A Micro Method for Separating Free and Esterified Vitamin A

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Glover, Goodwin & Morton (1947) have described a chromatographic method for separating free and esterified vitamin A, using bone meal as adsorbent. The method was developed for the separation of 600–1800 i.u. of vitamin A per column. For the estimation of free and esterified vitamin A in blood and lymph it is, however, often necessary to be able to separate smaller amounts, i.e. a few international units. Several other methods of separation have been published. In the earliest of these, phase

these carotenoids and the main problem was to find suitable 'indicators' or an alternative mode of recognition. Lycopene and Sudan III were tried but gave colours with antimony trichloride. Ultra-violet illumination also failed, as the amount of vitamin A present (a few micrograms) was not always sufficient to show a clear fluorescent zone on the column. Eventually, by using constant amounts of adsorbents and eluents it was possible to do without indicators. The efficiency of separation was

Table 1. *Adsorbents and eluting agents used for the separation of free and esterified vitamin A*

Adsorbent	Solvents and eluting agent for		Reference
	Vitamin A ester	Vitamin A alcohol	
Al ₂ O ₃	Hexane	Ethanol	Hoch & Hoch (1946)
Al ₂ O ₃	Acetone (2%) in hexane	Ethanol (8%) in hexane	Ganguly, Kon & Thompson (1947)
Al ₂ O ₃	Acetone (4%) in light petroleum	Estimated by deduction from total	Parrish, Wise & Hughes (1947)
Al ₂ O ₃	Ethylene dichloride	Estimated by deduction from total	Reed, Wise & Frunat (1944)
Al ₂ O ₃	Light petroleum	Acetone (20%) in light petroleum	Present investigation
Al ₂ (SO ₄) ₃ (on filter paper)	Light petroleum	Light petroleum	Datta & Overell (1949)
Bone meal	CHCl ₃ (2.5%) in light petroleum	Acetone	Glover <i>et al.</i> (1947)
Kieselguhr (Hyflo Super Cel)	Light petroleum	Light petroleum	Wilkes (1946)
Silicic acid	Light petroleum	CHCl ₃	Swain (1943)
MgO + celite	Light petroleum	CHCl ₃ (5%) in light petroleum	Narod & Verhagen (1948)

separation was used (for a review of the literature see Glover *et al.* 1947), but recently chromatography has been employed by most workers (Table 1). Molecular distillation (Gray, Hickmann & Brown, 1940) and fluorescence methods (Sobotka, Kann & Winternitz, 1944; Kascher & Baxter, 1945) have also been applied.

Of these various methods, chromatography is the most readily adapted to small-scale work. On a column of aluminium oxide, vitamin A alcohol travels with the hydroxy carotenoids, whereas esterified vitamin A is less strongly adsorbed, and moves with the hydrocarbon carotenoids. Vitamin A alcohol and its esters are almost colourless, but the accompanying carotenoids, when present, serve as a guide to separation. Hoch & Hoch (1946), working on human sera, were able to take advantage of this, but, in the present work, the extracts did not contain

checked in each case by collecting a middle fraction between the vitamin A alcohol and the ester fractions; the separation was only considered satisfactory when this fraction did not contain any vitamin A.

EXPERIMENTAL

Principle of method. A small column of Al₂O₃ was used in which vitamin A alcohol was adsorbed from a light petroleum solution, whereas the ester passed straight through and was collected as 'Fraction I'. To check whether all esters had been removed, a second fraction ('Fraction II') was collected by washing the column with light petroleum. Then the vitamin A alcohol was eluted with a solution of 20% acetone in light petroleum and collected as 'Fraction III'.

Preparation of the column. Glass tubes of internal diameter 6 mm. and height 100–130 mm. were plugged with cotton wool above a 1–2 mm. diameter constriction. Al₂O₃ (British Drug Houses Ltd.) and light petroleum were mixed

and poured into the tube to give on settling a 15 mm. layer of solid; the surface of the column was always covered with liquid, and the rate of flow without suction was 20–60 drops/min.

Chromatography. The extract containing the vitamin A (4–40 i.u.) was evaporated to dryness under reduced pressure. (For details of extracting vitamin A from biological tissues see Eden & Sellers, 1950.) The residue was made up in 1 ml. of light petroleum and the solution was transferred to the column. The flask was rinsed three times with 1, 2 and 2 ml. of light petroleum respectively. The filtrate from the original 1 ml. and the three washings were collected as 'Fraction I' containing the vitamin A esters. The column was then washed with an additional 2 ml. of light petroleum. If this middle fraction, containing the 2 ml. of washings, was found to contain no vitamin A, the separation was considered satisfactory. The third fraction containing the vitamin A alcohol was obtained by eluting the column twice with 2 ml. of 20% acetone in light petroleum. Each fraction was evaporated under reduced pressure and then dissolved in CHCl_3 .

Estimation of vitamin A. The intensity of the blue colour in the SbCl_5 reaction was measured in a single photocell absorptiometer (Eden & Sellers, 1949).

RESULTS

Efficiency of separation of artificial mixtures

Table 2 shows the separation obtained. The free vitamin A used was a crystalline product obtained

from Roche Products Ltd. and the vitamin A ester a concentrate prepared by Distillation Products Inc. Solutions of these compounds in light petroleum were mixed and subjected to chromatographic separation. The recovery of both vitamin A ester and alcohol was usually within 5% of the expected value and the 'middle' fraction contained no more than traces of vitamin A.

