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Received January 9, 1961. P.S.E.B.M., 1961, v107.

In vitro Metabolism of C-14 Labeled β-Carotene.* (26626)

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Previous studies in this laboratory(1) and others(2,3) on the *in vivo* metabolism of C-14 labeled β-carotene have indicated that radioactivity in the rat is found in the non-saponifiable fraction, fatty acid and water soluble fractions isolated from liver and extra-hepatic tissue. The expired CO_2 is also found to be radioactive.

It has been well demonstrated that β-carotene is converted to Vit. A by the intact animal but it is questionable if such a conversion can be demonstrated by isolated tissue preparation. In view of this possible difference between *in vivo* and *in vitro* metabolism of β-carotene it seemed worthwhile to investigate the *in vitro* metabolism of C-14 labeled β-carotene.

Materials and methods. The β-carotene† used in these experiments was produced by growing *Phycomyces blakesleeanus* in a suitable C-14 acetate medium(4). Carotene samples used were recrystallized from methanol and petroleum ether to a constant specific activity and they had an $E^{1\%}_{1\,cm}$ of 2535 at 450 mμ in light petroleum ether. Booth(5) suggests that 2550 is the representative value for pure β-carotene.

Carotene suspensions were prepared by using 40 mg of Tween 80 and an appropriate volume of buffer solution. The amount of carotene used in each incubation is given in the tables.

All animals were fasted 24 hours before use. Wistar strain, white female rats, weighing between 110-125 g, two rabbits weighing approximately 2 lb each, and four 2-week old chicks were used in these experiments. Animals were decapitated and allowed to bleed freely. The organs were quickly removed, blotted, and various types of homogenates prepared.

Whole homogenates were prepared by taking one-gram samples of organ and homogenizing it for 90 seconds in 2.5 ml of cold 0.25 M sucrose with a loose fitting glass Potter-Elvehjem homogenizer. Cell-free homogenates were prepared by centrifuging whole homogenates, as prepared above, at 800 × g at 0°C for 10 minutes and the supernatant fraction was used. Protein content of the latter fractions was determined by the method of Siekevitz(6).

Mitochondria samples equivalent to one gram of tissue were prepared according to the method of Schneider and Hogeboom(7) and suspended in 2.5 ml of 0.25 M sucrose.

Each standard incubation mixture contained 2 ml of a carotene suspension (see tables for amount of carotene used) in Tris buffer (veronal, Table I), 2 ml of tissue homogenate (representing 1 g of tissue or its equivalent) suspended in 0.25 M sucrose and 6 ml of 0.2 M Tris hydrochloride, pH 7.5 (veronal buffer pH 8.5 used for experiments in Table I). Two mg of penicillin G and

* Supported by a grant from Nat. Vitamin Foundation, New York.

† The labeled carotene was made available by a grant from Rockefeller Foundation to Dept. of Biochemistry and Plant Pathology, West Virginia Univ.

TABLE I. Destruction of C-14 Labeled Carotene and Incorporation of Radioactivity into Sterols, Saponifiable Material and Steam-Distillable Compounds by Whole Homogenates of Rat Organs.

The incubation media contained 800 μg (50,000 DPM) of C-14 labeled carotene, one g of whole organ homogenate in 10 ml of veronal buffer, pH 8.5 plus 0.05 M α-tocopherol and 0.1 M ascorbic acid. Flasks were shaken anaerobically in a water bath at 37° for 6 hr. All organs except adrenals came from the same rat. One g of the adrenal gland was obtained from pooled rats of similar strain, age and weight.

Organs used	Carotene recovered, μg	% destruction	Total N.S.*	DPS†	SF‡	SDF§	% activity recovered
			DPM				
Kidney	720	10	47,500	100	800	70	97
Skeletal muscle	680	15	47,000	110	1000	60	96
Pancreas	688	14	47,200	108	900	70	96
Adrenals	640	20	47,000	150	1500	80	97
Small intestine	656	18	46,800	112	1000	75	96
Spleen	672	16	47,100	107	1400	70	97
Liver	680	15	45,900	200	1500	85	95
No tissue	776	3	49,000	0	0	0	98

* Non-saponifiable material. † Digitonin-precipitable sterols. ‡ Saponifiable fraction. § Steam-distillable fractions.

