Brit. J. Nutr. (1960), **14**, 315

The uptake of radioactive β-carotene in the dog heart-lung preparation

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(*Received* 27 *October* 1959—*Revised* 18 *March* 1960)

The intestine is recognized as the major site for the conversion of orally administered β-carotene into vitamin A in the rat (Glover, Goodwin & Morton, 1947; Thompson, Ganguly & Kon, 1947; Mattson, Mehl & Deuel, 1947), and the rate of conversion in washed intestinal loops of the rat *in situ* is from five to ten times that required for maximum life maintenance (Olson, 1959, and unpublished observations).

Nevertheless, other tissues seem able to carry out the conversion. β-Carotene in Tween suspension is effectively converted into vitamin A when given intramuscularly (Tomarelli, Charney & Bernhart, 1946) or intravenously (Bieri & Pollard, 1954). Furthermore, complete removal of the kidneys, liver, stomach, intestine, pancreas, adrenals, thyroid and parathyroid glands, gonads, head, or lungs (Bieri & Pollard, 1954; McGillivray, Thompson & Worker, 1956; Worker, 1956, 1957) does not appreciably affect the increase in the vitamin A concentrations of plasma or liver after intravenous injection of β-carotene, and hence the conclusion was reached that all tissues, rather than any specific organ, are able to carry out the conversion.

Of many organs studied, the lung has been particularly mentioned as a possible site of conversion by Kon, McGillivray & Thompson (1955), and the suggestion has been supported by their findings that intravenously injected β-carotene is rapidly deposited in the lung, that the vitamin A content of lung increases rapidly after β-carotene injection, and that the concentration of both β-carotene and vitamin A in the lungs falls rapidly after reaching the maximum.

These events might, however, be a result of complex interactions within the animal rather than an indication of the actual conversion of β-carotene into vitamin A within the lung, and hence a system was sought that would allow virtual isolation of the lung but still maintain its physiological integrity. The Starling heart-lung preparation seemed to be suitable for this purpose, and this investigation deals with the metabolism of radioactive β-carotene in the dog heart-lung system.

EXPERIMENTAL

Radioactive β-carotene suspension. Uniformly labelled β-[^{14}C]carotene was extracted with diethyl ether and ethanol from cells of the photosynthetic organism, *Chlorella pyrenoidosa*, which had been grown on $^{14}CO_2$ as the sole source of carbon (Ellner, 1959). The lipid extract was saponified, extracted with hexane, and the washed

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concentrated hexane phase was chromatographed on Woelm alumina grade 1. The β-carotene fractions were pooled and rechromatographed. In the final preparation 71% of the radioactivity was in β-carotene, as shown by repeated recrystallization of a sample with non-radioactive, pure, crystalline β-carotene.

Plasma suspensions of β-carotene were prepared with and without Tween 20 (polyoxyethylene sorbitan mono-acylate, Atlas Powder Co., Wilmington, Delaware). For the former, a hexane solution of β-carotene containing 5 or 20 μg and 18000 or 20000 counts/min was evaporated to dryness under nitrogen, dissolved in 0·2 ml acetone, and 0·2 ml Tween 20 and 0·9 ml water were added. The acetone was evaporated, and the Tween 20 suspension was added to 20 ml dog plasma. For the latter without Tween 20, the acetone solution of β-carotene was added directly to 20 ml dog plasma, and the acetone was evaporated under nitrogen at room temperature. Both the Tween-plasma and acetone-plasma suspensions were clear, yellowish solutions. Neither suspension adversely affected the heart-lung preparation in any apparent way. Much higher Tween 20 concentrations (40 ml/l. blood), however, caused rapid haemolysis and immediate cardiac arrest in the preparation.

Heart-lung preparation. Mongrel dogs of both sexes, weighing 12–18 kg, were fasted 24 h before the experiment. They were anaesthetized with sodium pentobarbital (30 mg/kg) and the bilaterally vagotomized heart-lung system was set up in the usual manner (Knowlton & Starling, 1912). About 800 ml of additional whole blood for use in the blood reservoir was collected from a donor dog under thiopental (30 mg/kg) anaesthesia. Sodium polyanhydromannuronic acid sulphate (Mannuronate, Wyeth Laboratories) at a dosage of 80 mg/kg body-weight was used as anticoagulant in both the donor and heart-lung dog. In all preparations a cardiac output was selected which was constant within the range 300–800 ml/min, and arterial pressures of 15–40 mm Hg were used to prevent pulmonary oedema. The separation of the heart-lung circulation from that of the body was almost complete. The blood level in the venous blood reservoir remained constant throughout the experimental period, and the blood flow from a polyethylene catheter inserted in the femoral vein was not more than would be expected from passive drainage from the lower portion of the body. In most instances very little pulmonary oedema was observed.

