leave the undifferentiated nodal mass. Similarly, it is possible that synthesis of messenger-RNA in cells of the somite mesoderm is completed before segmentation. That a relatively stable messenger-RNA would have to be synthesized in the resistant cells seems to follow as a necessary consequence of these considerations. Such a suggestion appears feasible in view of the studies indicating that the stability of messenger-RNA varies in different cell systems (*11*). Alternative explanations for the regional specificity of the response of chick embryos with 11 to 13 somites to treatment with actinomycin D are also possible. Serious consideration should be given, for example, to the possibility of differential permeability to the antibiotic. Other investigators (*12*) have shown that, although *Escherichia coli* is resistant to actinomycin D, DNA-dependent RNA synthesis can be inhibited in protoplasts prepared from *E. coli*. The suggestion that the resistance of intact *E. coli* to actinomycin D may be related to the impermeability of the organism to this antibiotic is readily apparent (*13*).

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6 September 1963

Provitamin A_2 from Lutein

Abstract. *Alfalfa lipids were treated with* p-*toluenesulfonic acid in benzene under reflux; after saponification, chromatography of the unsaponifiable portion yielded a carotenoid having a single absorption maximum at 460 mμ in ethanol and hexane. Its properties correspond to those of the dehydration product of lutein, 3′-hydroxy-3,4-dehydro-β-carotene, for which the name "anhydrolutein" is proposed. Vitamin A depleted chicks convert this pigment to vitamin* A_2*, as shown by the high ratio of absorbancies at 693 and 620 mμ in a mixture of* $CHCl_3$ *and* $SbCl_3$ *and the absorption maximum at 350 mμ in hexane exhibited by the liver lipids. The possible role of this pigment in the biogenesis of vitamin* A_2 *is discussed.*

The predominance of vitamin A_2 over vitamin A_1 in fresh-water fish and some amphibians presents a puzzle to the biochemist. Whereas a number of carotenoids have been shown to exhibit provitamin A_1 activity in many animal species, no such precursors are known for vitamin A_2, the origin of which remains obscure. According to one report, β-carotene caused an increase in the amount of both vitamins A_1 and A_2 in certain fresh-water fishes (*1*), but the significance of this observation with regard to the biogenesis of vitamin A_2 is not clear.

The structural difference between the two A vitamins, and the fact that they are not interconvertible in vivo, suggest that a possible requirement for provitamin A_2 activity might be the presence of an unsubstituted 3,4-dehydro-β-ionone ring in the carotenoid molecule. Compounds of this type would be expected to be converted to vitamin A_2 in animals by the same series of reactions that lead to the formation of vitamin A_1 from its precursors. Furthermore, vitamin A_2 formation should then be possible in all those species in which vitamin A_1 is produced.

Several derivatives of 3,4-dehydro-β-carotene fulfilling the above structural condition were prepared by Zechmeister and his co-workers and supported growth in the vitamin A deficient rat (*2*, *3*). The most interesting of these compounds from the point of view of the biogenesis of vitamin A_2 is 3′ hydroxy-3,4-dehydro-β-carotene or "deoxylutein I" (*3*, *4*), which is formed from lutein by the loss of one molecule of water from the α-ionone ring, a reaction which might conceivably occur in nature. Zechmeister and Sease (*4*) reported yields of 3 to 4 percent provitamin, based on lutein, by melting a mixture of lutein, naphthalene, and boric acid (or tetraboric acid or boric anhydride). We have recently encountered the pigment in acidulated soybean soapstock (*5*) which had previously been reported to enhance the liver storage of vitamin A in chicks (*6*). Its presence in the soapstock was shown to result from the action of mineral acid on lutein during the industrial acidulation of raw soapstock. In fact, the reaction between lutein and acids proceeds readily and we have achieved considerably higher yields than those reported (*4*), by refluxing lutein or lutein-containing materials, such as dehydrated alfalfa meal, with benzene and *p*-toluenesulfonic acid. This has enabled us to prepare sufficient amounts of the provitamin for feeding tests with chicks. We now have evidence that the chick converts the pigment to vitamin A_2.

