CLXXVIII. THE DISTRIBUTION OF VITAMIN A BETWEEN LIGHT PETROLEUM AND AQUEOUS METHYL ALCOHOL.

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THE difference in behaviour on partition between light petroleum and 80–90 % aqueous methyl alcohol has for long provided a useful means of differentiation and separation of the hydrocarbon carotenoids from the corresponding hydroxylated compounds—the xanthophylls [Kraus, 1872; Borodin, 1883; Willstätter & Stoll, 1913]. The separation and extraction of carotenoids by this partition method can be controlled visually by the colour of the extract, but in analyses of biological materials containing carotenoids and vitamin A such as we have recently carried out on butter [Gillam *et al.* 1933], egg yolk [Gillam & Heilbron, 1935], and blood serum [Gillam & El Ridi, 1935], the question sometimes arises as to which phase contains the vitamin, which is, of course, colourless. In the analysis of butter it has usually been found that the vitamin A is divided between the petrol and alcohol phases rather irregularly, although Wolff *et al.* [1930] have shown that carotene and vitamin A can be separated by means of the phase test, the carotene going into the petrol phase whilst the vitamin goes into the alcohol phase. As however, the exact conditions for complete extraction were not specified and vitamin A has no colour by which its extraction can be followed visually, we have carried out a series of determinations of the partition coefficient of vitamin A between light petroleum and various accurately known concentrations of aqueous methyl alcohol, the conditions being strictly controlled.

EXPERIMENTAL.

Vitamin A. The material used throughout the experiments was a concentrate obtained by distillation of the sterol-free unsaponifiable matter of halibut liver oil [cf. Heilbron *et al.* 1932]. It was a pale amber-coloured viscous liquid having an intensity of absorption $E_{1\text{ cm.}}^{1\%}(328\text{ m}\mu) = 1200$ approx., i.e. it contained about 75 % of the vitamin.

Solvents. The methyl alcohol used (commercial pure) was further purified by refluxing with metallic sodium (20 g. in 2000 ml.) for 6 hours, followed by distillation, the first and last 100 ml. of distillate being rejected. The purified product was diluted with distilled water to the required density (by certified hydrometer) at 15°, the density-concentration values used being those quoted by Hodgman & Lange [1929]. The light petroleum was commercial material, B.P. 70–80°, redistilled.

Partition. 100 ml. of a solution of the vitamin A concentrate of suitable strength in light petroleum were transferred to a dry 250 ml. stoppered bottle. 100 ml. of the aqueous methyl alcohol were added and the mixture shaken vigorously by hand at room temperature. The bottle and its contents were then immersed in a thermostat at $20^\circ \pm 0.5^\circ$ and shaken occasionally during 1 hour. Portions of each phase were separated and their vitamin A contents determined.

Analysis of the two phases. Samples of each phase (2–5 ml.) were diluted with 25–100 ml. of ethyl alcohol (purified until completely transparent to ultra-violet rays) according to the amount of vitamin present, and the intensity of absorption of the solution, at 328 $m\mu$, determined spectrophotometrically, using a Hilger E_3 quartz spectrograph and Spekker photometer. The concentrations of vitamin A in each phase were calculated from the intensity of absorption, on the basis of a value of $E_{1\text{cm}}^{1\%} = 1600$ for the purest vitamin A, but the partition results—being relative—are independent of the absolute intensity of absorption of pure vitamin A.

The accuracy of the analytical method (photographic absorption spectrophotometry) is probably of the order of $\pm 2\%$.

Miscibility of light petroleum and aqueous methyl alcohol. The laws governing the distribution of solutes between two immiscible solvents are only strictly true when the solvents are perfectly immiscible. In the pair of liquids under discussion this ideal condition is apparently not realized but this does not affect the application of the phase test as a means of separation of, for example, carotene and lutein, or carotene and vitamin A, because the two phases are separated as such, irrespective of their composition. In the repeated extraction of a light petroleum solution with 90 % aqueous methyl alcohol the volume of light petroleum steadily diminishes by extraction into the alcohol phase.