Experimental period. The plasma suspension of radioactive β-carotene was added to the blood reservoir of the heart-lung preparation, and 5 min were allowed for complete mixing. At that time and at convenient intervals during the subsequent 2–4 h period, blood samples of 50 ml were withdrawn and analysed. At the conclusion of the experiment, the lungs were excised and individually analysed.

Analysis of blood. After centrifugation, 20 ml plasma were removed and shaken well with 20 ml 95% (v/v) ethanol and 40 ml hexane (Skellysolve B, Skelly Oil Co., El Dorado, Texas) for 5 min. From the hexane layer, 20 ml were decanted and dried over anhydrous Na_2SO_4. A 5 ml portion of the dry hexane extract was evaporated, dissolved in 1 ml chloroform, and analysed for vitamin A by the Carr–Price reaction (Thompson, 1949) in a Spectronic 20 (Bausch and Lomb) Spectrophotometer at 620 mμ (method A), and another 5 ml portion was evaporated to dryness on an aluminium planchette and counted in a windowless counter. The remaining hexane

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solution was evaporated to a small volume, β-carotene was determined at 450 mμ, and the solution was chromatographed on deactivated alumina (Thompson, Ganguly & Kon, 1949). Fractions were analysed for vitamin A by the Carr–Price reaction (method B) and counts were made for radioactivity. In several instances the aldehyde derivative was made as shown below.

Analysis of lungs. Each lung was weighed, homogenized in 200 ml water, and saponified with 200 ml 40% (w/v) KOH in methanol for 2 h at 70° under nitrogen. The solutions were extracted three times with diethyl ether, and the ether extracts were washed with water until clear, dried over anhydrous Na_2SO_4, evaporated to dryness under nitrogen, and dissolved in 10 ml hexane. The β-carotene content was measured at 450 mμ, vitamin A by the Carr–Price reaction, and the radioactivity by counting a sample. The remaining hexane solution was chromatographed on deactivated alumina. The column fraction containing vitamin A was evaporated to dryness under nitrogen, dissolved in 5 ml hexane containing 2 mg vitamin A alcohol as carrier, and oxidized in 90% yield to vitamin A aldehyde by shaking with 10 mg activated MnO_2 (Attenburrow, Cameron, Chapman, Evans, Hems, Jansen & Walker, 1952) in hexane for 3 h at 25° in the dark. The aldehyde solution was filtered and chromatographed on deactivated alumina. The spectrum of each fraction and its radioactivity were determined. In some instances vitamin A aldehyde semicarbazone was prepared (Ball, Goodwin & Morton, 1948), purified by chromatography and the radioactivity was measured.

Fractionation of plasma with ammonium sulphate. From a beagle dog fasted for 18 h, 50 ml fresh blood were drawn into a syringe moistened with a 1% solution of the anticoagulant sodium polyanhydromannuronic acid sulphate. The blood was centrifuged and the clear plasma was pipetted off. To a 5 ml portion of plasma, 20 μg β-carotene in either 0·4 ml acetone or 0·4 ml 10% Tween 20 were added slowly, and the volume was brought to 10 ml with 0·9% NaCl solution. After a very light precipitate had been centrifuged off, the solutions were treated at room temperature with a saturated solution of ammonium sulphate. Precipitates which formed at ammonium sulphate saturations of 0–25, 25–46, and 46–68% were centrifuged, transferred to a mortar and ground several times with anhydrous Na_2SO_4 and 10 ml portions of 50% (v/v) ethanol in diethyl ether until all pigment was extracted. The extract was evaporated to dryness under nitrogen and dissolved in 5 ml $CHCl_3$. β-Carotene was measured at 450 mμ and vitamin A was determined by the Carr–Price reaction. Native plasma, which did not contain added β-carotene, was fractionated in a like manner. In other experiments in which large amounts of Tween were used, precipitates obtained by ammonium-sulphate fractionation did not sediment readily.

