The conversion of 11-*cis* into all-*trans* vitamin A in the rat

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In conjunction with Professor G. Wald, we recently investigated the isomers of vitamin A_1 present in the marine euphausiid Crustacea *Meganyctiphanes norvegica* (M. Sars) and *Thysanoessa raschii* (M. Sars) and found that 11-*cis* vitamin A (neo-*b* vitamin A) was the predominant form, comprising some 80–90% of the total (Plack, Fisher, Henry & Kon, 1956; Wald & Brown, 1956–7; Fisher, Kon & Plack, 1957). Other marine Crustacea were also found to contain predominantly the 11-*cis* isomer (Wald & Burg, 1956–7; Fisher *et al.* 1957; Barnholdt & Hjarde, 1957).

The presence of this form of vitamin A in these euphausiids is of some importance, since they form part of the diet of several fish caught commercially and, together with *Thysanoessa inermis* Kröyer, the bulk of the food of northern baleen whales. Ponomareva (1949, 1954) also reports that *T. raschii* is a major component of the food of fish and whales in Far Eastern waters. Examination of the vitamin A from livers of Arctic blue- and fin-whales showed the presence of 80–90% of the all-*trans* isomer (Plack, 1956*a*), so that if the euphausiids constitute the sole source of vitamin A for baleen whales (Fisher, Kon & Thompson, 1952) then the whale either absorbs preferentially the small quantities of the all-*trans* isomer present in these animals or it converts 11-*cis* into all-*trans* vitamin A.

This paper reports the conversion of 11-*cis* into all-*trans* vitamin A in the rat and discusses the possible site of conversion. A preliminary account of the work has already been given (Plack & Thompson, 1958).

NOMENCLATURE

The Karrer system of numbering has been used in describing the isomers of vitamin A (see Plack, 1956*b*). Weights of vitamin A ester are given as the equivalent weight of vitamin A alcohol. The terms 'slow'-reacting isomers (11-*cis*, 13-*cis*, 9:13-di-*cis* and 11:13-di-*cis* vitamin A) and 'fast'-reacting isomers (9-*cis* and all-*trans* vitamin A) refer to the rates of reaction with maleic anhydride in benzene solution (see Plack, 1956*b*). In an absorption curve, the wavelength of maximum extinction is abbreviated to $\lambda_{max.}$.

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EXPERIMENTAL

Except for the dosing of rats and their dissection, and the opsin test, all work was carried out in a darkroom illuminated with a 60 W tungsten lamp covered with red Cellophane. In the opsin test, lighting conditions were more stringent, as described below.

General chemical methods

Extraction of vitamin A. The livers and gut walls of rats were extracted essentially as described by Thompson, Ganguly & Kon (1949). Intestinal contents and washings from the intestine (total volume 60 ml.) were added to 120 ml. ethanol and extracted in a 500 ml. separating funnel by shaking with two 200 ml. portions of light petroleum, b.p. 40–60°.

Samples of heparinized blood were centrifuged and 5 ml. plasma were taken with a pipette. When the sample did not yield 5 ml. plasma, the greatest possible volume was taken and made to 5 ml. with water. After the addition of 5 ml. ethanol, vitamin A was extracted with two 12·5 ml. lots of light petroleum, b.p. 40–60°, in a small separating funnel, shaken mechanically for 5 min periods.

In one instance vitamin A in the body of a rat was extracted. The body was heated with 500 ml. of 5% (w/v) aqueous KOH until dissolved (about 4 h). A 100 ml. sample was taken, 100 ml. ethanol were added and vitamin A was extracted with three 100 ml. lots of diethyl ether in a separating funnel. The combined extracts were washed with three 100 ml. portions of water and dried over anhydrous Na_2SO_4.

Chromatography. The method of Thompson *et al.* (1949) with the modification described by Kon, McGillivray & Thompson (1955) was used to separate vitamin A ester and alcohol and to purify the vitamin A alcohol obtained by saponification.

Saponification. The procedure was that of Plack (1956*b*) except that 60% (w/w) aqueous KOH was used.

Carr–Price reaction. The method and spectrophotometer of Thompson (1949) were used to measure the maximum intensities of the blue colours produced by vitamin A alcohol at $\lambda_{max.}$ 620 mμ and by vitamin A aldehyde at $\lambda_{max.}$ 664 mμ. The different isomers of vitamin A were assumed to have the same specific extinction coefficients (see Plack, 1956*b*) and the value $E^{1\%}_{1cm}$ (620 mμ) = 5000 has been used (Fisher, Kon & Thompson, 1956). With vitamin A aldehyde (prepared as described by Plack, Kon & Thompson, 1959) the value $E^{1\%}_{1cm}$ (664 mμ) = 3820 was found and used in this work. Hubbard, Gregerman & Wald (1952–3) and Robeson, Blum, Dieterle, Cawley & Baxter (1955) have shown that the different geometrical isomers of vitamin A aldehyde give similar specific extinction coefficients, but their mean values were slightly lower than the value above.

Preparation of substances for dosing

Saponification of 11-cis *vitamin A* p-*phenylazobenzoate.* The alcohol form of 11-*cis* vitamin A *p*-phenylazobenzoate (a synthetic material, given by Dr W. Oroshnik) was prepared by the method of Oroshnik (1957). About 0·75 g of the ester was weighed out and dissolved in a mixture of 12·5 ml. diethyl ether and 10 ml. ethanol. To this

solution were added 17·5 ml. of 5% (w/v) NaOH in ethanol, and the whole was left for 5–6 h at room temperature in the dark, with occasional shaking. The mixture was then transferred to 125 ml. water in a 500 ml. separating funnel and extracted with two 50 ml. lots of *n*-hexane. The combined extracts were dried over anhydrous Na_2SO_4, poured through a short column of the same material to remove suspended matter, and evaporated under reduced pressure at low temperature (20–30°) until 20–30 ml. were left. *n*-Hexane and ethanol were then added to give a final concentration of about 2 mg vitamin A/ml. and 2% (v/v) ethanol. A test for vitamin A ester by chromatography and the Carr–Price reaction showed that saponification was complete. The concentration of total vitamin A alcohol in the final solution was estimated by the Carr–Price test and the percentage of all-*trans* by the maleic-anhydride test. No all-*trans* vitamin A was detected, even after 3 weeks' storage in the dark.