For the preparation of the provitamin 30 g for a lipid extract from alfalfa (*7*), containing 3.87 mg β-carotene, 3.59 mg lutein and 0.38 mg zeaxanthin per gram, were refluxed for 30 minutes with 300 ml benzene and 2 g *p*-toluenesulfonic acid. Saponification was effected by adding to the mixture 300 ml of ethanol and 30 ml of KOH solution (50 g per 100 ml) and refluxing for 20 minutes. After cooling, water was added until separation of two phases occurred, the benzene phase was removed, and the aqueous alcohol phase was reextracted with benzene until the extracts were colorless. After washing and drying of the combined benzene solutions, and after removal of the

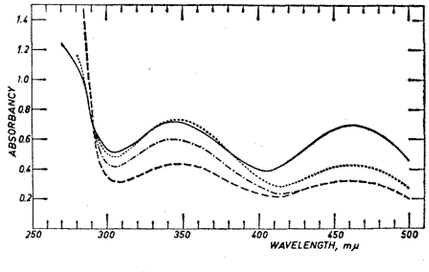

Fig. 1. Absorption spectra in ethanol of liver lipids from four vitamin A deficient chicks, after dosing with the following amounts of provitamin A_2: 5.4 mg (—·—· and — —), 8.4 mg (———), and 10.8 mg (– – – –).

Table 1. Vitamins A_1 and A_2 in livers from four chicks with provitamin A_2.

Provitamin supplied (mg)	E_{693}/E_{620}	Vitamin A_1 (μg/liver)	Vitamin A_2 (μg/liver)
5.4	2.00	18	313
5.4	1.78	19	198
8.4	2.25	4	286
10.8	2.56	Neg.*	338

* Negative value obtained by calculation.

benzene, the unsaponifiable matter was transferred to about 50 ml of hexane for chromatography on a column of MgO (40 × 400 mm) Hyflo-Super-cel 1:1; a mixture of acetone and hexane 15:85 by volume was used as the developing solvent. The provitamin formed an easily recognizable red band below the remaining lutein. The corresponding section of the adsorbent was removed mechanically and the pigment was eluted with ethanol. Its absorption spectrum, determined in a Beckman DU spectrophotometer, showed a single absorption peak at 460 mμ in ethanol or hexane and at 494 mμ in carbon disulfide. In addition to the red provitamin, the conversion products of lutein or lutein-containing materials always included two less strongly adsorbed yellow pigments with the same absorption spectrum as lutein and which were probably identical with the "deoxyluteins" II and III described by Zechmeister *et al.* (*3, 4*). A third yellow band situated between these two pigments was also observed. It exhibited absorption peaks at 417, 438, and 466 mμ in hexane. The yield of provitamin obtained in different batches was 21 to 23 mg, calculated from a molar extinction coefficient of 128,000 (*4*). This represents a reaction yield of 20 to 22 percent.

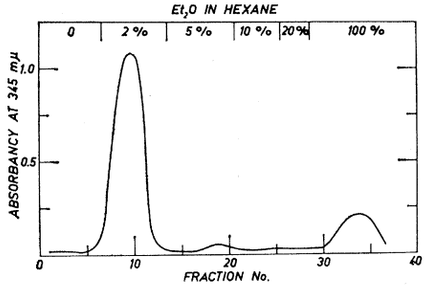

Fig. 2. Elution pattern of the pooled liver lipids from four chicks, after dosing with provitamin A_2. Absorbent, Al_2O_3 weakened with 5 percent water; eluant, increasing amounts of ethyl ether in hexane.

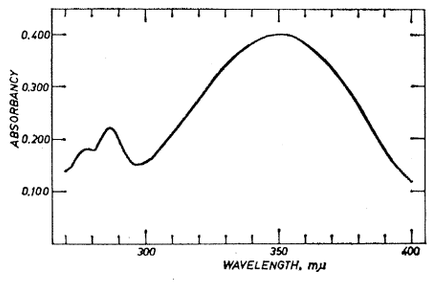

Fig. 3. Absorption spectrum in hexane of fraction 9 eluted from Al_2O_3 (Fig. 2).