The numerical results obtained in the partition experiments are recorded in Table I.

Extraction of vitamin A from one solvent by the other. Amongst other results of these determinations it is now possible to calculate how many extractions with a given concentration of aqueous methyl alcohol will be necessary to extract the whole of the vitamin A from a given solution in light petroleum. Thus, from the partition coefficient it follows that seven extractions of a light petroleum solution of vitamin A with 90 % methyl alcohol equal in volume to that of the light petroleum should be sufficient to remove all the vitamin A from the solution. We have tested this deduction experimentally and found the extraction of vitamin A to be almost quantitative.

When vitamin A is allowed to distribute itself freely between light petroleum and 90 % methyl alcohol the results show that the concentrations in each phase are approximately the same. This being so the reverse of the above procedure should also be true and seven extractions of the 90 % methyl alcohol solution with light petroleum should also remove practically the whole of the vitamin. This expectation has also been confirmed by experiment.

The effect of temperature on the distribution. All the determinations recorded were carried out at 20°, but in order to test the effect of variations of temperature, the 90 % methyl alcohol partition coefficients were also determined at 10 and 30°. The values were, within the experimental error, the same as those obtained at 20° (cf. Table I). It is therefore possible to work at any room temperature between 10 and 30° without affecting the results appreciably.

The effect of carotene. As it is frequently necessary to be able to separate carotene from vitamin A in biological material it was necessary to test whether small amounts of carotene affect the partition ratio. A standard solution of vitamin A in light petroleum was made up and pure β -carotene, approximately equal in weight to the vitamin A present, added. The mixture was extracted seven times with 90 % methyl alcohol, equal in volume to that of the light petroleum. The results showed that whilst the carotene remained in the petrol phase, 98 % of the vitamin A was recovered in the aqueous alcohol layers.

Table I. *Distribution of vitamin A between light petroleum and aqueous methyl alcohol (at 20°).*

Sp. gr. of methyl alcohol at 15° to water at 4°	Conc. of methyl alcohol % by wt.	Conc. of vitamin A in petrol phase (w/v %) C ₁	Conc. of vitamin A in methyl alcohol phase (w/v %) C ₂	Partition coefficient C ₁ /C ₂	Volume changes† in phases of light petroleum and aqueous alcohol after partition. Initially 100 ml. + 100 ml.		% of total vitamin A going into alcohol phase	
					Final volumes		Assuming complete immiscibility	Allowing for volume change
					Petrol	Alcohol		
0.810	95	0.0118	0.0177	0.67	—	—	59.3	—
		0.0159	0.0240	0.67	—	—	59.3	—
		0.0065	0.0102	0.64	—	—	61.0	—
		0.0138	0.0213	0.65	—	—	60.6	—
		—	—	0.66	77	123	60.0	70.5
0.824	90	0.0188	0.0194	0.97	—	—	50.8	—
		0.0177	0.0178	0.99	—	—	50.1	—
		*0.0203	0.0211	0.96	—	—	50.9	—
		*0.0206	0.0219	0.94	—	—	51.5	—
		0.0159	0.0160	0.99	—	—	50.1	—
		0.0175	0.0175	1.00	—	—	50.0	—
		†0.0191	0.0200	0.96	—	—	51.1	—
		†0.0188	0.0195	0.96	—	—	50.9	—
		0.0180	0.0190	0.95	—	—	51.3	—
		0.0177	0.0183	0.97	—	—	50.8	—
—	—	0.97	90	110	50.8	55.7		
0.838	85	0.0258	0.0181	1.43	—	—	41.2	—
		0.0250	0.0189	1.32	—	—	43.0	—
		0.0195	0.0138	1.42	—	—	41.4	—
		0.0195	0.0148	1.32	—	—	43.1	—
		0.0206	0.0151	1.36	—	—	42.4	—
		0.0188	0.0141	1.33	—	—	42.9	—
		—	—	1.36	94	106	42.4	45.3
0.850	80	0.0270	0.0101	2.67	—	—	27.2	—
		0.0270	0.0106	2.55	—	—	28.2	—
		0.0152	0.00583	2.60	—	—	27.7	—
		0.0270	0.00958	2.82	—	—	26.2	—
		0.0270	0.0096	2.81	—	—	26.2	—
		—	—	2.69	96	104	27.1	29.3
0.875	70	0.0359	0.00444	8.09	—	—	11.0	—
		0.0344	0.00437	7.87	—	—	11.3	—
		0.0362	0.00442	8.19	—	—	10.9	—
		0.0352	0.0042	8.38	—	—	10.7	—
		—	—	8.13	97	103	11.0	11.5

