8. Preliminary work on whale-liver phospholipins suggests that kitol is not present in the true acetone-insoluble lipid, but that it may occur in more than one type of combination.

9. Vitamin A and possibly kitol is present in fin-whale milk.

A small sample of kitol was kindly placed at our disposal by Dr Baxter of Distillation Products Inc. We are indebted to the Ministry of Food and the Medical Research Council for grants in aid of the work. In obtaining material we have had most friendly help from the whaling industry and many scientists engaged in it both in this country and abroad.

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Studies in Vitamin A

13. THE ALLEGED FORMATION OF VITAMIN A FROM β -CAROTENE TREATED WITH IODINATED CASEIN

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Kaplansky & Balaba (1946) claimed that β -carotene in colloidal solution when incubated with either thyroglobulin or iodinated casein was converted into vitamin A in good yield; a typical experiment resulted apparently in the production of 60 μ g. of vitamin A from 150 μ g. of β -carotene. If it be true that thyro-active compounds can effect such a conversion, the finding is of major importance to the biochemistry of carotene and vitamin A. An investigation of Kaplansky & Balaba's claim was therefore undertaken. In the first part of the work the experimental procedure of Kaplansky & Balaba was followed as closely as possible, but when efforts to produce vitamin A failed, the conditions were then varied. A short report of the results of this work has already appeared (Cama & Goodwin, 1948).

EXPERIMENTAL

Preparation of iodinated casein. NaHCO_3 (3.6 g.) was added to skimmed milk (500 ml.) freed from fat by repeated centrifugation, and the liquid heated to 40°. Small portions of I_2 were added with constant stirring until a slight excess remained at the bottom of the beaker (about 8.0 g. of I_2 were required). The temperature was then raised to 65° for 24 hr., the liquid being constantly stirred. After cooling, the intensely yellow solution was filtered and the filtrate brought to pH 4.5 with acetic acid. The precipitated iodinated casein was washed, dissolved in very dilute alkali and reprecipitated with acetic acid. This process was repeated three times. The I_2 content of the product, determined according to the

micro method of Groák (1926) was 8.45–8.67%. Kaplansky & Balaba (1946) report 0.85%, but this appears to be a mistake.

Preparation of colloidal solutions of β -carotene. Crystalline β -carotene (approx. 3 mg.) was dissolved in a small volume of freshly redistilled diethyl ether (20 ml.) and 96% (v/v) ethanol added (5 ml.). The ether was removed by distillation on a water bath at 50–60°; the distillation was continued until a test portion of the distillate no longer separated into two layers after the addition of a small volume of water. The ethanol solution of β -carotene was then diluted with distilled water and rapidly filtered; the colloidal solution was placed in a boiling water bath to remove the ethanol and any residual ether. The remaining concentrated colloidal solution was filtered again, diluted with water to approximately the desired concentration and used without delay.

Incubation of solutions. The details of Kaplansky & Balaba's (1946) method are as follows: To a known volume of colloidal solution containing 150 μ g. β -carotene is added iodinated casein (30 μ g.) dissolved in weak alkali; the reaction mixture, after being adjusted to pH 7.3 with acetic acid, is incubated for 2 hr. at 37°; controls consisting of β -carotene solutions only or β -carotene solution + boiled iodinated casein adjusted to pH 7.3 are treated in the same way.

In the first stages of the present investigation the above procedure was followed as closely as possible. (Kaplansky & Balaba did not specify the concentration of the weak alkali or the acetic acid; we used 0.01 N- NaHCO_3 and 0.01 N-HOAc.) The procedure was later modified by altering (a) the pH (by the use of phosphate buffers), (b) the incubation times, and (c) the amounts of substrate and 'enzyme', and (d) by using desiccated thyroid or minced fresh thyroids instead of iodinated casein.

Determination of carotene and vitamin A. Kaplansky & Balaba used the method of Rachevski for the determination of carotene; no reference or details were given, but a search of the literature indicates that the method used can only be that of Rachevski & Troitaki (1938). It is not based on absorption spectrophotometry as are most methods of carotenoid analysis, but on iodometry. A known amount of iodine is added to a colloidal solution of carotene and the amount not absorbed is measured in the usual way by titrating with thiosulphate. Such a method, as will be noted later, has serious drawbacks when used in experiments of the type under discussion. We preferred the method in use in this laboratory, as it is unambiguous and well authenticated.

A measured volume of the colloidal solution is extracted three times with an equal volume of freshly redistilled diethyl ether; the ether extracts are combined and the solvent removed on the water bath; the residue is dissolved in a known volume of cyclohexane and the absorption spectrum recorded using a Beckman photoelectric spectrophotometer. cycloHexane is chosen as the solvent so that vitamin A determinations, making use of both ultraviolet absorption and the SbCl_3 colour test, could be carried out on the same solution; this eliminates further manipulations involving heat, and minimal heat treatment is always to be aimed at when dealing with carotenoids.

