LII. THE MOLECULAR WEIGHT OF VITAMIN A.

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INTRODUCTION.

RECENT experiments on the problem of the relation between carotene and vitamin A bore evidence in favour of the view that in the animal body carotene is being transformed into vitamin A [Moore, 1929; Capper, 1930; Wolff *et al.* 1930]. This being so, carotene would be the vegetable source from which vitamin A is built up and should be considered as a "provitamin." An artificial conversion has not yet been obtained. Euler, Euler and Karrer [1929] have suggested that vitamin A might be a partly hydrogenated carotene, but no evidence has been found about the nature of their chemical relation.

It seemed important to us to determine the relative molecular size of the two substances. We therefore determined their diffusion constants in the same solvent. Were these constants found to be of almost the same order of magnitude, then the conclusion could be drawn that a simple chemical relation (*e.g.* a hydrogenation or an oxidation) existed.

However, the result was the reverse. The diffusion constants of carotene and vitamin A showed such a difference that their chemical relation cannot be a simple one. If the idea that one substance is converted into the other is right, the conversion cannot consist in simple hydrogenation, but must be a more radical transformation of the molecule. It must be stated here, that what we considered as "vitamin A," was determined by the reaction of Carr and Price (the blue colour developed by a solution of SbCl_3 in chloroform). We started from the point of view that up to now there has been evidence enough to consider this reaction as specific for the substance with vitamin properties when the absorption band is at $610\mu\mu$.

In this connection we should like once more to draw attention to the fact that the transformation *in vivo* was also detected by the increase of the blue colouring capacity of liver extracts after animals had been fed with carotene.

Materials.

Vitamin A preparations were obtained from the unsaponifiable part of sheep-liver extracts. After saponification, the light petroleum extract was concentrated, cooled and filtered. The solution now contained about 0.1 mg.

of solid *per* rat unit of vitamin A¹ and was by no means a pure vitamin solution as the actual active constituent was perhaps 2 % of the solid residue. Since indifferent substances do not appreciably influence each other's rates of diffusion provided that their solutions are not too concentrated, it seemed possible to work with these impure preparations. By evaporating the light petroleum *in vacuo* in a carbon dioxide atmosphere, adding xylene and evaporating half of the latter, a clear solution in xylene was obtained. Crystalline carotene was prepared from carrots and showed a m.p. of 174°. Considering the desirability of avoiding convection currents, when bringing the pure solvent above the very dilute carotene solution in order to effect diffusion, it was necessary to bring the solution to a higher specific gravity than that of the pure solvent. Therefore a certain amount of cholesterol was added. This amount was in Exps. 1 and 2 about 6 %, in Exps. 3 and 4 about 3 %.

The ratio of carotene to the total solid was in Exp. 2 1:630, in Exp. 3 1:60. The solutions of carotene in xylene proved extremely unstable, even when kept hermetically closed in a dark room. To get rid of this inconvenience in the second series of experiments a small quantity of pyrogallol was added to the solution which proved to be very effective in keeping the quantity of carotene unchanged for a considerable time if preserved in the dark.

Method of measuring the diffusion velocity.

In our experiments we used the diffusion apparatus of Cohen and Bruins [1923]. This consists of six glass discs, which may be rotated around a common axis, all being of exactly the same thickness. With the exception of the lowest and uppermost, all the discs are provided with three round perforations fitting exactly upon each other. When the discs are put together these perforations make three cylindrical spaces. The actual experiment is carried out by filling the lowest section of these cylinders with the solution to be investigated and the three higher compartments with the pure solvent. By suitably and very carefully rotating the discs, the upper three compartments are now brought above the lower one. After the diffusion has taken place for a certain time, the four compartments are again separated by rotating two alternate discs (Nos. 2 and 4). In this way the column of liquid is divided into four equal parts. Every one of these parts is analysed separately. From the ratio of the concentrations of the dissolved substance in the four layers the diffusion constant may be calculated. For a detailed description of the apparatus and of the calculation the original literature should be consulted.

A few simplifications of the original procedure were introduced². As our analytical methods did not permit a very high exactitude, part of the precautions usually exercised in diffusion experiments were not considered im-

¹ We have to thank N. V. Organon, Oss, for having supplied us with this preparation.

² The diffusion apparatus was placed in the cellar of the van 't Hoff Laboratory, Utrecht (Director Prof. Dr E. Cohen).

perative in this case. In the first series of experiments, with both substances, we did not work with a thermostat, but wrapped the diffusimeter in cotton wool and took care that no sudden changes of temperature should occur. During the second series the apparatus was placed in the air-thermostat [Cohen and Bruins, 1923, p. 371], but the water in the thermostat did not circulate and therefore no auxiliary thermostat was needed. The rotation of the discs was not performed mechanically but very carefully by hand. Before starting the diffusion process, the apparatus was kept for 3 hours in the thermostat.

In all our experiments we used pure *m*-xylene (B.P. 139–139.5°) as a solvent. The discs were lubricated with glycerol in the first series. As this took up moisture from the air very easily, the edges were greased with soft soap. In the second series the discs were lubricated by a hydrogel of glycerol-amylum.

Calculation.

Each diffusion experiment gave us three concentration values for each cylindrical section, the average of which was used for the calculation of the diffusion constant. To this end the tables of Kawalki [1894] were used.

Intermediate values, not recorded in these tables, were found by linear interpolation. In this way we obtained from each experiment four values of x in the formula:

$$x = \frac{h'^2}{D \cdot t}$$

where h' is the half of the height of one section, D is the diffusion constant, t is the time of diffusion. From these four values the average was calculated for each diffusion-experiment, their relative "weights" being taken into account [for details see Cohen and Bruins, 1923].