RESULTS

β-Carotene uptake in the heart-lung preparation. In early experiments, unlabelled crystalline β-carotene (350 μg) in acetone-plasma suspension was used. The results of a typical experiment are shown in Fig. 1. On the addition of β-carotene to the blood reservoir, the plasma β-carotene concentration rose quickly to a maximum.

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It is apparent that the β-carotene was evenly distributed in the blood within 5 min, which is in keeping with the calculated time required for complete mixing in the reservoir at the flow rate used. As discussed later, the immediate small increase in vitamin A level with β-carotene addition may be attributed to the reactivity of β-carotene and its oxidation products in the Carr–Price reaction rather than to formation of vitamin A. During the subsequent experimental period, the plasma vitamin A concentration remained constant while that of β-carotene decreased slightly but significantly. At the conclusion of the experiment, after 80 min, the lungs, which weighed 60 and 56 g, contained 13·5 and 10·0 μg β-carotene, and 8·3 and 6·8 μg vitamin A (method A), respectively.

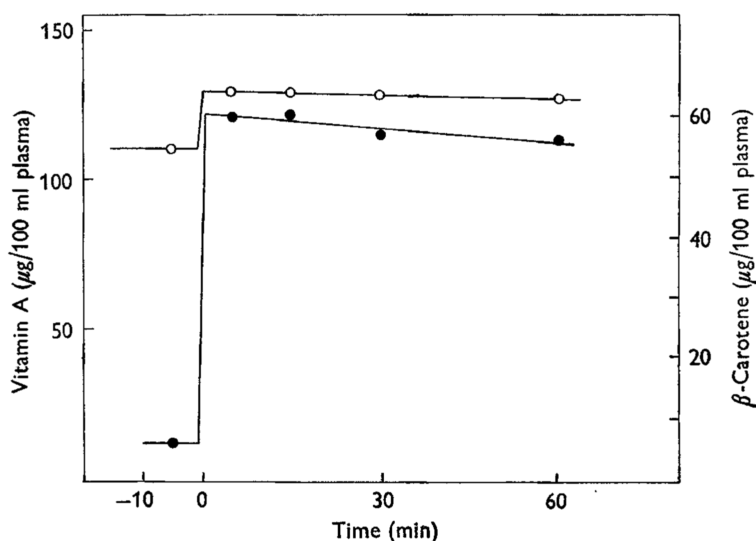

Fig. 1. Plasma vitamin A and β-carotene in the circulating blood of a dog heart-lung preparation after the administration of 350 μg β-carotene in acetone-plasma suspension. ○, vitamin A (method A); ●, β-carotene.

In order to determine whether β-carotene was taken up by the lung, or whether the amount found was due solely to blood present in the tissue, the ratios of β-carotene to vitamin A in fresh normal lung, blood plasma and lung from the heart-lung preparation were compared. Lungs of normal fasted dogs contained 4–7 μg β-carotene and 8–10 μg vitamin A/100 g fresh tissue, with β-carotene:vitamin A ratios of 0·5–0·7. The blood of normal dogs contains almost no β-carotene (ratio 0·1), and the β-carotene:vitamin A ratio of the plasma used in the heart-lung preparation was 0·45. The lungs of the heart-lung preparation, on the other hand, had ratios of 1·63 and 1·47, which clearly showed that preferential uptake of β-carotene had occurred. The total uptake in both lungs, after correction for the β-carotene of blood in the tissues, was 17 μg; the amount calculated to have disappeared from the circulating blood was approximately 25 μg. We were unable to account for the other 8 μg.

The effects were more pronounced when small amounts (5–20 μg) of β-[^{14}C]-carotene in acetone-plasma suspension were used. In a typical experiment (Expt 6)

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the total plasma radioactivity fell by 50% during the experiment, as shown in Fig. 2. The activity of the plasma β-[^{14}C]carotene ran parallel with the total activity, and was probably lower owing to the lack of quantitative recovery from the alumina column as well as to oxidation of some β-carotene during its isolation. The concentration of vitamin A in the plasma did not change during the experiment, and no radioactivity was found in the vitamin A fraction of plasma.

A large portion of the dose administered accumulated in the lung during the acetone-plasma experiments, as shown in Table 1. Chromatographic analysis showed that the major component was β-carotene, with some activity in the vitamin A fraction. Since the vitamin A fraction is often contaminated with oxidation products of β-carotene which are formed during purification, vitamin A aldehyde was prepared.