Oily solutions. For feeding experiments, the 11-*cis* vitamin A alcohol was dissolved with the aid of chloroform in arachis oil containing 0·01% quinol. The concentration was adjusted so that 1 mg of vitamin A was contained in four drops (78·4 mg) of oil from a standardized dropper. The vitamin A content of the oily solution was determined directly on a sample in chloroform solution by the Carr–Price test. Solutions of crystalline all-*trans* vitamin A alcohol (Roche Products Ltd) in arachis oil were similarly prepared.

Samples of four drops of these solutions were saponified and chromatographed, the residues from the eluates with 8% (v/v) ethanol in *n*-hexane being dissolved in 25 ml. *n*-hexane for isomer analysis.

Aqueous dispersions. These were prepared with the surface-active agent Tween 40 (polyoxyethylenesorbitan monopalmitate, a gift from Honeywill–Atlas Ltd, London). For injection into the jugular vein, 2 mg vitamin A were required in 0·4 ml. water containing 10% (v/v) Tween 40. Vitamin A (53 mg, that is 6% excess) was dissolved in 2 ml. chloroform and 1 ml. Tween 40 was added. The chloroform was removed, the solution of vitamin A in Tween 40 heated to about 70° and 9 ml. of water at about 70° were added. A clear dispersion resulted on cooling. For injection into the intestine, the dispersion was diluted ten times with water, giving 2 mg vitamin A in 4 ml. water containing 1% (v/v) Tween 40.

The vitamin A from a sample of the dilute dispersion was obtained by adding 2 ml. to 6 ml. ethanol in a 100 ml. separating funnel and extracting with three 20 ml. lots of light petroleum. The combined extracts were dried over anhydrous Na_2SO_4, the solvent was removed and the residue taken up in 25 ml. *n*-hexane. Two 1 ml. samples of this solution were used for estimation of the vitamin A content of the dispersion and the remainder was reserved for the analysis of isomers.

Emulsions in water of oily solutions of vitamin A. Crude lecithin (given by J. Bibby and Sons Ltd, Liverpool) was dissolved in arachis oil with the aid of chloroform to give a 4% (w/w) solution. When the chloroform had been removed, the mixture emulsified well on shaking with water and, although larger particles came to the top after 2 h, there was no sign of an oily layer.

For injection into the intestine, doses of 2 mg vitamin A in eight drops (156·8 mg) of oil from the standardized dropper were required as an emulsion in 4 ml. water.

The amount of oil in the emulsion then corresponded to that in the 2 mg dose given by mouth. Vitamin A sufficient for eight doses (about 17 mg, 6% in excess) was dissolved in 1·5 ml. chloroform and sixty-four drops of the solution of lecithin in arachis oil were added. The chloroform was removed, 30·6 ml. water were added (the volume of oil being about 1·4 ml.) and the whole was shaken for 5 min to give an emulsion. For estimation of vitamin A and of isomer content, a 2 ml. sample of the emulsion was added to 30 ml. ethanol and 15 ml. water in a separating funnel and extracted with four 100 ml. lots of light petroleum. If the emulsion did not break down, a small volume of 60% aqueous KOH was added. The residue from the combined extracts was saponified and chromatographed and material remaining after removal of the 8% ethanol in *n*-hexane was taken up in 25 ml. *n*-hexane. Samples of 1 ml. were taken for the Carr–Price test and the remainder was kept for the analysis of isomers.

Dosing of rats and removal of tissues

Vitamin A-deficient rats. The rats used in this work were prepared as described by Plack *et al.* (1959). For most experiments, male rats were used, weighing 210–340 g. In some instances, female rats of weight 180–220 g were used.

Dosing by mouth. Before dosing, rats were given free access to diet 213 (Henry, Kon, Mawson, Stanier & Thompson, 1949) which contains about 3 μg carotene/100 g but no vitamin A. The rats were dosed by holding their jaws open with one hand and dropping the oily solution into their throats or on to their tongues. After dosing, the rats were placed in separate cages and offered *ad lib.* diet 172*a* (Clarke & Todd, 1957), completely deficient in vitamin A. At suitable times after dosing, the rats were anaesthetized with diethyl ether, killed by bleeding from the heart, and the liver was removed, cut into small pieces and placed in a mixture of 30 ml. ethanol and 15 ml. water. If required, other organs were put into similar mixtures.

Injection into the intestine. For these experiments, the technique was essentially that of Davidson & Garry (1939–40). Vitamin A-deficient rats were given skim milk on the penultimate day and water only on the day before the experiment to eliminate coarse material from the intestine. In later experiments, milk was given on the day before only. The rats were anaesthetized by a subcutaneous injection of a 50% (w/v) solution of urethane (0·25 ml./100 g rat). A midline ventral incision was made and the stomach and caecum ends of the small intestine were brought to the surface and ligatured. In some experiments, the intestinal contents were washed out of the gut with 30 ml. of Krebs–Ringer bicarbonate (modified as described by Bleehen & Fisher (1954) and gassed with a mixture of 95% O_2 with 5% CO_2); in others, this step was omitted. The vitamin A preparation (4 ml.) was injected into the intestine at the cranial end, the intestine replaced in the abdominal cavity, the edges of the wound were clipped together and the rat was placed in a bath of 0·9% aqueous NaCl, maintained at 37°, so that its head remained above the surface.

After 2 or 4 h, during which the rat remained under the anaesthetic, the intestine was again exposed, the contents were washed out with 0·9% aqueous NaCl and the liquid remaining in the intestine was blown through with air. The combined washings (about 60 ml.) were added to 120 ml. ethanol. The rat was then killed by bleeding from

the heart and the small intestine and liver were removed, cut into small pieces and placed in flasks containing mixtures of 30 ml. ethanol and 15 ml. water.

