and continued until papillomas of normal appearance resulted.

The incidence of immunologic regression was unaffected by vitamin A treatment. Combining the data of all the domestic rabbits, of 17 rabbits treated with vitamin A which lived 90 days or longer after virus inoculation, 3 showed complete regression of all papillomas. Papillomas did not return on these rabbits after vitamin treatment was stopped. Of 24 corresponding rabbits treated with control solution, 3 showed similar permanent papilloma regression. These regression frequencies are less than expected because of the inclusion of the Group III rabbits, on which the papillomas were already about 2 months old at the start of the experiment. Spontaneous regression occurs infrequently beyond this period.

The histologic changes caused by vitamin A consisted of shortening and ultimate disappearance of the fronds of viable papilloma tissue and thinning of the nonkeratinized epidermal portion of the papilloma. These changes are illustrated in Fig. 5 (compare with Fig. 6) and in Fig. 7. No change was noted in the histologic appearance of the keratin. No qualitative change was noted in the stroma. A decrease in amount of stroma occurred in association with the shortening and eventual loss of the papillary fronds.

The virus assay data suggest that vitamin A-treated papillomas contain a somewhat higher concentration of infective virus than control papillomas. Papillomas grew at all 24 sites inoculated with extracts of papillomas from the wild rabbits treated with vitamin A. Papillomas grew at 15 of 18 sites inoculated with extracts from the control wild rabbit papillomas. The incubation periods of the papillomas arising on sites inoculated with extracts from vitamin A-treated papillomas tended to be shorter than those of papillomas arising on sites inoculated with the control extracts. This comparison is shown in Chart 3.

The data of Table 1, based upon the time of appearance of papillomas in the Group V rabbits, show that vitamin A prolongs the incubation period by a small but statistically significant amount when concentrated virus inocula are used.

Serum vitamin A levels are shown together with height measurements of fleshy papilloma in Table 2 for 4 vitamin-treated rabbits of Group V. The sera were obtained after papilloma atrophy was well established. Peak values, obtained 2 hr after vitamin injection, ranged from 46 to 223 μg/100 ml. (Pilot determinations on 2 rabbits which were not in the experimental series indicated that the serum vitamin A level is maximal approximately 2 hr after i.p. injection of vitamin A for the dose used with the rabbits of Group V.) Samples drawn 48 and 72 hr

### TABLE 1

<table>
<thead>
<tr>
<th>Dilution of virus inoculum</th>
<th>Treatment</th>
<th>Mean incubation period (days)*</th>
<th>S.D. of incubation period (days)</th>
<th>Difference of means (vitamin A minus control)</th>
<th>S.E. of difference of means</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>Vitamin A</td>
<td>15.75</td>
<td>1.89</td>
<td>2.33</td>
<td>0.97</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>13.42</td>
<td>1.62</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:10</td>
<td>Vitamin A</td>
<td>20.08</td>
<td>3.58</td>
<td>4.50</td>
<td>1.77</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>15.58</td>
<td>2.44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td>Vitamin A</td>
<td>25.08</td>
<td>6.75</td>
<td>1.66</td>
<td>3.26</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>23.42</td>
<td>4.30</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Calculated by averaging the means of the incubation periods for each rabbit for that inoculum strength.

b d. f., degrees of freedom.
after injection showed vitamin A levels in the range of 23–48 μg/100 ml. Control sera from non-vitamin-treated rabbits had a mean level of 17 μg/100 ml with a range of 6–26 μg/100 ml.

The data shown in Table 2 indicate that the vitamin A levels decreased sufficiently rapidly, even after about a month of treatment, that the vitamin A concentration in the serum was within the normal range during substantial portions of the treatment period. Comparison of the fleshy heights of the papillomas with the vitamin levels suggests that the variation among rabbits in the degree of papilloma atrophy may be explainable by differences in the degree of hypervitaminemia achieved.

**Discussion**

The histologic alterations caused by excess vitamin A suggest that the reduced growth and ultimate atrophy of the fleshy portion of the papilloma are brought about either by an acceleration of differentiation and keratinization of the epithelial papilloma cells or by a decrease in their mitotic rate. Either change, or a combination of these effects, would produce the observed decrease in quantity of the nonkeratinized epithelial component of the papilloma. As no qualitative changes were observed in the stroma, it is thought unlikely that the epithelial change is secondary to any direct effect of vitamin A on the stroma. Rather, the ultimate decrease in stroma is considered to be secondary to the decrease in the epithelial component of the papilloma.