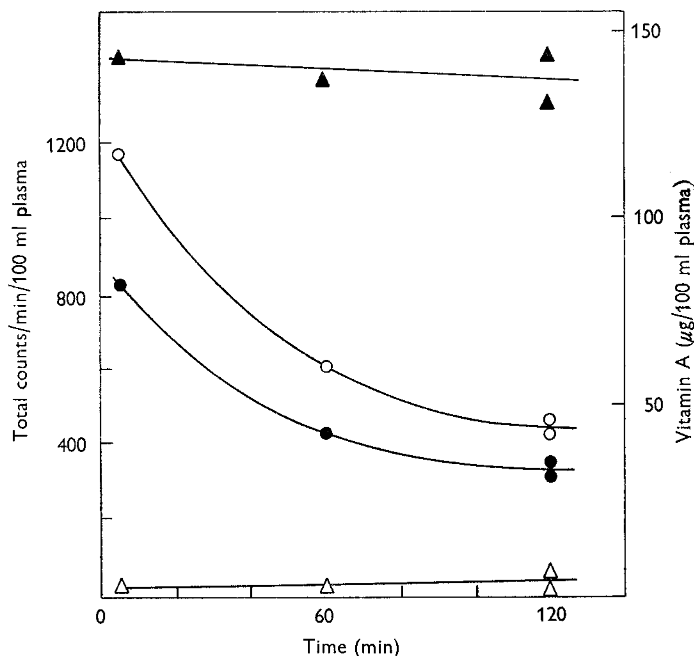

Fig. 2. Plasma radioactivity and vitamin A in the circulating blood of a dog heart-lung preparation after the administration of an acetone-plasma suspension of β-[^{14}C]carotene (10000 counts/min in 20 μg). Two fasted 14 kg dogs were used. Average cardiac output 600 ml/min; average blood pressure 15/8 mm Hg. ○, non-saponifiable fraction, total counts/min; ●, β-carotene, total counts/min; △, vitamin A, total counts/min; ▲, vitamin A, μg/100 ml (method A).

In most instances radioactivity in the aldehyde was negligible. In one experiment (Expt 6), however, the aldehyde contained about 100 counts/min, corresponding to 0·2 μg vitamin A.

When Tween-plasma suspensions of β-[^{14}C]carotene were used, the radioactivity in the blood remained high during the experiment and only minimum uptake by the lung was observed (Table 1, Expts 8 and 9). In both experiments, however, the β-carotene (counts/min): vitamin A ratio was significantly higher in the lungs than

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Table 1. *Radioactivity in lung tissue after introduction of β-[^{14}C]carotene into the circulating blood of dog heart-lung preparations*

Expt no.	Suspension	β-[^{14}C]carotene added: total counts/min	β-[^{14}C]carotene added: μg	Duration (min)	Distribution of radioactivity (total counts/min) in: Total non-saponifiable matter	β-Carotene fraction	Vitamin A fraction	Vitamin A aldehyde derived from vitamin A
5	Acetone-plasma	10 000	20	120	1 230	890	97	0
6	Acetone-plasma	10 000	20	120	5 870	5 050	510	108 (96)*
7	Acetone-plasma	10 000	20	180	2 460	2 290	81	10
8	Tween-plasma	20 000	20	180	250	140	75	0
9	Tween-plasma	18 000	5	180	590	480	35	10

* Vitamin A aldehyde semicarbazone.

Table 2. *Distribution of vitamin A and added β-carotene in ammonium-sulphate fractions of dog plasma*

Fraction	Vitamin A in native plasma (μg/100 ml): Expt 12*a*	Expt 12*b*	β-Carotene (μg), In acetone-plasma: Expt 13*a*	Expt 13*b*	In Tween-plasma: Expt 13*c*	Expt 13*d*
Original plasma	80·6	76·1	20	20	20	20
Precipitate from saturation with ammonium sulphate						
0–25 %	≤5	≤5	6·5	6·5	0	0
25–46 %	23·3	22·8	3·5	3·0	10	11·5
46–68 %	48·8	40·7	1·8	0·8	0·5	1·8
Supernatant liquid	≤5	≤5	< 1·0	< 1·0	< 1·0	< 1·0
Recovery	≤82·1	≤73·5	12·8	11·3	11·5	14·3

https://doi.org/10.1079/BJN19600041 Published online by Cambridge University Press

in blood; in Expt 8 the ratio was 13·3 in lung and 4·6–5·5 in blood, and in Expt 9 the ratio was 19·8 in lung and 10·8 in blood. Hence, the radioactivity of the lung cannot be explained on the basis of blood content alone, and a preferential uptake of β-carotene must have occurred. Conversion into vitamin A, however, was negligible; the aldehyde derivatives prepared from the vitamin A fractions of lung and plasma contained < 10 counts/min.

