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American Cockroach (*Periplaneta americana*) Synthesizes Carotenoids from the Precursor [^{14}C]Mevalonic Acid Pyrophosphate

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Abstract—The level of an important carotenoid (β-carotene) in the gut of *Periplaneta americana* depends on the content of the carotenoid in food: a carotenoid-fortified diet causes accumulation of β-carotene up to 10 μg/g wet weight, while on a carotenoid-deficient diet the level of this substance is low (~0.7 μg/g wet weight). In the eye, in contrast to the gut, a constant level of β-carotene (1.3-1.4 μg/g wet weight) is found regardless of the diet. This phenomenon remained unchanged over three years of feeding of the cockroaches with the carotenoid-deficient diet, suggesting that *P. americana* produces carotenoids by *de novo* biosynthesis. This suggestion was confirmed in experiments using intraperitoneal injection of the exogenous carotenoid biosynthesis precursor [^{14}C]mevalonic acid pyrophosphate followed by extraction of carotenoid and chromatographic purification of the labeled product. Injection of 3.4 nmoles [^{14}C]mevalonic acid pyrophosphate transiently increased the β-carotene content in eyes on days 2 and 4 after injection of the label. Purification of radiolabeled carotenoids from eye and gut by the transfer of carotenoids into a less polar solvent, alkaline hydrolysis (saponification), and chromatography on alumina and cellulose columns decreased the specific radioactivity to a constant level that cannot be further decreased by repeated chromatography. The elution profile of these purified preparations of β-carotene after chromatography is characterized by coincidence of symmetric peaks of count and absorption. We suggest that to create the optimal carotenoid concentration in the eye, *P. americana* uses two biochemical mechanism: 1) it accumulates carotenoids in reserve in the gut when abundant supplies of carotenoids are available in the diet; 2) it synthesizes carotenoids *de novo* when its food is deficient in these compounds.

Key words: American cockroach, *Periplaneta americana*, accumulation and biosynthesis of carotenoids, column chromatography on alumina and cellulose, [^{14}C]mevalonic acid pyrophosphate, β-carotene, eye, gut

It is generally accepted that carotenoids are the metabolic precursors of visual pigment chromophores. In insects a clear correlation between chemical structures of carotenoids and chromophores (retinoids) has been found: in eyes with 11-*cis*-3-hydroxyretinal chromophore, xanthophylls (zeaxanthin, lutein, or β,β-cryptoxanthin) usually dominate, and a correspondingly high level of β-carotene in the eye occurs when the visual pigment chromophore is 11-*cis*-retinal [1]. According to present knowledge insects, like animals in general (including human beings), cannot synthesize carotenoids *de novo* and therefore depend on carotenoids in their diet [1]. Numerous experiments on several species of *Diptera* and *Lepidoptera* have shown that these insects, when reared on carotenoid (retinoid)-deficient diets, have drastic changes in their visual system: visual sensitivity is diminished by several log units [2-4], rhodopsin (and the apoprotein opsin) content is reduced [4-6], and two proteins relevant to visual function, phospholipase C and retinoid binding protein, are decreased [7]. Most of these visual deficits can be prevented or completely abolished by carotenoid (retinoid) "therapy" including appropriate carotenoids (retinoids) in the diet or by introducing these substances directly into the eye [4, 6, 8, 9]. Recent reports widely discuss the role of carotenoids and retinoids at the level of opsin gene transcription, namely their action on the synthesis of opsin mRNA in *Diptera* [7, 10-12]. The use of various *Drosophila* mutants with genetic defects in the eye demonstrates a new approach to the study of chromophore metabolism in insects [13, 14].

Studies of the visual system in cockroaches is of interest since these evolutionarily ancient animals might possess specific biochemical mechanisms that could be retained, lost, or functionally modified during evolu-

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tion. Therefore, the study of the relationships between carotenoids and the visual system in *Periplaneta americana* has particular interest. However, before studying the role of carotenoids as precursors of the chromophore of visual pigments in insects, we should answers some questions. These are the following. 1. Does *P. americana*, like some other species of *Diptera* and *Lepidoptera*, depend on exogenous carotenoids, and can it accumulate them? 2. What organs and tissues accumulate carotenoids? 3. What are the quantitative and qualitative characteristics of carotenoids at the sites of their accumulation under surplus and deficiency in the diet? 4. How long can a colony of cockroaches exists on a carotinoid-deficient diet? 5. Can *P. americana* synthesize endogenous carotenoids from the exogenous radiolabeled precursor [^{14}C]mevalonic acid pyrophosphate? 6. What is the chemical nature of the labeled substance after injection of a radiolabeled precursor? These questions are addressed and answered in this communication.