1 mg of streptomycin phosphate were added to each homogenate, placed in a 50 ml Erlenmeyer flask and shaken in water bath at 37° for 6 hours. Determinations were made in duplicate.

The non-saponifiable fraction (NSF) was obtained by saponifying with 10 ml of 2 N alcoholic KOH for 2 hours, adjusting the alcohol concentration to 50%, and extracting with light petroleum ether. To insure complete separation of the non-saponifiable fractions the above procedure was repeated and appropriate extracts combined.

Digitonin-precipitable sterols (DPS) were isolated from the non-saponifiable fractions according to the procedures of Kelsey(8). Extinction values of the petroleum ether solution from the non-saponifiable fraction were measured at 450 mμ and amount of carotene calculated.

After the non-saponifiable material was extracted, the saponifiable fraction (SF) was prepared according to the procedure of Srere *et al.*(9). The extracted saponified product was repeatedly resaponified, acidified, extracted and washed to a constant specific activity. The saponifiable content was determined by weight.

The remaining acidified water soluble material was subjected to steam distillation and the steam-distillable fractions collected (SDF).

Aliquots of the above fractions were oxidized to CO_2, counted in a Model 6000 Dynacon Electrometer and reported in disintegrations per minute (DPM) per flask. Technical difficulties made it impractical to count the water soluble residue remaining after steam distillations.

Suitable controls were run with each set of experiments. One set of controls was run without tissue and another set used tissue previously heated to 100°C for 5 minutes. In these controls no significant radioactivity was detected in the digitonin-precipitable sterols, saponifiable material or steam-distillable fractions. Carotene recovery in the controls was approximately 98%.

The CO_2 liberated (Table III) was measured by connecting the incubation flasks directly to the gas chamber of the Dynacon. The entire system was under reduced atmospheric pressure throughout the incubation period. The pH of the mixture at end of incubation was 6 and addition of acid did not liberate additional labeled CO_2.

Results. The destruction of carotene by various whole tissue rat homogenates incubated for a 6-hour anaerobic period ranged between 10 and 20% (Table I). Preliminary studies showed that shorter incubation time resulted in less carotene destruction and that more carotene was destroyed when larger amounts of tissue were used. Adrenal and

TABLE II. Effect of Adding Various Cofactors to Cell-Free Rat Liver Homogenates on Destruction of Carotene.

Each incubation mixture contained 388 μg of carotene and a cell-free homogenate prepared from one g of rat liver (156 mg of protein). The medium was buffered with 0.2 M Tris hydrochloride to a pH 7.5 and made to a final vol of 10 ml. When cofactors were added their concentrations were: .05 M α-tocopherol; .1 M ascorbic acid; .0008 M DPN; .0015 M ATP; .0048 M $MgCl_2$ and .03 M nicotinamide. All incubation flasks were shaken aerobically in a water bath at 37° for 6 hr.

Incubation conditions	Carotene recovered, μg	% destroyed
Cell-free homogenate + Tris buffer	347	10.5
Idem + ascorbic acid	349	10.0
" + α-tocopherol	352	9.3
" + nicotinamide	347	10.5
" + ATP	340	12.4
" + DPN	342	11.9
" + $MgCl_2$	347	10.5
" + ATP + DPN + $MgCl_2$ + nicotinamide	328	15.5
" + α-tocopherol + ascorbic acid + ATP + DPN + $MgCl_2$ + nicotinamide	329	15.2

intestinal homogenates showed the greatest destructive activity. More activity was found in the saponifiable portion than in the digitonin-precipitable sterol or steam-distillable fractions.

To acquire optimum conditions for carotene destruction various cofactors were added to cell-free liver homogenates (Table II). The combined addition of ATP, DPN, nicotinamide and $MgCl_2$ produced maximum carotene destruction over a 6-hour period. The amount of carotene destroyed was approximately the same at a pH of 7.4 or 8.5.

The destruction of β-carotene and incorporation of radioactivity into the digitonin-precipitable sterols, saponified material and steam-distillable fractions by cell-free homogenates were not appreciably different for either aerobic or anaerobic conditions.

Equivalent amounts of whole liver homogenate, cell-free rat liver homogenate, and mitochondria appeared to be equally effective in destroying carotene and in their ability to incorporate radioactivity into the different isolated fractions.