Injection into the jugular vein. The rat was anaesthetized with diethyl ether and the dose (0·4 ml.) injected into the exposed jugular vein. After the incision had been sewn up, the rat was allowed to recover from the anaesthetic. It was again anaesthetized with diethyl ether 2 or 4 h after dosing and a blood sample was taken first from the heart and then from the pleural cavity after cutting the heart, a trace of heparin being used as anticoagulant. The liver was then removed, cut into small pieces and laced in a mixture of 30 ml. ethanol and 15 ml. water.

Measurement of vitamin A

Estimation. The rat organs were extracted as already described and, in the feeding experiments and certain of the injection experiments, the residue from the extract after removal of solvent was saponified and chromatographed. The residue from the eluate with 8% ethanol in *n*-hexane, which contained vitamin A in the alcohol form, was dissolved in 25 ml. *n*-hexane. In some of the injection experiments, the residue from the light-petroleum extract was dissolved in a few ml. of *n*-hexane and chromatographed to separate the ester and alcohol forms of the vitamin A. The ester fractions were saponified and chromatographed and the ester and alcohol fractions were finally dissolved in 25 ml. portions of *n*-hexane. Samples (1 ml.) of the *n*-hexane solutions were taken for the Carr–Price test and the total vitamin A content of the organ was calculated.

The vitamin A extracts from blood samples were taken to dryness and chloroform was added for the Carr–Price test. In the experiment on the distribution of vitamin A in the rat (see p. 121), all samples were saponified and chromatographed and, with the exception of those from the liver, the residues from the eluates with 8% ethanol in *n*-hexane were put into suitable volumes of chloroform for the Carr–Price test. The residue from the liver eluate was put into 25 ml. *n*-hexane and a 1 ml. sample taken for the test.

Maleic-anhydride test. The general procedure of Plack (1956*b*) was used, but the concentrations of vitamin A in benzene were adjusted according to the quantity of vitamin A available for the test, and the readings were restricted to 0, 3, $4\frac{1}{2}$ and 6 h after addition of the benzene solution of maleic anhydride.

Opsin test. Cattle opsin combines with 11-*cis* vitamin A aldehyde to give the light-sensitive pigment rhodopsin, with $\lambda_{max.}$ 500 mμ when dispersed in digitonin solution, whereas 9-*cis* and 9:13-di-*cis* vitamin A aldehyde give a similar pigment, isorhodopsin, with $\lambda_{max.}$ 486–487 mμ, and the other isomers of vitamin A aldehyde do not combine (Hubbard & Wald, 1952–3; Oroshnik, 1956). Hubbard *et al.* (1952–3) suggested the use of this reaction as an analytical procedure, and the techniques used in the present work were as follows.

(a) Preparation of digitonin solution. A 2% (w/v) solution of digitonin (Roche Products Ltd) in 0·067 M phosphate buffer, pH 6·47, was prepared by bringing the suspension slowly to boiling, boiling for a few minutes until clear, and cooling rapidly. The solution was filtered through a no. 4 porosity sintered-glass funnel before use.

(b) Preparation of opsin. Fresh cattle eyes were obtained from the local abattoir and the retinas removed as described by Collins & Morton (1950), but in daylight to bleach the rhodopsin. Batches of fifteen retinas were placed in Polythene bottles and stored at $-20°$ until required. Eight batches were usually extracted at one time by modifications of the methods of Collins, Love & Morton (1952) and Wald & Brown (1951–2). After thawing, each batch of fifteen was vigorously shaken with 5 ml. of a 45% (w/v) solution of sucrose in 0·067 M phosphate buffer, pH 6·47, and the suspension filtered through a 40-mesh stainless-steel gauze, in the shape of a cone, placed in a glass funnel. The solid residue was shaken with a second 5 ml. sucrose solution and filtered, and the operation repeated a third time. The combined sucrose suspension was centrifuged at 1200 *g*, when the retinal rods remained in suspension and other cell debris was deposited. After removal by pipette, the supernatant liquid was diluted with five times its volume of 0·067 M phosphate buffer, pH 6·47, and the whole centrifuged at 20,000 *g* for 30 min at 5° in a Spinco ultracentrifuge, Model L. The deposit of rods was suspended in a small volume of phosphate buffer, distributed equally between four 25 ml. centrifuge tubes and again spun at 20,000 *g* for 30 min at 5°. The supernatant liquid was discarded and the deposit of rods was mixed with 8 ml. digitonin solution per tube and left for 1 h at room temperature. The mixture was then centrifuged at 20,000 *g* for 45 min at 5° and the slightly yellow, opalescent dispersion of opsin in digitonin solution was carefully poured off and stored at $-20°$.

(c) Preparation of vitamin A aldehyde. This work was carried out in a darkroom illuminated by a safelight containing a 40 W bulb and a filter transmitting light of wavelength greater than 605 mμ.

As reference substance, a crystal of pure 11-*cis* vitamin A aldehyde (from a sample kindly given by Professor G. Wald) was dissolved in two drops of ethanol and 2 ml. digitonin solution were added. Samples of 0·1 ml. of this stock dispersion were diluted as required.

The mixture of vitamin A isomers to be tested, in the alcohol form, was oxidized chemically to the aldehyde form. Samples of 50 μg were usually taken and dissolved in a mixture of 4·9 ml. *n*-hexane and 0·1 ml. ethanol. This solution was passed under reduced pressure in about 10 min through a 10 × 6 mm column of precipitated manganese dioxide (Attenburrow, Cameron, Chapman, Evans, Hems, Jansen & Walker, 1952). The column was washed with 2·5 ml. 8% ethanol in *n*-hexane and the combined eluate, containing about 70% of the vitamin A in the aldehyde form, was evaporated to dryness under reduced pressure. A considerable quantity of sterols was present when vitamin A from rat tissues was used, and the addition of 0·1 ml. ethanol did not always ensure solution of the solid matter. To the ethanol solution or dispersion, 5 ml. digitonin solution were added and the whole was left for 15 min. The digitonin precipitated further sterols from solution and these adsorbed a considerable amount of vitamin A aldehyde but, after filtration through a no. 4 porosity Allihn-type sintered-glass funnel, the clear filtrate usually contained enough vitamin A aldehyde in dispersion for the test.