MATERIALS AND METHODS

Wild-type and white-eyed *Periplaneta americana* colonies were used in our study. The white-eyed animals have no screening ommochrome pigment in their ommatidia and are characterized as simple autosomal recessives [15]. The cockroaches were maintained on a natural photoperiod cycle at 25°C. Two basic diets were used to produce two different levels of carotenoids: carotenoid-deficient (white bread, oat flakes, sugar, water) and carotenoid-fortified (white bread, oat flakes, sugar, carrot juice, water).

The radiolabeled carotenoid precursor 5-pyrophosphate R[5-^{14}C]mevalonic acid (Amersham, England, specific activity 58 mCi/mmol) was used. The label in 10 μl (0.2 μCi, 3.4 nmoles) was injected laterally between the third and fourth abdominal segments of the cockroach using a microsyringe. For controls, animals of the same size were injected with the same volume of saline. Animals injected with labeled precursor were used for carotenoid extraction on the morning of day 1, 2, 3, 4, and 7. All procedures of extraction, saponification, and purification of carotenoids by adsorption chromatography were carried out under dim red light to prevent the photodestruction of carotenoid. Animals (injected or controlled) were killed by decapitation, and the eyes were removed with a sharp razor and collected in a small plastic tube. The body was cut longitudinally, and the gut was transferred into saline and perforated with a scissors. The gut tissue was separated from the gut contents by washing carefully with saline, and then the tissue was dried on filter paper. The collected tissues (10-120 eyes, 5-60 guts) were weighed and kept at −15°C until analysis. The frozen tissues were allowed to thaw and ground to powder with anhydrous Na_2SO_4 in a small mortar. The powder was extracted six times with 1-2 ml of acetone. To the combined acetone extracts, 2-4 ml hexane or petroleum ether (40-70°) and 2-3 ml of 5% NaCl were added, then the mixture was gently stirred and centrifuged at 5,000*g* for 5 min at room temperature. After centrifugation, the epiphase was transferred to a tube and the aqueous phase was extracted three times with hexane (or petroleum ether). The combined epiphase was washed three times with 5% NaCl to remove acetone, concentrated under vacuum to 400-3000 μl, dried over anhydrous Na_2SO_4, and then used as the crude carotenoid extract. The crude extract was saponified, its carotenoid content and radioactivity were determined, and then it was applied to columns containing aluminum oxide or cellulose.

Due to relatively low carotenoid level in the eye of the carotenoid-deficient and carotenoid-fortified animals and in the gut of carotenoid-deficient animals, the following methodical device was used. On fourth day after radioactive label injection, eyes or guts from injected carotenoid-deficient animals were isolated and then mixed with 3-4-fold excess of the corresponding tissues dissected from intact carotenoid-fortified animals. The combined preparations were subjected to extraction, saponification, and chromatographic purification. This mode of artificial enrichment of radioactive material with non-radioactive material finally provides a high level of carotenoids in samples and gives the possibility to use multiple chromatographic runs of individual carotenoids.

For saponification, the crude carotenoid extracts (3-4 ml) were mixed with an equal volume of 10% methanolic KOH solution. The mixture was allowed to stand at room temperature for 1-1.5 h, then the reaction was stopped by the addition of 2 ml of 5% aqueous NaCl solution. After centrifugation and separation of the phases, the epiphase was removed and the aqueous phase was extracted three times with hexane (or petroleum ether). The combined hexane (ether) extracts were washed three times with aqueous NaCl solution to remove methanol and then concentrated and dried under anhydrous Na_2SO_4.