Kaplansky & Balaba stated that they determined vitamin A by the Carr-Price method (SbCl_3 reagent), and, in one experiment, by the spectrophotometric determination of the absorption maximum at $328 \text{ m}\mu$, but no details of the method were given. In the present investigation two procedures for determining vitamin A were used: (a) the Morton & Stubbs (1946) method of direct ultraviolet spectrophotometry, and (b) the SbCl_3 colour test. The Morton & Stubbs method is a well-authenticated method in which allowance can be made for absorption not due to vitamin A. The SbCl_3 colour test was carried out using a Hilger-Nutting visual spectrophotometer; this instrument allows the differentiation of the SbCl_3 -vitamin A absorption maximum at $617 \text{ m}\mu$ from SbCl_3 -carotene absorption maximum at $590 \text{ m}\mu$, even when carotene occurs in considerable excess over vitamin A.

RESULTS

Thirty-six experiments were carried out under varying conditions, and in no case was the formation of vitamin A unequivocally demonstrated after incubation of β -carotene with either iodinated casein, desiccated thyroid, or fresh thyroid brei; in one case only the Morton & Stubbs (1946) correction procedure pointed to the presence of a trace of vitamin A, but this was not confirmed by the antimony trichloride colour test.

In the first series of experiments Kaplansky & Balaba's (1946) conditions were reproduced as closely as the published directions permitted; later the conditions were varied and Table 1 indicates the limits of variation.

In Fig. 1 are shown the full absorption curves obtained in three typical experiments in which β -carotene was incubated: (a) alone, (b) with iodinated casein, and (c) with desiccated thyroid. It will be noted that although the curves do not

coincide, owing to the difficulty of obtaining colloidal carotene solutions of the same concentration (see p. 319), they are almost identical in shape; thus no differential changes in the β -carotene have occurred

Table 1. Variations in the experimental conditions used in attempting to convert β -carotene into vitamin A by incubating with iodinated casein or desiccated thyroid

Variable	Limits of variation
β -Carotene	2.5-30 μg .
Incubation time	2-2.5 hr.
pH	4-8
Iodinated casein	50-60 μg .
Desiccated thyroid	30-60 μg .
Temperature	Not altered (37°)

by incubation under different conditions. The appearance of a small band at $336 \text{ m}\mu$ is due to *trans-cis* isomerization of the carotenoid produced under the action of heat; the $336 \text{ m}\mu$ band is Zechmeister's (1944) *cis* peak.

Fig. 1. The absorption spectrum of β -carotene: A, after incubation alone for 2.5 hr. at 37° ; B, after incubation with iodinated casein for 2.5 hr. at 37° ; C, after incubation with desiccated thyroid for 2.5 hr. at 37° .

DISCUSSION

It will be seen that we have not, in this laboratory, been able to detect the formation of vitamin A when colloidal solutions of β -carotene are incubated with iodinated casein or desiccated thyroid. The apparatus used is known to be capable of detecting small amounts of vitamin A in the presence of carotene so there is no doubt that vitamin A was not present; it was not a matter of failure to detect it.

Kaplansky & Balaba (1946), on the other hand, report three experiments in which the yield of vitamin A was 45%. We have thus to attempt a reconciliation between contradictory experiences. It may be that favourable conditions are elusive, and

that although we tried to follow Kaplansky & Balaba's methods exactly we failed to achieve such conditions; it seems, however, that a method which produces a 45 % yield of vitamin A in the hands of one set of workers should in another laboratory at least yield detectable amounts. It is interesting to note that Wiese-Lowry (1949) has informed us that she too was unable to repeat Kaplansky & Balaba's work. It is possible to criticize Kaplansky & Balaba's work from a technical viewpoint, and it is then clear that their claims are not securely based. Their technique may be considered under three heads:

(i) *Estimation of β -carotene.* The iodometric method used by Kaplansky & Balaba is at best unspecific, and it is difficult to understand why it was used if an optical instrument were available (as apparently there was, because in one experiment E 328 $m\mu$. was measured). The iodometric method presents a problem which Kaplansky & Balaba have not discussed. For example, the results of a typical experiment recorded by them indicates that 150 μg . of β -carotene after incubation with iodinated casein yielded 86 μg . of β -carotene and 55 μg . of vitamin A. Now, vitamin A is an unsaturated compound and will itself absorb iodine; thus, unless this is allowed for, the iodometrically determined β -carotene content of a mixture of β -carotene and vitamin A will be incorrect. Assuming this correction has not been made, and there is no internal evidence that it has, the results just quoted are open to two serious objections: (a) If the β -carotene not converted into vitamin A is reasonably stable to incubation, as they claim it is in the case of boiled iodinated casein, and as we find it is in all cases, then the iodometric method should give a value for ' β -carotene' still in the neighbourhood of 150 μg . (b) If the value 86 μg . was the determined uncorrected value then this must represent ' β -carotene and vitamin A', and as 55 μg . of vitamin A are reported only about 30 μg . of β -carotene have been accounted for out of an expected remainder of about 100 μg .