(d) General method. The following procedures were also carried out in the darkroom with a red safelight, except when otherwise stated. The reaction was followed in a

Beckman model DU quartz spectrophotometer which, to conserve material, was adapted so that 1 ml. of solution sufficed for measurements. In front of the slit aperture, a hole 0·5 mm in diameter in a sheet of metal was arranged so that the maximum intensity of light fell on the photocell. The normal 1 cm light-path quartz cells were supported in the cell-holder on blocks of cork so that the light from the hole passed centrally through 1 ml. of liquid in the cells. Thus equipped, the spectrophotometer could be used with the tungsten-lamp light source at wavelengths down to 350 mμ. Once the cells were in the light-proof compartment of the spectrophotometer and other solutions in a light-proof box, the instrument could be worked in normal lighting.

The extinction of the dispersion of vitamin A aldehyde in digitonin solution was determined by mixing 0·5 ml. of the dispersion with 0·5 ml. digitonin solution and reading at 385 mμ against digitonin solution. The dispersion was diluted as necessary to give the required extinction (see below under (e)). A 2 ml. sample of the final dilution was added to 4 ml. ethanol and extracted with three 10 ml. volumes of light petroleum. The combined extracts were dried over anhydrous Na_2SO_4, the solvent was removed and chloroform added to the residue for the Carr–Price test, which gave the concentration of vitamin A aldehyde in the digitonin solution irrespective of the isomers present.

In an experiment, the control cell contained 1 ml. digitonin solution and the experimental cell 0·5 ml. of the dispersion of opsin and 0·5 ml. of the dispersion of vitamin A aldehyde, which were mixed together at a noted time. The cells were placed in the spectrophotometer compartment and left for 3 h at room temperature for the reaction to proceed. To break down fortuitous vitamin A-aldehyde complexes and combine with excess aldehyde, 0·1 ml. of a freshly prepared molar solution of hydroxylamine hydrochloride (brought to pH 6·5 with 6N aqueous NaOH) was then added to each cell in red light (Wald & Brown, 1951–2). After a further 5 min, the absorption curve of the solution between 460 and 540 mμ was determined. Any light-sensitive pigments were then bleached by placing the cells 15 cm from a 60 W clear glass electric-light bulb. Bleaching was usually complete after 2 min but an exposure of 8 min was given before the absorption curve was read again over the range 460–540 mμ. The difference curve obtained by plotting the differences of extinction before and after bleaching represented the absorption of rhodopsin or isorhodopsin produced in the reaction. The curve was analysed by the method of Hubbard (1955–6) to give the extinctions of rhodopsin and isorhodopsin present at their respective maxima.

(e) Standardization of opsin. For purposes of calculation, it was necessary to determine the ratio of the extinction of rhodopsin at 500 mμ to the extinction of 11-*cis* vitamin A aldehyde at 385 mμ which gave rise to the rhodopsin when opsin was present in excess. This ratio was determined by allowing a series of dispersions of pure 11-*cis* vitamin A aldehyde of decreasing concentration to react with the dispersion of opsin as described under (d). The extinction of rhodopsin thus determined was for a volume of 1·1 ml., since 0·1 ml. of hydroxylamine solution was added. Before determination of the ratio, the value for the extinction of the vitamin A-aldehyde dispersion, measured in 1·0 ml. as described under (d), had therefore to be adjusted by a factor of 10/11. Values of the ratio were found to increase with decreasing extinction of the

dispersion of 11-*cis* vitamin A aldehyde until a steady value was obtained, which varied between 1·3 and 1·7 for different preparations. This ratio is always below the theoretical value of 1·92 (Wald & Brown, 1956). The highest extinction of 11-*cis* vitamin A aldehyde to give this constant ratio was the highest extinction that could be used experimentally and was about 0·1. With dispersions containing mixtures of all-*trans* and 11-*cis* vitamin A aldehyde, the composition of which was approximately known (e.g. by the maleic-anhydride test), the concentration was adjusted so that the extinction due to the estimated content of the 11-*cis* isomer did not exceed this value. The ratio was usually determined in each experiment.

(f) Estimation of 11-*cis* vitamin A in a mixture of isomers. The extinction of the dispersion of the aldehyde form of the isomers (p. 117, (d)) was adjusted as described above, and the experimental procedure set out under (d) was followed. Two methods of calculating the percentage by weight of 11-*cis* vitamin A in a mixture were used. In both it was assumed that the same proportion of isomers was present in the original alcohol form as in the aldehyde form tested.

Method A was a direct comparison of the concentration of 11-*cis* vitamin A aldehyde present, derived from the extinction of rhodopsin produced, with the concentration of total aldehyde measured by the Carr–Price test. A value for 11-*cis* vitamin A aldehyde dispersed in digitonin solution of $E^{1\%}_{1cm}$ (385 mμ) = 746 was assumed (Wald & Brown (1953–4, 1956) give this value for 384 mμ) and the 'factor' was the ratio, ($E_{\text{rhodopsin}}$):($E_{\text{11-cis vitamin A aldehyde}}$), determined as described above, for the sample of opsin used. The calculation was then as follows:

$E_{\text{rhodopsin}}$ in 1·1 ml. in cell, as calculated under (d), (1)

$$\text{Concentration of 11-cis vitamin A aldehyde in cell} = \frac{(1)}{\text{factor}} \times \frac{10{,}000}{746}\ \mu\text{g/ml.}, \quad (2)$$