After saponification, the extracts (2.0 ml) were applied to columns (1 × 18 cm, V_0 ~ 10 ml) of aluminum oxide (grade 2, Soyuzreactive, Russia) or cellulose (Sigmacell, Sigma, USA) that had been pre-equilibrated with petroleum ether (or hexane). The adsorbed materials were eluted with 30 ml of petroleum ether (or hexane) followed by 45 ml 3% acetone in petroleum ether (or hexane). One-milliliter fractions were collected, and their absorption (A_{450}) and absorption spectra were recorded on a Hitachi 150-20 (Japan) recording spectrophotometer using 400-μl microcuvettes. Individual carotenoids were identified according to their chromatographic behavior, absorption spectra, and partition coefficient using the tables of Foppen [16] and Liaaen-Jensen and Jensen [17]. The individual carotenoids were collected, concentrated,

and again subjected to second, third, and fourth chromatographic procedures under the same conditions as described above to attain the maximal purification grade. A single symmetrical carotenoid peak that could be superimposed with a single count peak was pooled and used as the purified labeled carotenoid.

To detect radioactivity in the samples of the crude and purified carotenoids, aliquots (400-1000 μl) were transferred to scintillation vials, evaporated to dryness, and dissolved into 400 μl of acetone. Then the samples were mixed with 5 ml of HiSafe scintillation cocktail (LKB, Sweden), and the radioactivity was counted for 10 min in a Rackbeta 1209 counter (LKB, Sweden). The final count (cpm) was the difference count between the samples and background. The results were expressed as cpm or as specific radioactivity (cpm/μg carotenoid).

The total content of carotenoids was calculated according to the formula: $C = A \cdot V \cdot f \cdot 10/2500$ [18], where C is total carotenoid content in milligrams, A is the sample absorption at 450 nm, V is the total volume in milliliters, f is the dilution factor for the sample actually measured, and 2500 is an average extinction coefficient for carotenoids ($A^{1\%}_{1\,cm,\,450}$). The carotenoid content in tissue is expressed as μg carotenoid per g wet weight.

RESULTS AND DISCUSSION

Upon preliminary screening of the cockroach tissues, it was found that the maximal carotenoid content occurs in the eye and gut. For further characterization, these tissues were subjected to detailed examination. Analysis of the crude eye and gut carotenoid extracts isolated from the carotenoid-fortified animals showed that the basal substance (~80%) of the gut extracts was a relatively nonpolar carotenoid with low fine structure (λ_{max} in hexane at 425, 450, and 476 nm) which was identified as the bicyclic compound β-carotene. Two minor carotenoids, the less polar and more rapidly migrating bicyclic α-carotene with low fine structure (λ_{max} in hexane at 420, 442, and 472 nm) and the more polar and slowly migrating aliphatic ζ-carotene with clear fine structure (λ_{max} in hexane at 380, 400, and 425 nm), could be separated from the major peak after repeated chromatographic runs (data not shown). It must be emphasized here that no carotenoid except β-carotene was found in the eye of carotenoid-fortified animals or in the gut and eye of carotenoid-deficient animals.

In the animals maintained for six months on the carotenoid-fortified diet, in comparison with the carotenoid-deficient animals, a 13-fold increase in carotenoid content in the gut was found (Fig. 1a). In contrast, in the eye no difference in the carotenoid content between the two groups of animals was detected (Fig. 1b). These data suggest that *P. americana* may accumulate substantial quantities of the carotenoids and, using an unknown mechanism, support a stable physiological level these substances in the eye that does not depend on the carotenoid supply from external sources.