Fractionation of β-carotene suspensions. The aggregate size of β-carotene particles and the nature of their interaction with plasma proteins might be expected to influence the uptake of β-carotene by the lung in the heart-lung system. Since large differences in particle size or in binding to specific proteins might be revealed by step-wise precipitation of the plasma proteins, dog plasma to which β-carotene had been added in acetone or Tween was fractionated with ammonium sulphate.

From Table 2, it is clear that the distribution of β-carotene among the different protein fractions depended on the mode of suspension. With the acetone-plasma suspensions, the fraction precipitating at 0–25 % saturated ammonium sulphate contained the greater part of the β-carotene added. When Tween was present, however, the 0–25 % fraction was colourless and β-carotene appeared mainly in the 25–46 % fraction. Upon denaturing the protein with ethanol and centrifuging, the β-carotene remained with the protein precipitate, which suggests that β-carotene was bound to protein in both instances.

Ammonium-sulphate fractions of native plasma and of the Tween-plasma and acetone-plasma suspensions were analysed for vitamin A as well. The results with native plasma are given in Table 2. With all three the distribution was the same, one-third to one-half of the vitamin A being found in the 25–46 % (globulin) fraction. A similar distribution of vitamin A was reported to occur in the plasma of chickens, cows and pigs (Ganguly, Krinsky, Mehl & Deuel, 1952).

DISCUSSION

In the past, studies on the conversion of β-carotene into vitamin A in specific organs have been hampered by the difficulty of demonstrating the transformation in vitro. Consequently, information regarding organs (other than the intestine) which carry out the conversion has been gathered from studies on the accumulation of β-carotene and vitamin A in various tissues of intact animals after dosing with β-carotene, and from an examination of the vitamin A content of blood and other tissues after the surgical removal of one or more organs. Clearly, these approaches are indirect; the accumulation of carotenoids and vitamin A in a given tissue may be unrelated to the site of conversion, the excision of a major organ may unpredictably affect the metabolic activities of an animal, and small changes in the apparent levels of vitamin A in the blood may not be a good measure of vitamin A formation.

In order to investigate directly the role of the lung in β-carotene metabolism, the heart-lung preparation seemed to be an excellent tool. The essential requisites of tissue isolation and physiological integrity were met and, in addition, the processes of β-carotene uptake, vitamin A formation, and vitamin A release into the blood could

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be independently assessed. Furthermore radioactive β-carotene of high specific activity can be used at very low concentrations and without appreciable dilution by β-carotene already present in the blood. The formation of as little as 0·05 μg of radioactive vitamin A from β-carotene was detectable under these conditions.

In our experiments a striking difference was noted in the behaviour of the two suspensions used; namely, that uptake of β-carotene by the lung was much greater from acetone-plasma suspensions than from Tween-plasma suspensions. Kon *et al.* (1955) similarly found that colloidal β-carotene, injected intravenously into rats, rabbits and calves, was more rapidly removed from the blood and concentrated in the lungs and liver than β-carotene dispersed in Tween 40. Concentrations of Tween detergents similar to those used in this investigation invoke in the canine family a general allergic response which is characterized by a fall in blood pressure, flushing, urticaria and vomiting (Krantz, Carr, Bubert & Bird, 1949). Since Kon *et al.* (1955) found that the uptake of β-carotene in rats and rabbits, which tolerate Tween, was similar to that in calves, which react allergically, it is unlikely that this factor significantly affected the results. Rather, these workers suggested that the uptake of β-carotene was a function of particle size, and our present findings on ammonium-sulphate fractionation of plasma containing carotene support their interpretation. In every instance β-carotene suspended with acetone appeared mainly in the 0–25% fraction, whereas Tween-suspended β-carotene was found largely in the 25–46% fraction. In both, carotene was bound to protein and it is possible that the attachment to a specific protein as well as the size of the complex particle are important factors in determining the fate of β-carotene in plasma. The existence of specific carrier proteins for vitamin A and carotenoids in plasma and liver has been suggested (Krishnamurthy, Mahadevan & Ganguly, 1958; Ganguly & Krinsky, 1953; High & Wilson, 1956). It is difficult to ascertain how closely the Tween and acetone suspensions used in the present study resemble natural complexes of β-carotene in plasma, but there is no question that their behaviour in the heart-lung preparation is like that of similar preparations which were intravenously injected into whole animals.