(ii) *Colloidal carotene solutions.* Although following precisely the directions given by Kaplansky & Balaba, we found the production of the colloidal solutions to be much less reproducible than they did. For example, according to their table they used for each experiment exactly 150 μg . of carotene obtained by diluting a concentrated colloidal solution a known amount. We could not make this method work, for, no matter how carefully the water was added to the concentrated carotene solution, a little was always thrown out of solution and had to be filtered off; thus it was impossible to obtain a solution of colloidal carotene of known strength merely by dilution; the content of each colloidal solution had to be measured each time.

(iii) *Measurement of vitamin A.* Kaplansky & Balaba give no details of how they measured the

SbCl₃ colour test. How they differentiated between the carotene-SbCl₃ colour test and the vitamin A-SbCl₃ colour test, especially in a mixture of two, is not stated, so that it is impossible to assess the evidence.

Regarding the determination of vitamin A in the presence of excess of β -carotene by ultraviolet spectrophotometry, β -carotene itself absorbs in the 300–340 $m\mu$. region and this has to be allowed for before the contribution of vitamin A to the absorption of a solution can be evaluated. This is even more important when β -carotene solutions have been incubated, as increased absorption appears in the 300–400 $m\mu$. region owing to the formation of *cis* isomers from all *trans*- β -carotene. When a photoelectric spectrophotometer is available the correction procedure of Morton & Stubbs (1946) allows the determination of the vitamin A contributed.

Fig. 2. The absorption spectra of β -carotene and mixtures of β -carotene and vitamin A in the spectral region 300–350 $m\mu$.: A, pure β -carotene; B, β -carotene after incubation at 37° for 2.5 hr.; C, β -carotene after incubation at 37° for 2.5 hr. with iodinated casein; D, a computed curve for a mixture of vitamin A and incubated β -carotene, containing about 30 % of vitamin A; E, pure vitamin A.

The appearance of a carotene *cis* peak (Zechmeister, 1944) in the region of 330–335 $m\mu$. after incubation could easily be mistaken for vitamin A absorption, especially when a spectroscope with a low dispersion is used. To illustrate this point a number of absorption curves are recorded in Fig. 2. The following points will be noted: (a) the reality of the *cis* peak of incubated carotene; (b) the difference between the curve of isomerized β -carotene and that of a mixture of this β -carotene and vitamin A (approx. 30 % vitamin A) is slight but well marked, and the presence of absorption due to the vitamin A in an incubated β -carotene solution could be detected even without application of the Morton & Stubbs (1946) correction if the curve were measured

photoelectrically; (c) that this difference might be missed using old (photographic) methods, for the changes, involving a shift of the band maximum only 1–2 $m\mu$. and a small decrease in the slope on the short wave side of the maximum, are nearing the limits of detection; (d) that the curve of the β -carotene incubated alone is little different qualitatively from that obtained after incubation with iodinated casein.

Although experiments using more refined techniques have failed to substantiate earlier claims that thyroactive compounds catalyse the *in vitro* conversion of β -carotene into vitamin A, it is not to be assumed that the thyroid has no action on the *in vivo* metabolism and vitamin A. In fact, it has recently been shown in this laboratory that desiccated thyroid

enhances and thiouracil reduces the absorption of β -carotene from the intestinal tract (Cama & Goodwin, 1949).

SUMMARY

1. It has not been possible to substantiate the claim that iodinated casein and desiccated thyroid are active in converting β -carotene into vitamin A *in vitro*.

2. The most likely error leading to this conclusion is the failure to distinguish the *cis* peak of isomerized β -carotene from the 326 $m\mu$. absorption band of vitamin A.

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The Pyruvate-oxidase System in Brain and the Tricarboxylic Acid Cycle*

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The term pyruvate-oxidase system has been used in this laboratory to denote the complex of enzymes in brain which induce oxidation of added pyruvate. In earlier work (Long, Ochoa & Peters, 1939; Banga, Ochoa & Peters, 1939) the idea that this system in pigeon brain involved a tricarboxylic acid cycle (Krebs & Johnson, 1937; Krebs & Eggleston, 1940) was not supported. One of the main reasons was that, in contrast with kidney preparations, the increased oxygen uptake observed on addition of fumarate as well as pyruvate was not observed with citrate, which could not therefore be an intermediate; but, since then, the role of citrate has been changed to that of a side product rather than that of an

intermediate (Wood, Werkman, Hemingway & Nier, 1941). It has always been difficult to account for the action of fumarate upon any other view than that of a tricarboxylic acid cycle, and we now think that the pyruvate-oxidase system in our homogenates does in fact include much of this cycle when fumarate is present; we are giving our evidence for this, though all details are not yet clear. The most important new point is that citrate and a substance, which is estimated as α -ketoglutarate, accumulate during the oxidation of pyruvate in the presence of fumarate and when malonate is also present.

METHODS

Estimations

Pyruvic acid. The Neuberg-Case method (Case, 1932) has been used as modified by Peters & Thompson (1934), the final colour of the hydrazone in ethanolic KOH being

* A preliminary account was given to the Biochemical Society on 22 January 1949 (Coxon, Liébecq & Peters, 1949).

† With the technical assistance of R. W. Wakelin.