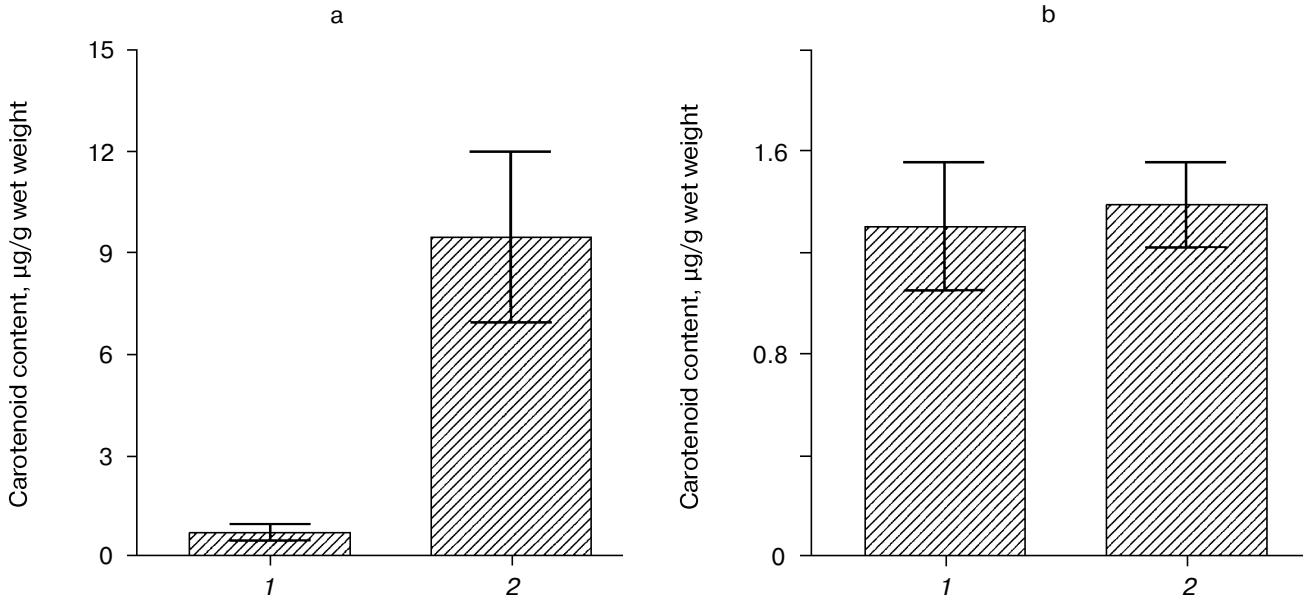

Fig. 1. Carotenoid content in gut (a) and eye (b) of cockroaches fed for six months on carotenoid-deficient (*1*) and carotenoid-fortified (*2*) diets. Mean values from 4-5 determinations ± SEM with 15-30 animals are given.

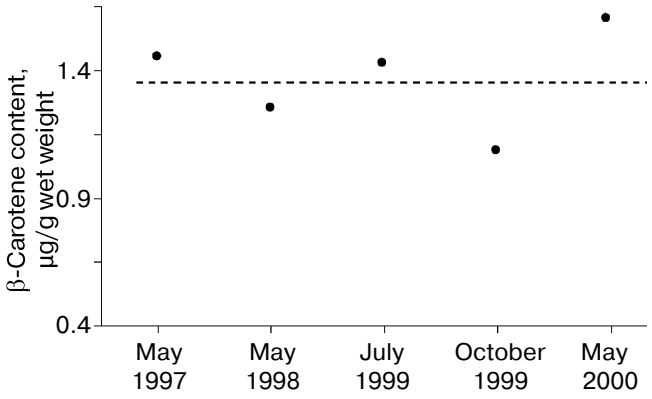

Fig. 2. β-Carotene content of eyes of cockroaches maintained on a long-term carotenoid-deficient diet. Mean values from 3 determinations, each using 15 animals.

Comparison of these data with those obtained earlier for *Calliphora erythrocephala* (*Diptera*) [6] showed the following differences: 1) the major carotenoid of *Periplaneta americana* is β-carotene rather that the xanthophyll zeaxanthin found in *C. erythrocephala*; 2) it is impossible to change the content of carotenoid in the eye of *P. americana* by changing the carotenoid content in the animal's diet, in contrast to the case of *C. erythrocephala*.

Considering the latter fact, we investigated in subsequent experiments how long *P. americana* can support a constant carotenoid level in the eye while on the carotenoid-deficient diet. We found that the cockroaches on the carotenoid-deficient diet for three years (at least 3-4 generations, the lifetime of individual being 9-13 months) did not show a decreased carotenoid level in the eye (Fig. 2) or gut (data not shown). This fact may have to be explained by an ability of the cockroach to synthesizes carotenoids *de novo*, although the "maternal effect" described in *Diptera* [2] could make a contribution to supporting the stable level of carotenoids by the delivery of some part of carotenoid for the next generation through the egg. However, this endogenous supply must be depleted in subsequent cockroach generations, and this was not seen over three years (Fig. 2).