Concentration of total vitamin A aldehyde in dispersion in digitonin solution, determined by Carr–Price test, in μg/ml., (3)

$$\text{Concentration of total vitamin A aldehyde in cell} = (3) \times \frac{0{\cdot}5}{1{\cdot}1}\ \mu\text{g/ml.}, \quad (4)$$

$$\text{Percentage of 11-cis vitamin A aldehyde} = \frac{(2)}{(4)} \times 100.$$

Method B dispensed with the Carr–Price test, but assumed that the mixture of isomers consisted essentially of all-*trans* and 11-*cis* vitamin A. The molecular extinction coefficients given by Wald & Brown (1953–4, 1956), 38,300 for all-*trans* vitamin A aldehyde at 389 mμ and 21,200 for the 11-*cis* isomer at 384 mμ for dispersions in digitonin solution, were assumed to apply with negligible error to the wavelength 385 mμ. From these values, a curve (Fig. 1) was plotted relating the ratio,

($E_{\text{11-cis vitamin A aldehyde at 385 m}\mu}$):($E_{\text{total vitamin A aldehyde at 385 m}\mu}$),

to the percentage by weight of 11-*cis* vitamin A aldehyde in a mixture containing this isomer and all-*trans* vitamin A aldehyde. The method of calculation was then as follows, the 'factor' being the ratio, ($E_{\text{rhodopsin}}$):($E_{\text{11-cis vitamin A aldehyde}}$) as before:

$E_{\text{rhodopsin}}$ in 1·1 ml. in cell, as calculated under (d), (5)

$$E_{\text{11-}cis\text{ vitamin A aldehyde at }385\,\text{m}\mu} \text{ in } 1{\cdot}1 \text{ ml.} = \frac{(5)}{\text{factor}}, \quad (6)$$

$$E_{\text{total vitamin A aldehyde at }385\,\text{m}\mu} \text{ in } 1{\cdot}0 \text{ ml. as measured,} \quad (7)$$

$$E_{\text{total vitamin A aldehyde at }385\,\text{m}\mu} \text{ in } 1{\cdot}1 \text{ ml.} = (7) \times \frac{1{\cdot}0}{1{\cdot}1}, \quad (8)$$

$$\text{Ratio, } \frac{E_{\text{11-}cis\text{ vitamin A aldehyde at }385\,\text{m}\mu}}{E_{\text{total vitamin A aldehyde at }385\,\text{m}\mu}} = \frac{(6)}{(8)}.$$

The percentage of 11-*cis* vitamin A aldehyde was then read from the curve of Fig. 1.

This method was used only when an error occurred in the extraction of vitamin A aldehyde for the Carr–Price test, so that method A could not be used, and insufficient material was available for another determination.

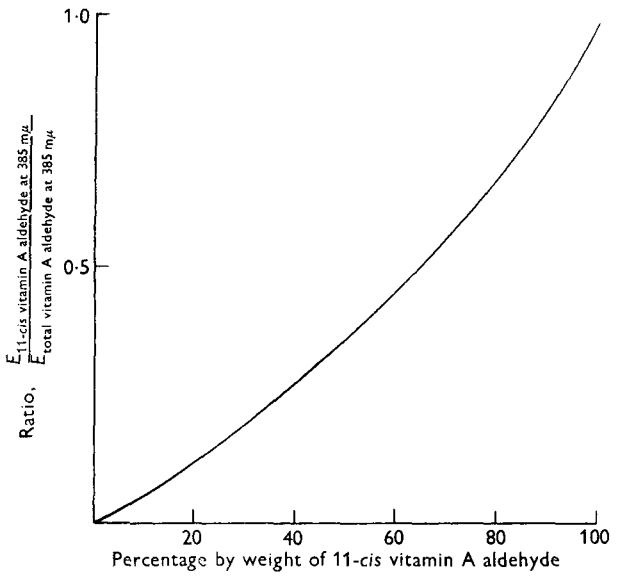

Fig. 1. Theoretical values of the ratio $(E_{\text{11-}cis\text{ vitamin A aldehyde at }385\,\text{m}\mu}):(E_{\text{total vitamin A aldehyde at }385\,\text{m}\mu})$ for dispersions in digitonin solution of mixtures of 11-*cis* and all-*trans* vitamin A aldehyde.

RESULTS

Precision of the opsin test

The precision was estimated by assaying known mixtures of all-*trans* and 11-*cis* vitamin A. Solutions of the two isomers were mixed to give 35 and 69% by weight of 11-*cis* vitamin A. From the assay, calculation by method A gave 35 and 73%, and by method B 33 and 67%. In the previous work on samples from marine Crustacea (Fisher *et al.* 1957), the percentages of 11-*cis* vitamin A were between 89 and 107% of the values for the percentages of 'slow'-reacting isomers in the maleic-anhydride test, which may possibly have included 13-*cis*, 9:13-di-*cis* and 11:13-di-*cis* vitamin A in addition to the 11-*cis* isomer. The precision of the test is therefore of the order of ±10%.

Dosing with 11-cis *vitamin A by mouth*

Liver storage. Storage from doses of 1, 2 and 5 mg 11-*cis* vitamin A was compared with that from similar doses of all-*trans* vitamin A. Only the results for 2 mg doses are considered here since at the other levels the general picture was the same. Fig. 2 shows the liver storage at intervals up to 7 days after doses of 2020 μg 11-*cis* vitamin A or 2040 μg of the all-*trans* isomer. The storage was at a maximum 4 days after dosing, that from 11-*cis* vitamin A being about 10% of the dose and one-quarter of the amount stored from all-*trans* vitamin A.

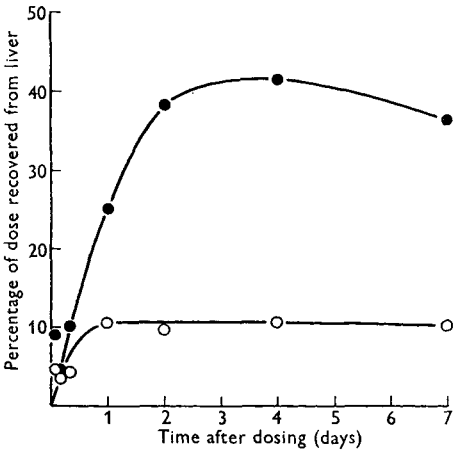

Fig. 2

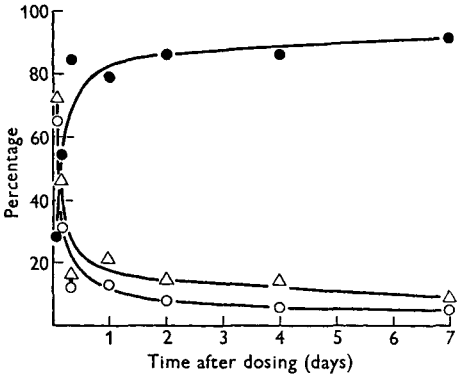

Fig. 3

Fig. 2. Vitamin A in the livers of deficient male rats given by mouth 2020 μg 11-*cis* vitamin A alcohol (○—○), or 2040 μg all-*trans* vitamin A alcohol (●—●).