* Both these sets of values were obtained at 30° (cf. text).

† These two sets of values were obtained at 10° (cf. text).

‡ These values were the means of actual volumes measured during the vitamin A distribution experiments but are not accurate to more than $\pm 1\%$.

The effect of sterols. In the biological material in which vitamin A occurs, there are always present relatively large quantities of sterols and the question therefore arises as to whether the partition coefficient of the vitamin is altered by the presence of sterols. In order to answer this question partition coefficients were determined as usual for light petroleum—90% methyl alcohol, except that to the petrol solution of vitamin A was first added 1.2% of cholesterol in one case and 2% in another. The results are given in Table II.

Table II. *Effect of cholesterol on the partition coefficient of vitamin A between light petroleum and 90% methyl alcohol.*

% of cholesterol in petrol solution (at beginning of exp.)	% of vitamin A in petrol phase C_1	% of vitamin A in alcohol phase C_2	C_1/C_2	% of total vitamin A in alcohol phase $C_2 \times 100^*$ $(C_1 + C_2)$
2.0	0.0131	0.0085	1.54	39.3
2.0	0.0127	0.0088	1.44	40.9
1.2	0.0188	0.0161	1.16	46.1
1.2	0.0180	0.0145	1.23	44.6
0.0	0.0177	0.0183	0.97	50.8

* Assuming complete immiscibility of the two phases (cf. Table I).

It is thus clear that the presence of sterols affects the partition coefficient considerably, in the direction of increasing the fraction of the vitamin remaining in the petrol phase. This is presumably due to the fact that the vitamin is extracted more effectively by a petrol solution of cholesterol than by petrol itself, for we know that in the partition the sterol goes preferentially into the petrol phase. Thus when use is made of the observed partition coefficients for work of a quantitative nature sterols should be removed as far as possible. It is probable that small amounts of sterol do not affect the partition seriously for it should be noted that in the above experiments the sterol: vitamin A ratio was of the order of 100–200 : 1.

SUMMARY.

In view of the usefulness of the Kraus distribution method (usually known as the "phase test") for separating carotenes from xanthophylls and the lack of accurate knowledge of the way vitamin A distributes itself under varying conditions when it occurs with carotenoids, a study of the partition coefficient of vitamin A has been made. The two solvents chosen have been those commonly used, i.e. light petroleum and aqueous methyl alcohol over the range 70–95% alcohol. The results show that whilst with 90% alcohol the vitamin distributes itself almost equally between the two phases, with 70% alcohol the partition ratio is about 8 : 1 in favour of the light petroleum. It has been shown that it should require at least seven extractions of a light petroleum solution of vitamin A with an equal volume of 90% methyl alcohol each time, to extract the whole of the vitamin from the light petroleum. This has been confirmed experimentally, as has also the conclusion that with this concentration of alcohol the extraction can be made as effectively from alcohol to petrol as in the opposite direction. The partition coefficient is unaffected by temperature changes between 10 and 30° but the presence of cholesterol alters it appreciably.

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