Newly hatched cockerels (New Hampshire crossed with White Leghorn) were kept on a basal sorghum-soybean meal diet deficient in vitamin A, for 16 days, after which time the amount of vitamin A in each liver of five control chicks was 10 to 30 IU. Two of the chicks then received 5.4 mg provitamin, one was given 8.4 mg and one 10.8 mg. The provitamin was supplied as a solution in refined soybean oil (0.76 mg/g) which was mixed with 100 g feed. After each chick had consumed its portion, which took 4 to 5 days, it was given the basal diet for another day. The chicks were then killed and the liver lipids were extracted (*8*). The antimony trichloride reaction was carried out on the liver lipids and the absorbancy of the blue color measured at 620 and 693 mμ. The amount of vitamins A_1 and A_2 were calculated by the formula of Plack and Kon (*9*).

Table 1 reveals that the absorbancy is about twice as high at 693 mμ as at 620 mμ for all four liver extracts. Calculation shows that the livers contained considerable amounts of vitamin A_2 with only minor amounts of vitamin A_1. It should be pointed out that the determination of small amounts of vitamin A_1 in the presence of large amounts of vitamin A_2 is obviously inaccurate.

In Fig. 1 the absorption spectra of the liver extracts in ethanol are shown. Besides the maximum at 460 mμ characteristic of the provitamin, which is apparently stored in the livers, an absorption peak is observed at about 345 mμ indicative of vitamin A_2. No maximum or inflection is seen in the 325 to 330 mμ region.

Definite proof of the presence of vitamin A_2 was obtained by chromatography of the pooled liver extracts on water-weakened alumina (*10*). Figure 2 shows the curve obtained by collecting 10 ml fractions of eluent and measuring the absorbancy of each at 345 mμ. The material eluted during the early part of the chromatography is undoubtedly vitamin A_2 ester, as seen by the spectrum shown in Fig. 3. An absorption maximum occurred at 350 mμ, a secondary peak at 287 mμ, and a third, minor, peak at 278 mμ. Besides the vitamin A_2 elution peak (Fig. 2), a small amount of material appeared in the eluent, but it did not exhibit any specific absorption. The last elution peak, obtained with ether, was due to the provitamin pigment, which at this relatively high concentration contributes to the absorption at 345 mμ, probably through its *cis*-peak.

The fact that the chick is able to convert the provitamin to vitamin A_2 indicates that the conversion step is probably a general one, not restricted to certain species, such as fresh-water fish. Any specificity in vitamin A_2 formation in animals would then be determined by either of the two following factors: (i) availability in the food of a suitable provitamin such as the one formed from lutein; or (ii) ability of the animal itself to carry out the dehydration of lutein (or other suitable xanthophylls) to the provitamin A_2.

In the former case the provitamin A_2 should be detectable in lower animal or vegetable organisms, particularly those found in fresh-water; while in the latter case, lutein or other suitable plant xanthophylls would act as provitamin A_2 in animals such as fresh-water fish. Both of these possibilities deserve further exploration.

Although 3′hydroxy-3,4-dehydro-β-carotene has not been reported to occur naturally, De Nicola (*11*) has described a pigment with a single absorption maximum at 460 mμ and with a chromatographic behavior very similar to our compound, and which was found in the carapace of the echinoderm *Ophidiaster ophidianus,* where it accompanies astaxanthin.

If 3′-hydroxy-3,4-dehydro-β-carotene occupies a central position in the biogenesis of vitamin A_2, it would be desirable to give it a shorter name. The term "deoxylutein I" used by Zechmeister and Petracek (*3*) is based on an earlier incomplete characterization of the compound (*4*). We propose the name "anhydrolutein," because it better reflects the structure and origin of the provitamin (*12*).

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Starch Synthesis in Excised Lemon Fruit Tissue Growing in vitro

Abstract. *Significant quantities of starch accumulation were evident in excised mature lemon fruit tissue growing in vitro on a nutrient solution of mineral salts and sucrose. Positive evidence for the presence of starch in the growing cells was obtained with iodine staining and polarizing microscopy. Starch was observed in all growing cultures regardless of age.*

Vesicle stalks freshly excised from mature yellow lemon fruits [*Citrus limon* (L.) Burmann, variety Eureka] squashed in dilute iodine solution showed no signs of starch-containing bodies. This is consistent with the observation that most of the starch has disappeared from mature fruits except in seed tissue and occasional grains in the outer layers of the yellow peel (*1*) and in masses of small cells in or near the center of each juice vesicle (sac portion) (*2*).