Fig. 3. Percentage of 11-*cis* vitamin A (○—○), all-*trans* vitamin A (●—●), and 'slow'-reacting isomers (△—△), in the liver vitamin A of deficient male rats given by mouth 2020 μg 11-*cis* vitamin A alcohol.

Isomer composition of the liver vitamin A. Fig. 3 shows the percentage composition of the liver vitamin A after dosing with 2020 μg of the 11-*cis* isomer, in terms of 11-*cis* vitamin A, 'slow'-reacting isomers and all-*trans* vitamin A. The last was estimated as 'fast'-reacting isomers in the maleic-anhydride test, but since insignificant amounts of 9-*cis* vitamin A were found by the opsin test, all-*trans* vitamin A was the only 'fast'-reacting isomer present. Each point in Fig. 3 is the mean result for samples from three rats, but 2, 4 and 8 h after dosing, the maleic-anhydride and opsin tests were done on different samples, since the quantity of vitamin A from one liver was not sufficient for both tests. The percentage of 11-*cis* vitamin A in the liver was high 2 and 4 h after dosing, falling to 5% after 4 days. A similar curve was given by the 'slow'-reacting isomers, but the corresponding percentages were slightly greater. The curve for all-*trans* vitamin A was complementary to that for the 'slow'-reacting isomers and, from a low initial value at 2 h, the percentage content rose rapidly to a maximum of about 90.

With all-*trans* vitamin A (2040 μg), the percentage of this isomer in the liver vitamin

A remained constant at between 93 and 96 over 7 days. At 1 and 7 days, 3% of 11-*cis* vitamin A was found to be present, but this quantity is not significant within the precision of the opsin test.

Distribution in organs of vitamin A after a dose of the 11-cis *isomer.* One day after 4640μg of 11-*cis* vitamin A in twenty drops of oil had been given to a rat by mouth, the rat was killed and the vitamin A content of various organs examined. Only 26% of the dose was recovered, distributed as follows: liver 15%; kidneys 0·2%; contents of stomach, small intestine and caecum, and faeces 4·4%; walls of small intestine 1·2%; remainder of body, including contents of large intestine, 5·3%. The two other rats in the same group each had 11% of the dose in their livers. Extraction of the eyes of all three rats yielded very little vitamin A, at most 1μg in all.

Injection of 11-cis *vitamin A into the intestine*

Table 1 shows the results with 11-*cis* vitamin A placed in the small intestines of anaesthetized rats. Since no 9-*cis* vitamin A was found in these samples, the 'fast'-reacting isomers in the maleic-anhydride test were taken to consist of all-*trans* vitamin A. After 2 h, the total recovery of the dose was 50–60%, the amount falling to 33% after 4 h with the aqueous dispersion (Exp. 3) but being maintained with the oil emulsion (Exp. 4). The vitamin A was found mainly in the wall and contents of the small intestine, with little in the liver, although the storage and percentage of all-*trans* vitamin A in this organ were similar to those found after dosing by mouth (Figs. 2 and 3). The presence of all-*trans* vitamin A in the intestinal wall and contents indicated that conversion of the 11-*cis* isomer had taken place. The higher proportion of the all-*trans* form in the wall than in the contents suggested that the intestinal wall was the site of conversion.

The vitamin A was separated into ester and alcohol fractions in Exps. 3 and 4 and each was examined separately. In the intestinal wall, there was more ester than alcohol, but the proportion of all-*trans* vitamin A in each was about the same. The alcohol form was predominant in the intestinal contents, with very little ester. In the liver, the quantities of ester and alcohol were similar, but the alcohol fraction contained about twice the proportion of all-*trans* vitamin A found in the ester fraction. The values for the ester and alcohol fractions have been combined in Table 1 and, for all-*trans* vitamin A, they have been weighted for the quantity of vitamin A and the percentage of the all-*trans* isomer present in each fraction.

Control experiments with all-*trans* vitamin A instead of the 11-*cis* isomer showed no significant difference between the two isomers in the total vitamin A content of the intestinal wall or of the liver. The differences in liver storage from the two isomers were not yet apparent 2 and 4 h after dosing (cf. Fig. 2).

Intravenous injection of 11-cis *vitamin A*

Table 2 shows the recovery of vitamin A from the liver and blood after injection of aqueous dispersions of 11-*cis* vitamin A into the jugular vein of rats. A negligible amount of vitamin A remained in the blood after 2 h and even less after 4 h. Only a small amount of the dose appeared in the liver and most of it had been converted

Table 1. *Conversion of* 11-cis *into all-*trans *vitamin A in the small intestine of anaesthetized vitamin A-deficient rats*

(Mean values for two rats)

Exp. no.	Sex	Experimental conditions	Dose: Total vitamin A (μg)	Dose: All-*trans* vitamin A (%)	Time after dosing (h)	Intestinal wall: Recovery of dose (*a*) (%)	Intestinal wall: All-*trans* vitamin A in (*a*) (%)	Intestinal contents: Recovery of dose (*b*) (%)	Intestinal contents: All-*trans* vitamin A in (*b*) (%)	Liver: Recovery of dose (*c*) (%)	Liver: All-*trans* vitamin A in (*c*) (%)	Total recovery of dose (%)
1	♀	Intestine washed out. Dose in aqueous dispersion with Tween 40	1910	0	2	14	17	32	6	3	—	49
2	♂	Intestine washed out. Dose in aqueous dispersion with Tween 40	1340	3	2	26	14	26	11	3	40	55
3	♂	Intestine not washed out. Dose in aqueous dispersion with Tween 40	2020	0	2	33	5	24	9	3	41	60
			2020	0	4	25	18	5*	14*	3	38	33
4	♂	Intestine not washed out. Dose in oily solution emulsified with water	2140	0	2	7	16	41	9	0	—	48
			2140	0	4	12	15	54	5	2	27	68

* Value for one rat only.

into the all-*trans* form (the other 'fast'-reacting isomer, 9-*cis* vitamin A, was again absent).