Formation of vitamin A in our preparations was negligible. In the lung less than 0·05 μg vitamin A was found in four experiments out of five, but in one 0·2 μg was found. The possibility that vitamin A was being excreted into the blood as quickly as it was formed can be ruled out, since radioactive vitamin A was never detected in the circulating blood. It should be noted, however, that a total of 0·2 μg might escape detection in plasma analysis. Pollard & Bieri (1958) have recently shown that β-carotene and vitamin A are rapidly destroyed by haemolysed red cells, but in our experiments very little haemolysis occurred and the concentration of vitamin A in the plasma remained constant during the experiments. Finally, the normal concentrations of β-carotene in dog lung and plasma are low and, hence, appreciable dilution of the dose administered is not a serious consideration. If several assumptions are made, the possible physiological role of the lung in vitamin A formation can be roughly estimated. If a minimum daily requirement of 7 μg vitamin A/kg is accepted for the dog (Moore, 1957), if the physiological rate of conversion of β-carotene into vitamin A is relatively constant, and if the lungs are fully active under the experimental condi-

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tions used, then less than 5% of the required daily dose of vitamin A would be provided by the cleavage of β-carotene in the lung. In this regard it is interesting that dog heart-lung preparations are also unable to synthesize cholesterol from radioactive acetate, whereas rapid cholesterol formation occurs in the heart-lung-liver system (Tennent, Zanetti, Atkinson, Kuron & Opdyke, 1957). It must be noted, however, that only dogs were used in this study, and that species differences in β-carotene metabolism have been reported (Kon *et al.* 1955).

In the early stages of the present investigation, the determination of vitamin A was rendered difficult by the instability of β-carotene during the isolation procedure. Even after the addition of non-radioactive carrier β-carotene, as much as 10% of the dose given was oxidized to a product which accompanied vitamin A alcohol in chromatography on deactivated alumina. This oxidation product gave a blue colour in the Carr–Price reaction and was probably responsible for the apparent slight increase in plasma vitamin A content which was found in early experiments (Fig. 1). The difficulty was overcome by oxidizing vitamin A alcohol with manganese dioxide to vitamin A aldehyde, which could be readily separated from the β-carotene artifacts on alumina, and which gave a quantitative measure of the amount of vitamin A formed. On conversion into the semicarbazone, the specific activity of the derivative agreed closely with that of the aldehyde. Since the amount of vitamin A actually formed in these and other experiments was only a small fraction of the oxidized β-carotene artifact, measurements by chemical or radioactive methods of vitamin A in eluates from an alumina column were seriously in error unless further purification of vitamin A derivatives was carried out.

With the same methods attempts are now being made in this laboratory to investigate directly the role of other organs in the conversion of β-carotene into vitamin A. The recent observation that β-carotene in the presence of sodium glycocholate is readily converted into vitamin A by longitudinally cut intestinal sections in vitro (Olson, 1960) may open the way to a series of studies with other tissues in vitro.

SUMMARY

1. The metabolism of radioactive β-carotene was studied in the dog heart-lung preparation, which possesses the requisites of virtual organ isolation and of physiological integrity. Methods were developed for the accurate measurement of small amounts of radioactive vitamin A.

2. Radioactive β-carotene in acetone-plasma suspension was rapidly concentrated from the blood into the lung of a dog heart-lung preparation. When Tween 20 was used as the suspending agent, however, very little β-carotene accumulated in the lung.

3. β-Carotene suspended in dog plasma with acetone was mainly in the protein fraction precipitated by 25% saturation with ammonium sulphate, whereas Tween 20-dispersed β-carotene appeared largely in the 25–46% fraction. Vitamin A was found in the 25–46 and 46–68% fractions.

4. In four experiments out of five, less than 0·05 μg vitamin A was formed from

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5–20 μg β-carotene in the heart-lung preparations. In the fifth, 0·2 μg was formed. It is concluded that the lung is probably not an important organ for conversion of β-carotene into vitamin A.

This investigation was supported by PHS research grant A-1278 from the Committee on Arthritis and Metabolic Diseases of the National Institutes of Health, U.S. Public Health Service.

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Printed in Great Britain

https://doi.org/10.1079/BJN19600041 Published online by Cambridge University Press