If *P. americana* call in fact synthesize carotenoid, then an injected labeled precursor should be involved in the synthetic reaction, and incorporation of the label into newly formed carotenoid should be observed. Indeed, the injection of 3.4 nmoles of the labeled precursor mevalonic acid pyrophosphate induced a cyclic change of β-carotene content in the eye with two maxima occurring on days 2 and 4 after the injection (Fig. 3). It remains difficult to explain the reason for these fluctuations during the formation of the final synthesis product. It is obvious that the injection of the exogenous mevalonic acid pyrophosphate at the concentration used (probably non-physiological), which is transformed to isopentyl diphosphate, the basic structural element of the skeleton of all terpenoids [18], acts in a complicated manner on the subsequent dimerization, dehydration, and oxidation reactions that finally lead to the formation of the individual carotenoids. Nevertheless, it seems to us that the increase carotenoid content in the eye on days 2 and 4 after injection of the precursor indicates the synthesis of carotenoids *in vivo* in *P. americana*.

If *P. americana* incorporates the radioactive label into the carotenoid skeleton, it should be impossible to separate the labeled from unlabeled carotenoid by means of various purification procedures. Also, it would be expected that the specific radioactivity in the samples after repeated chromatographic procedures would have a relatively constant value in each experiment. Figure 4 shows that relatively high specific activity of the acetone extract that might be related to radioactive impurities (mevalonic acid pyrophosphate is also a precursor in the synthesis of such polar substances as ubiquinone, cholesterol, and dolichol [18]) was substantially decreased following the transfer of the carotenoid into the less polar solvent as well as after saponification, where the alkaline hydrolysis these impurities might proceed. Following the first run on the column with aluminum oxide, the specific activity of the non-saponifiable β-carotene reached a value that could not be significantly decreased on further chromatographic purification on aluminum oxide and on cellulose (Fig. 4). The elution profile of the β-carotene from the eye (Fig. 5a) and gut (Fig. 5b) showed that after

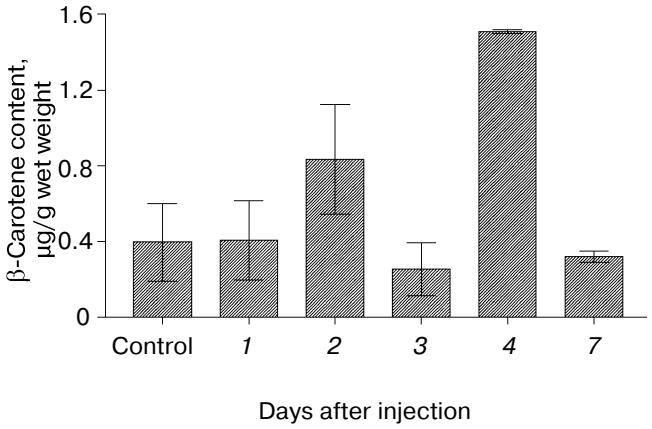

Fig. 3. Changes in the content of β-carotene in the eye following intraperitoneal injection of 3.4 nmoles of [^{14}C]mevalonic acid pyrophosphate into experimental animals. Mean values from 3-4 determinations ± SEM using 15-30 animals for each determination.

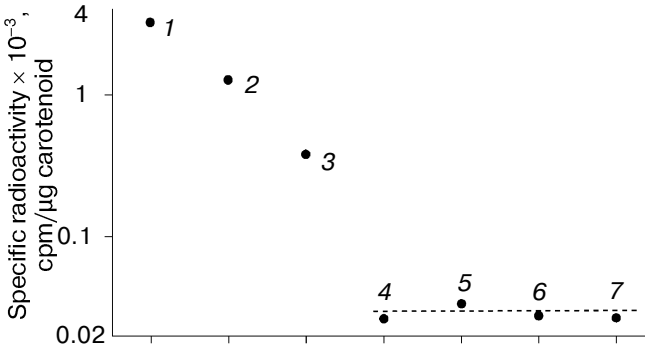

Fig. 4. Effect of various purification procedures on stabilization of the level of specific radioactivity of the β-carotene from gut: *1*) crude acetone extract of carotenoids; *2*) after transfer of carotenoids from acetone to hexane; *3*) after saponification of the hexane fraction of carotenoids; *4*) after the first chromatographic run of the non-saponifiable β-carotene on aluminum oxide; *5*) after the second chromatographic run of β-carotene on aluminum oxide; *6*) after the third chromatographic run of β-carotene on aluminum oxide; *7*) after the fourth chromatographic run of β-carotene on cellulose. Mean values from three determinations for a typical experiment. The ordinate axis is in logarithmic scale.

multiple chromatographic purifications of the samples, it is impossible to separate the radioactive label from the colored carotenoid (the coincidence of the count peak with the absorbance peak). This proves that the physical and chemical properties of the substance containing the radioactive label are identical to the colored carotenoid, implying that the labeled compound is the carotenoid.