Whole liver homogenates from several different species exhibited little difference in their ability to metabolize carotene.

The incubation of cell-free rat liver homogenate with labeled β-carotene produced some labeled CO_2 (Table III).

All measurements reported in tables are averages from duplicate incubations. The maximum variation between duplicate samples for carotene determination was 5% and 10% for all radioactive measurements in terms of DPM.

Discussion. The incubation of C-14 labeled β-carotene with various tissue preparations resulted in a negligible oxidation of carotene to CO_2 and incorporation of radioactivity into saponifiable material, digitonin-precipitable sterols, and steam-distillable compound(s). The saponifiable material from several incubation experiments was combined and the lead salts isolated according to the technic of Twitchell(10). The lead salts so isolated were found to have appreciable radioactivity.

It is recognized, in reporting the per cent carotene destroyed in the incubation experiments, that these values may not represent the amount of pure or unaltered carotene remaining after incubations. Since the amount of carotene present is determined spectrophotometrically at 450 mμ, it is possible that some early degradation product may also be affecting the light absorption at this wave length. The amount of yellowish non-saponifiable material present in rat liver did not appreciably affect the recovery of carotene

TABLE III. Liberation of CO_2 by Anaerobic Incubation of Cell-Free Rat Liver Homogenate.

The incubation mixture contained 500 μg (70,000 DPM) of C-14 labeled carotene, cell-free rat liver homogenate from one g of rat liver (170 mg protein), 0.2 M Tris hydrochloride buffer at pH 7.5 to a vol of 10 ml and the following added: .0048 M $MgCl_2$; .03 M nicotinamide; .0008 M DPN; and .0015 M ATP.

Incubation time, hr	CO_2 released DPM
1	30
2	180
3	60
4	40
5	20
6	0
Total period	330

as demonstrated by the approximate 98% recovery of added carotene to various controls.

The examination of radioactivity of the non-saponifiable fraction after 6 hours of incubating carotene with cell-free liver homogenate reveals considerable activity in a fraction which does not contain carotene or digitonin-precipitable sterols. It was not possible under these conditions to recover C-14 labeled Vit. A. Work is now in progress to isolate and characterize the various radioactive products present in this non-saponifiable fraction.

The liberation of $C^{14}O_2$ early in the incubation periods indicates that some cleavage of the carotene molecule takes place early in the degradation phase.

Addition of ATP, DPN, nicotinamide and $MgCl_2$ accelerated the destruction of carotene. Although there was considerable variation between one rat liver and another, the addition of these cofactors did accelerate the destruction of carotene by any one liver preparation. The amount of carotene destroyed by cell-free rat liver homogenates appeared to be approximately the same under aerobic and anaerobic conditions.

Summary. Ten to 20% of β-carotene was destroyed by incubating C-14 labeled β-carotene with various tissues from the rat. All tissues studied had the ability to transfer radioactivity from β-carotene to sterols, saponifiable material and steam-distillable compound or compounds. ATP, DPN, nicotinamide and $MgCl_2$ were cofactors that increased β-carotene destruction by cell-free homogenates. A negligible amount of CO_2 was liberated during incubation.

The author thanks Mrs. Phyllis Spiker for technical assistance.

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Received January 18, 1961. P.S.E.B.M., 1961, v107.

Action of Synthetic Lysine Polypeptides on Isolated Guinea Pig Ileum.* (26627)

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Synthetic lysine polypeptides or polylysines are polycations which readily interact with many polyanionic substances. *In vitro,* they agglutinate bacteria(1,2), viruses and virus deoxyribosenucleic acid(3), and red blood cells(4,5). Lysine polypeptides inhibit pepsin(6) and fibrinolysis(7), but enhance conversion of fibrinogen to fibrin(8), and are hydrolyzed by trypsin(9). *In vivo,* polylysine preparations possess antiviral(10) and

* This report is based on a portion of a Ph.D. thesis by T. Richardson and was supported in part by research grant from Nat. Microbiol. Inst., N.I.H., U.S.P.H.S., and the Research Committee, Graduate School, Univ. of Wisconsin, from funds supplied by Wisconsin Alumni Research Foundation. Approved for publication by Director of Wisconsin Agr. Exp. Station.

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