Recovery and separation of vitamin A alcohol and ester added to plasma extracts

Extracts of plasma, intestinal contents and mucosa in light petroleum were chromatographed after addition of small quantities of vitamin A alcohol or ester. Control extracts without the addition of vitamin A were put through the same procedure. Table 3 shows that the separation is also satisfactory in the presence of biological extracts.

SUMMARY

1. A micro method using aluminium oxide as adsorbent has been devised for separating vitamin A alcohol from the esterified form. Under the conditions of the method, vitamin A ester in light petroleum passed through the column, whereas vitamin A alcohol was adsorbed and could be eluted with 20% acetone in light petroleum.

Table 2. *Chromatographic separation of vitamin A alcohol and esters*

Vitamin A ester			Vitamin A alcohol			Percentage of total vitamin A in the 'middle' fraction
Added (i.u.)	Recovered (i.u.)	Recovery (%)	Added (i.u.)	Recovered (i.u.)	Recovery (%)	
15.0	14.4	96	Nil	Nil	—	0.0
11.0	11.2	102	Nil	Nil	—	1.0
10.1	9.4	93	Nil	Nil	—	0.7
5.1	5.2	102	3.15	3.1	98	0.0
5.1	5.13	101	6.3	6.6	104	0.0
2.52	2.56	102	6.3	5.8	92	0.1
10.97	10.75	98	20.15	19.1	95	0.4
Nil	Nil	—	20.15	19.8	98	0.1
Nil	Nil	—	9.8	10.2	104	0.0

Table 3. *Recovery of vitamin A alcohol and ester added to biological extracts*

Nature of extract	Vitamin A ester				Vitamin A alcohol			
	Original (i.u.)	Added (i.u.)	Found in ester fraction (i.u.)	Recovery of added vitamin A (%)	Original (i.u.)	Added (i.u.)	Found in alcohol fraction (i.u.)	Recovery of added vitamin A (%)
Plasma	0.11	11.5	11.59	101	1.42	0	1.40	—
"	1.51	10.1	9.24	91	6.79	0	6.99	—
"	0.07	7.6	7.12	94	12.7	0	12.3	—
"	0.07	9.1	8.9	98	12.3	0	11.7	—
"	0.33	0	0.41	—	5.20	12.7	17.0	93
"	3.18	0	3.49	—	7.19	25.4	35.9	113
Intestinal contents	1.87	9.3	9.3	100	1.68	23.4	22.6	97
Intestinal mucosa	0	22.7	22.5	99	0	13.5	13.2	99

2. The separation proved to be satisfactory either for artificial mixtures or biological extracts fortified with vitamin A alcohol or ester.

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Hydrolysis and Esterification of Vitamin A during Absorption

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The absorption of vitamin A by the lymph stream has been demonstrated in several species, including man (Drummond, Bell & Palmer, 1935), dogs (McCoord, Breese & Baum, 1943), rats (Popper & Volk, 1944; Radice & Herraiz, 1947), sheep (Eden & Sellers, 1949), and bovines (Eden & Sellers, 1949; Goodwin & Gregory, 1948). Further studies on the mechanism of vitamin A absorption have been made by Gray, Morgareidge & Cawley (1940) who have shown that vitamin A esters are hydrolysed in the intestinal tract of the rat. In the present work the fate of free and esterified vitamin A during absorption by bovines and sheep has been studied.

EXPERIMENTAL

The procedure adopted was to determine the relative concentration of vitamin A alcohol and ester in the intestinal content, mucosa, lymph and blood after dosing with one or other form of the vitamin. The interval between dosing and slaughter was chosen as 4 hr., as previous experiments (Eden & Sellers, 1949) had shown that, although the rate of absorption of vitamin A was variable, it most commonly reached its maximum then.

The experimental animals were ten adult sheep and eleven calves. All the sheep and seven of the calves were about a year old; four newborn calves

were also studied, to see if absorption was the same in them as in older animals.

Preparation of dose. Vitamin A ester concentrates (from fish-liver oils) in which over 95 % of the vitamin was esterified, were obtained from Distillation Products Inc. Vitamin A alcohol was prepared by saponification of halibut-liver oil, and was compared chromatographically with a crystalline preparation of vitamin A alcohol obtained from Roche Products Ltd. Over 95 % of the vitamin A in the liver oil unsaponifiable fraction was free alcohol. The materials were made up for dosing by emulsifying the vitamin A with reconstituted separated milk and arachis oil in a Waring blender. The animals received about 5000 i.u. of vitamin A/kg. of body weight, and the total volume of the dose was 200–400 ml. according to the size of the animal.

Collection of materials for estimation. Before dosing and at slaughter, samples of jugular blood were collected. At slaughter the intestinal lymph glands were removed, sliced and centrifuged, the supernatant liquid drawn off and its vitamin A content determined. The intestinal contents were obtained from the duodenum and the jejunum. These portions of intestine were cut open and washed repeatedly with 0.9 % saline in order to remove any adhering contents. The samples of mucosa were removed with a scalpel from the connective and muscular tissue.