Although lemon fruits belong to the class of plant organs which are without starch reserve at maturity (*3*), excised vesicle stalks from mature Eureka lemons are apparently capable of indefinite starch synthesis when grown in vitro on a nutrient solution consisting of mineral salts and sucrose. Starch synthesis has been shown by iodine staining and polarizing microscopy. Hence the genetic capacity for starch synthesis is not irreversibly altered in this tissue during growth and maturation of the fruit in vivo.

Vesicle stalks were excised from mature Eureka lemon fruits and planted in vitro as described previously (*4, 5*). The cultures were maintained in constant darkness at room temperature. After 2 to 3 weeks' growth in vitro, proliferating stalks were examined with a Mineralite shortwave ultraviolet hand lamp (253 mμ), and all invariably showed the characteristic yellow-green fluorescence (*6*). Living proliferating material was squashed in sterile nutrient solution and examined with bright field and polarizing microscopy, respectively. Relatively large numbers of round or slightly acentric bodies were present in the solution external to the squashed cells as well as in intact cells. When examined between crossed polarizers, these bodies exhibited birefringence and the polarization cross characteristic of starch granules (Fig. 1). The symmetry of the starch granules in the proliferating fruit tissue under polarized light was similar to the symmetry of starch granules of waxy and normal maize (*7*, Figs. *8–11*; *8*, Fig. 3; *9*, Fig. 40). The addition of a dilute aqueous iodine solution (0.3 percent I_2 in 1.5 percent KI) to the squash preparations resulted in a deep blue coloration of the acentric bodies—that is, the bodies gave a positive color reaction for starch. The birefringence and polarization cross were still clearly evident in the blue, iodine-stained bodies. The unstained and iodine-stained bodies, respectively, were optically positive between crossed polarizers.

Proliferating fluorescent stalks, when dropped into the dilute iodine solution, manifested an extensive blue coloration soon after immersion. Hand sections as well as squash preparations of the iodine-treated proliferating tissue revealed relatively large numbers of the blue-stained bodies under bright field microscopy. These blue-stained bodies were anisotropic; they showed the polarization cross characteristic of starch granules and were optically positive between crossed polarizers. Subcultures of proliferating lemon fruit tissue (3½ years old) which fluoresced yellow-green under ultraviolet light also gave the same specific color reaction with iodine and the same birefringent bodies. These bodies, like those in the 3-week-old stalks, showed the polarization cross and were also optically positive. Hence the innate capacity to synthesize starch does not decrease with increasing age of the growing culture. Cultures which no longer manifested visible signs of cellular growth and which did not fluoresce under ultraviolet light showed very few bodies that stained blue with iodine, thus indicating an apparent cessation of starch synthesis in quiescent or dying tissue. The specificity of the blue coloration with iodine for starch in the growing cells is shown by its complete restriction to specific bodies which are birefringent and which show the distinct polarization cross characteristic of starch granules (*8–10*).

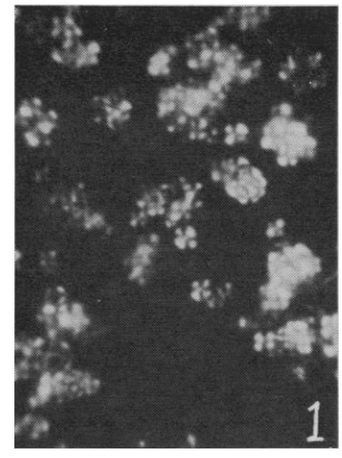

Fig. 1. Starch granules in an unstained section of excised lemon fruit tissue proliferating in vitro as observed between crossed polarizers. (× 750)

The ability of excised lemon fruit tissue to synthesize starch on a medium of minerals and sucrose affords a possible tool for studying the transformation of sucrose, the main transport sugar in plants (*8*), into starch under relatively simple and precisely standardized into vitro conditions (*5; 11*).

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