Thus, based on the present results, it can be concluded that: 1) in contradistinction to other insects, *P. americana* does not need exogenous carotenoids because it can synthesize these substances; this allows the animals to exist for an unlimitedly long time on the carotenoid-deficient diet while maintaining a stable carotenoid level in the eye; 2) the surplus of carotenoids in food results in an accumulation a markedly high amounts of these compounds in the gut, suggesting that this organ, in addition to its well-known digestive and biosynthetic functions, is also serving as a storage organ; 3) the major carotenoid in the eye and the gut is β-carotene, which is radiolabeled after injection of [^{14}C]mevalonic acid pyrophosphate into experimental animals. It is obvious that, in contrast to studied representatives of *Diptera* and *Lepidoptera*, *Periplaneta americana* has two unique mechanisms which probably act to provide optimal visual function. On one hand, with an excess of carotenoids in food, the cockroach accumulates a large store of carotenoids in the gut and thus becoming independent from its supply in food. On the other hand, on a carotenoid-deficient diet for three years, *P. americana* switches to the synthesis of these compounds and is thus independent of carotenoids in the diet. It seems probable that both mechanisms guarantee a stable carotenoid level in the eye that does not change in either carotenoid abundance or shortage. It remains unclear whether the cockroach has a biogenic carotenoid-synthesizing enzyme set, or perhaps the carotenoid producers are the enzymes of symbiotic bacteria living in the alimentary tract of the cockroach. A role of microorganisms in carotenoid biosynthesis in insects, e.g., in *Musca domestica*, was proposed 37 years ago by Goldsmith et al. [2]. To resolve this question in relation to *P. americana* requires further experiments.

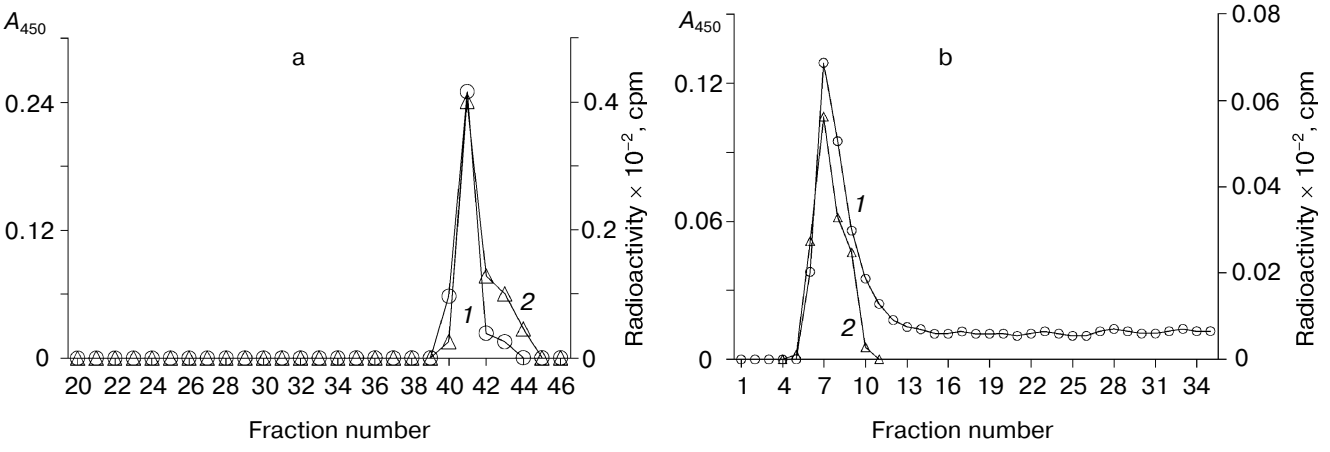

Fig. 5. a) Elution profile of β-carotene extracted from eyes after a single chromatographic run on aluminum oxide. b) Elution profile of β-carotene extracted from gut after the fourth chromatographic run on cellulose (the sample was previously subjected to threefold chromatography on aluminum oxide): *1*) A_{450}; *2*) radioactivity.

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