Only 1 vitamin-treated rabbit and 1 control rabbit provided adequate histologic material for measurement of mitotic rates in the papillomas. No significant difference in mitotic rate was observed. This is not regarded as adequate evidence against the possibility that a reduced mitotic rate is responsible for the effects observed. High levels of vitamin A are known to suppress mitosis of chick embryo corneal epithelium (1) and of the corneal epithelium, tracheal epithelium, and epidermis of the rat (14). It is entirely possible, therefore, that suppression of mitosis is the mechanism responsible for the effects we have observed. The suppression of mitosis by excess of vitamin A is dependent on the degree of excess of vitamin A (1, 14). Mildly increased vitamin A levels may, in fact, stimulate epithelial mitosis (14). In our experiments, mitosis-inhibiting levels might have been achieved only intermittently during the treatment period. Inhibition of mitosis might well have been absent at the particular time when the papillomas were removed for measurement of the mitotic index; yet intermittent mitotic inhibition might nevertheless have been responsible for the observed effects. It appears that any further study of the effect of vitamin A on the mitotic rate of the papilloma cells should use tissue culture because of the many in vivo problems, e.g., (a) the instability of serum vitamin A levels (see Table 2); (b) the variation in serum vitamin A levels among individual rabbits (see Table 2); (c) the known critical dependence of mitotic suppression on the degree of vitamin A excess, cited above; and (d) the rapid response of mitotic rate to changes in vitamin A level (14).

Other known changes associated with excess vitamin A which might play a role in bringing about the observed effects on the papillomas are release of proteolytic enzymes from lysosomes (7, 15), swelling of mitochondria (10), and alteration of membrane permeability (5).

That the effects observed were merely a consequence of weight loss by the rabbit due to the vitamin A treatment appears ruled out by the failure of similar weight loss to affect the growth of the papillomas on the rabbits tested on the quantitatively restricted, but otherwise normal, diet (Chart 2).

The evidence shown in Chart 3 for an increase in concentration of infective virus in vitamin A-treated papillomas is scanty and only suggestive of an increase of moderate proportion. It is possible that vitamin A might have a specific effect on virus yield. It appears reasonable, however, that the moderate increase suggested by these data could be explained more simply as resulting from the increased proportion of keratinized material in the vitamin A-treated papillomas; mature virus is known to be present mainly in the keratinizing or keratinized portions of Shope papillomas (12).

The lengthening of the incubation period by vitamin A we attribute to retardation of growth of the papilloma, resulting in prolongation of the time required for the papilloma to be macroscopically visible. This delay in papilloma appearance may support the hypothesis that vitamin A produces its effect on the papilloma by reducing the mitotic rate of the papilloma epithelial cells, rather than by accelerating keratinization, since keratinization is not a prominent feature at this early stage of papilloma development. The failure to demonstrate a lengthening of the incubation period on sites where the least concentrated virus inoculum was used is attributed to obscuration of the effect by the wide variation in incubation periods in both vitamin-treated and control animals at this inoculum concentration.

**Acknowledgements**

The author wishes to thank Mr. Najam Kahn for performing vitamin A assays and Mrs. Jacqueline Pearson for able technical assistance.

**Addendum**

Since submitting this report, it has come to our attention that large doses of Vitamin A damage membrane structures of rat dermal fibroblasts (see Lucy et al., Nature, 206:156, 1964); that it alters the chemical composition of rat dermis (Chung et al., Proc. Soc. Exptl. Biol. Med., 115: 631, 1964), and that it inhibits new blood vessel formation in the rabbit (Nelken et al., Nature, 206:

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**Table 2**

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Average Height of Papillomas (mm)</th>
<th>Serum Vitamin A (μg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26 days after virus inoculation</td>
<td>40 days after virus inoculation</td>
</tr>
<tr>
<td>205</td>
<td>2 dead</td>
<td>dead</td>
</tr>
<tr>
<td>206</td>
<td>1 2</td>
<td>46 29</td>
</tr>
<tr>
<td>213</td>
<td>1 0.5</td>
<td>223 32</td>
</tr>
<tr>
<td>212</td>
<td>0.5 0.5</td>
<td>185 48</td>
</tr>
</tbody>
</table>
These findings increase the possibility that the changes we have observed might be initiated by an effect of vitamin A on the papilloma stroma rather than on the epithelial component.

REFERENCES


Fig. 1.—Vitamin A-treated papilloma, Group III, 90 days after virus inoculation; 27 days after start of treatment. Note atrophy of fleshy portion (compare with Fig. 2).

Fig. 2.—Control papilloma, Group III, 90 days after virus inoculation; 27 days after start of treatment.

Fig. 3.—Shed portion of papilloma (base of previous attachment presenting at lower portion of photograph), Group III, 137 days after virus inoculation; 74 days after start of vitamin A treatment.

Fig. 4.—Papilloma site from which material shown in Fig. 3 was shed, same day.
Fig. 5.—Vitamin A treated papilloma, Group II, 66 days after start of treatment; 92 days after virus inoculation. Note atrophy of papillary fronds (compare with Fig. 6). × 37.

Fig. 6.—Control papilloma, Group II, 66 days after start of treatment; 92 days after virus inoculation. × 37.

Fig. 7.—Vitamin A-treated papilloma, Group III, 96 days after start of treatment; 167 days after virus inoculation. Viable papilloma tissue no longer visible grossly. Note absence of papillary fronds and marked thinning of epidermal component of papilloma. × 150 (approximately).

Fig. 8.—Skin from non-papilloma-bearing area of same rabbit as in Fig. 7, for comparison. No striking effects of vitamin A were noted in non-papilloma-bearing areas of skin. × 150 (approximately).
Inhibition of Growth of Shope Rabbit Papilloma by Hypervitaminosis A

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