The liver vitamin A in Exp. 6 was separated into ester and alcohol fractions. At 2 h the ester represented 57% of the total vitamin A, and at 4 h 66%. The proportions of all-*trans* vitamin A in each fraction were similar. In Table 2, the values for the two fractions have been combined.

Table 2. *Conversion of intravenously injected* 11-cis *vitamin A into the all-*trans *form by vitamin A-deficient rats*

(The dose, dispersed in 0·4 ml. water containing 10% (v/v) Tween 40, was injected into the jugular vein. Mean values for two rats)

					Liver	
Exp. no.	Sex	Dose* (μg)	Time after dosing (h)	Blood vitamin A (μg/ml. plasma)	Recovery of dose (*a*) (%)	All-*trans* vitamin A in (*a*) (%)
5	♀	1910	4	—	6·1	72
6	♂	2020	2	1·82	3·0	51
		2020	4	0·89	2·9	71

* All-*trans* vitamin A was absent from the doses.

DISCUSSION

This work was largely based on two experimental techniques for the identification and estimation of geometrical isomers of vitamin A. One, the maleic-anhydride test, has already been described and discussed in detail (Plack, 1956*b*). It enables the percentages by weight of two groups of isomers—'fast'-reacting (9-*cis* and all-*trans* vitamin A) and 'slow'-reacting (11-*cis*, 13-*cis*, 9:13-di-*cis* and 11:13-di-*cis* vitamin A)—to be estimated. Its precision depends on that of the Carr–Price test and is of the order of ± 5%. The other, the opsin test, is based on the work of Professor G. Wald and his colleagues. As used in the work reported here, it is specific for the estimation of 11-*cis* vitamin A, since no significant quantities of isorhodopsin, derived from 9-*cis* or 9:13-di-*cis* vitamin A, were ever formed.

In the preparation of opsin, the step involving tanning of the rods with potash alum (Wald & Brown, 1951–2) was omitted since it was found by experiment that, although tanning produced a purer preparation, there was a considerable loss of activity. Radding & Wald (1955–6) have shown that on both sides of the pH range 5·5–7·0 opsin rapidly loses its capacity to regenerate rhodopsin. The tanning step involves a pH below this range. In earlier experiments with this test (Fisher *et al.* 1957), the opsin was extracted from the rods from batches of fifteen eyes with 4 ml. of digitonin solution, and the maximum extinction of 11-*cis* vitamin A aldehyde that could be used in an experiment was about twice that with the large-scale preparation described here, which indicates that, with the increase in scale of preparation, less opsin was extracted from the same number of eyes. An increase in concentration of the opsin dispersion above that used in these experiments would increase the accuracy of the test. Hubbard (1958) recently reported some experiments on the thermal stability of aqueous solutions

of opsin (presumably dispersions in digitonin solution) and found that denaturation was half complete at 39·6° in 10 min, but combination with 11-*cis* vitamin A aldehyde increased the stability. The opsin dispersions prepared for my work suffered little or no loss in activity after storage for some months at −20°. They were stored in small volumes so that the numbers of thawings and exposures to room temperature were reduced to a minimum.

The efficiency of conversion of vitamin A alcohol into aldehyde varied with the preparation of manganese dioxide used. With material prepared by the method of Attenburrow *et al.* (1952), 90% conversion was sometimes obtained, but the usual value with crude extracts of vitamin A was about 70%. The aldehyde was not separated from residual alcohol since chromatography of the vitamin A aldehyde might involve some isomerization and vitamin A alcohol seemed only to interfere with the Carr–Price estimation of the aldehyde. At 664 mμ, the extinction of the blue colour due to vitamin A alcohol is 10% of its extinction at 620 mμ, so that with 70% conversion, the 664 mμ reading would be about 5% too high. The possibility of preferential conversion of one isomer of vitamin A into the aldehyde must also be considered, but the results with known mixtures of isomers (p. 119) seem to exclude it for the all-*trans* and 11-*cis* forms. Another possible source of error with natural extracts is the preferential adsorption of one isomer of vitamin A aldehyde on the sterol precipitate not taken up in ethanol or produced on addition of the digitonin solution (p. 116, (c)). A comparison of the proportions of 11-*cis* vitamin A and of 'slow'-reacting isomers in some previous results (see p. 119) indicates that this effect is negligible or non-existent.

The reaction between cattle opsin and 11-*cis* vitamin A aldehyde goes more or less to completion in 2 h (Wald & Brown, 1956). In my work, a temperature-controlled cell-holder for the spectrophotometer was not available and, in some earlier experiments, low results were obtained when the temperature of the darkroom was about 15°. These may have been due to the reaction's not being completed in 3 h. Since then, as described in the experimental section, a control reaction with 11-*cis* vitamin A aldehyde and opsin has been done with the experimental preparations as a check.

In a previous paper (Fisher *et al.* 1957), the effects of various chemical manipulations on the isomer composition of an extract containing predominantly 11-*cis* vitamin A were reported. The only serious factor was exposure to daylight; to counteract it, I carried out the chemical work in a darkroom illuminated with red light.

The vitamin A storage in the livers of rats 4 days after dosing with 2 mg of 11-*cis* or all-*trans* vitamin A alcohol was 10·5 and 41·5% of the dose (Fig. 2), so that liver storage from 11-*cis* was 25% of that from all-*trans*. This result agrees with the value of 23% for the relative biological potency of 11-*cis* vitamin A acetate in terms of all-*trans* vitamin A acetate (Ames, Swanson & Harris, 1955). These authors found two liver-storage assays and one growth assay of this isomer in fair agreement. My analysis of other parts of the body after a dose of 11-*cis* vitamin A showed no substantial storage other than in the liver.

One day after dosing with 2 mg 11-*cis* vitamin A alcohol, the liver vitamin A of a rat was very largely in the all-*trans* form (80%) and the proportion of this isomer had become 90% at 7 days after dosing. This finding is a clear indication of the conversion

of 11-*cis* into all-*trans* vitamin A in the rat. With a similar dose of all-*trans* vitamin A alcohol, this isomer accounted for 93–96% of the liver vitamin A in the period 1–7 days after dosing. These results are contrary to the findings of Ames *et al.* (1957), who gave all-*trans*, 9-*cis* and 9:13-di-*cis* vitamin A acetate to rats and found that with each the vitamin A stored in the liver, when extracted and given to other rats, had a biological potency of about 64%. This result indicated that, whatever isomer was given, the rat isomerized it to a mixture of isomers of more or less constant composition, of which all-*trans* vitamin A constituted less than 64%. Murray & Campbell (1958), who gave all-*trans* and 13-*cis* vitamin A to rats, also disagree with these authors.

The injection of 11-*cis* vitamin A dispersions into the small intestine of anaesthetized vitamin A-deficient rats resulted in some conversion into the all-*trans* form. In general, the proportion of all-*trans* was higher in the vitamin A from the intestinal wall than in that from the contents, which possibly indicates conversion in the wall, although the same effect might be produced by conversion in the contents and preferential uptake of the all-*trans* form. The values for liver storage and for the proportion of all-*trans* in the liver vitamin A were similar to those obtained after dosing by mouth. When the dose was placed in the intestine as a water emulsion of an oily solution, conversion still took place but was somewhat slower, as indicated by the figures for liver storage (Table 1). In their study of the isomerization of 13-*cis* vitamin A in vivo, Murray & Campbell (1958) found some conversion into the all-*trans* form in the stomach and a further increase in the proportion of all-*trans* in the intestinal wall. Workers on carotenoids have also found that isomerization of these pigments occurs in the digestive tracts of rats, chicks and hens (Kemmerer & Fraps, 1945; Deuel, Ganguly, Koe & Zechmeister, 1951).

The digestive tract seems not to be the only place where conversion of 11-*cis* into all-*trans* vitamin A can occur, since intravenous injection of the 11-*cis* isomer gave rise to all-*trans* vitamin A in the liver, though in small amounts. Little vitamin A remained in the blood 2 h after the injection and there would seem to be considerable destruction, as reported by Pollard & Bieri (1958).

These findings establish that the biological activity of 11-*cis* vitamin A is due to its isomerization predominantly to the all-*trans* form, and not to an inherent activity of the isomer itself. This same conclusion could be reached indirectly from the agreement between the liver-storage and growth assays of vitamin A, established by Ames *et al.* (1955) and Ames & Harris (1956). At the moment, there is some disagreement about the composition of the mixture of isomers stored in the liver from doses of one particular isomer, even from all-*trans*, but it is safe to say that all-*trans* vitamin A is the major component. The low biological activity of 11-*cis* vitamin A might result from an isomerization reaction 23% efficient followed by normal uptake of the all-*trans* vitamin A produced, or from a 23% uptake of 11-*cis* as compared with all-*trans* vitamin A, followed by complete conversion, or from a combination of the two. There are no definite pointers from the results reported here. The storage of 11-*cis* vitamin A in the liver 2 and 4 h after an oral dose of this isomer indicates that uptake can take place, but the large dose of 2 mg may have swamped the normal mechanism. From

Figs. 2 and 3, the total quantity of 11-*cis* vitamin A in the liver may be calculated, and the slight fall during the period of the experiment indicates a possible conversion into all-*trans* vitamin A in the liver. Since the values for the various time intervals are based on results for different rats, the evidence is not conclusive. In the experiments in which the dose was put into the tied-off intestine there was some conversion in the intestinal contents, which provides evidence for conversion followed by uptake of the all-*trans* form.

Whatever may be the mechanism, it seems unlikely that all animals will take up or isomerize 11-*cis* vitamin A with the same efficiency, so that the biological activity of the isomer for rats may be different from that for other species. The baleen whale, for instance, which probably obtains most of its vitamin A as the 11-*cis* isomer, may be better adapted than the rat to utilize this form and thus to store in the liver as all-*trans* vitamin A proportionately more than a rat given the 11-*cis* isomer.

SUMMARY

1. In a study of the conversion of 11-*cis* into all-*trans* vitamin A in the rat, the reactions of geometric isomers of vitamin A alcohol with maleic anhydride, and of those of vitamin A aldehyde with cattle opsin, have been used to estimate the quantities of the two forms of vitamin A.

2. Liver storage from a 2 mg dose of 11-*cis* vitamin A alcohol was 25% of that from the same dose of all-*trans* vitamin A alcohol.

3. Rats given 2 mg of 11-*cis* vitamin A alcohol by mouth finally stored 10% of the dose in their livers, of which 80–90% was in the all-*trans* form. About 4% by weight of the dose was recovered from the liver 2 and 4 h after dosing. After 2 h, 65% of this liver vitamin A was in the 11-*cis* form and after 4 h, 31%.

4. Aqueous dispersions, and aqueous emulsions of oily solutions, of 11-*cis* vitamin A alcohol placed in the tied-off small intestine of anaesthetized rats gave rise to all-*trans* vitamin A in the walls and contents of the intestine, and in the liver.

5. Aqueous dispersions of 11-*cis* vitamin A alcohol injected into the jugular vein of rats produced a small storage of vitamin A in the liver, most of which was in the all-*trans* form.

6. It is suggested that the biological activity of 11-*cis* vitamin A in the rat is due to its conversion predominantly into the all-*trans* form and that this conversion takes place in the small intestine, though other sites of conversion are also indicated.

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