The time of diffusion in all experiments was about the same, hence the following values for the "weight" p of the various sections were used, the relative variations in the concentrations of the four sections being between $x = 0.1296$ and $x = 0.1936$, i.e. the part of the tables used in these calculations.

Section 1	$p = 529$	
2	$p = 312$	
3	$p = 178$	
4	$p = 660$	
	$p = 1679$	$x_{av} = \frac{\sum px}{\sum p}$

The diffusion constant is found from the formula:

$$D_{av} = \frac{h'^2}{x_{av} \cdot t}$$

For our diffusimeter $h'^2 = 0.2376$ [Cohen and Bruins, 1924], D is expressed in cm.² per 24 hours.

For the calculation of the diffusion constant at 20° we used a temperature

coefficient of 1.4 % per ° C. This is the temperature coefficient of the viscosity of *m*-xylene calculated from the data given by Thorpe and Rodger (Landolt-Börnstein tables).

Method of analysis.

In these diffusion experiments only the relative concentrations in single sections are to be determined, and it is not required to know the absolute concentration of the substance investigated. This is very important for the reason that only in view of this fact may we use without any objection the reaction of Carr and Price [1926] for our vitamin A determinations. However doubtful the absolute value of this reaction is, the units by which we express the concentration value of the cylindrical sections may be quite arbitrary.

A. Vitamin A determination.

The reaction was carried out in the usual way (0.2 cc. of the solution, to which two drops of acetic anhydride were added to prevent precipitation, were mixed with 2 cc. of a saturated solution of SbCl_3 in chloroform [Bertram, 1929], and the blue colour measured in the Lovibond colorimeter.

The colour expressed in Lovibond units not being a linear function of the dilution, a standard curve of this function was made.

The solutions to be investigated were always brought to such dilutions that the reaction of Carr and Price gave a blue colour corresponding to about 5 units. Two other dilutions were made, if possible of about 4 and 6 units. From these three determinations the average value was obtained. The values recorded in the Tables are these averages calculated in rat units of Rosenheim per cc. solution (5 Lovibond units are equal to 5 rat units). As already stated, the absolute value of the units is of no importance. The error in our series of experiments amounts to about 5–10 %. Errors of this range of magnitude seemed rather disturbing for the reliability of the diffusion constants to be calculated; therefore the average of three concentration measurements was used in these calculations.

B. Carotene determinations.

The determinations of carotene were carried out colorimetrically with the stuphometer of Zeiss, by means of a standard curve established in a preliminary series with solutions of known concentration. Carotene having an absorption band at 490–475 $\mu\mu$ the filter S_{47} was used in these determinations. Solutions of carotene containing 0.001–0.01 mg. per cc. can be determined very exactly with the photometer. The probable error in these estimations does not exceed 3 %. In the following tables the concentrations are recorded in 0.001 mg. per cc. These figures also represent the average of three determinations.

RESULTS OF THE EXPERIMENTS.

Vitamin A; first series.

I.

Diffusion time: 3.030 days; temp. 16.3°–15.0°; average 15.7°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	73	—	72	72	1109	0.1477	78.13	
2	127	—	123	125	1926	0.1397	43.59	
3	188	—	193	191	2943	0.1226	21.82	$x_{av} = \frac{244.26}{1679} = 0.1455$
4	269	—	253	261	4021	0.1526	100.72	
				649	9999		244.26	

 $D = 0.540 \text{ cm.}^2/24 \text{ hours; } D_{20} = 0.572 \text{ cm.}^2/24 \text{ hours.}$

II.

Diffusion time: 3.018 days; temp. 16.1°–18.2°; average 17.2°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	—	47	48	47.5	1237	0.1343	71.05	
2	—	77	62	69.5	1811	0.1613	50.33	
3	—	111	102	106.5	2773	0.0835	14.86	$x_{av} = \frac{248.18}{1679} = 0.1478$
4	—	157	164	160.5	4180	0.1696	111.94	
				384.0	10001		248.18	

 $D = 0.531 \text{ cm.}^2/24 \text{ hours; } D_{20} = 0.552 \text{ cm.}^2/24 \text{ hours.}$

III.

Diffusion time: 3.015 days; temp. 18.9°–19.5°; average 19.2°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	58	58	—	58	1090	0.1495	79.09	
2	107	107	—	107	2012	0.1222	38.13	
3	158	157	—	158	2971	0.1242	22.11	$x_{av} = \frac{235.16}{1679} = 0.1401$
4	208	210	—	209	3929	0.1452	95.83	
				532	10002		235.16	

 $D = 0.564 \text{ cm.}^2/24 \text{ hours; } D_{20} = 0.570 \text{ cm.}^2/24 \text{ hours.}$ *Vitamin A; second series.*

I.

Diffusion time: 2.950 days; temp. 19.3°–19.7°; average 19.5°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	33	32	34	33.0	1235	0.1347	71.26	
2	52.5	52.5	52.5	52.5	1965	0.1310	40.87	
3	77.5	77.5	79	78.3	2932	0.1151	20.49	$x_{av} = \frac{224.89}{1679} = 0.1339$
4	105	105	100	103.3	3868	0.1398	92.27	
				267.1	10000		224.89	

 $D = 0.602 \text{ cm.}^2/24 \text{ hours; } D_{20} = 0.606 \text{ cm.}^2/24 \text{ hours.}$

II.

Diffusion time: 2.840 days; temp. 16.8°–17.3°; average 17.0°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	25	27	29	27.0	1049	0.1540	81.47	
2	46.5	48	51.5	48.5	1885	0.1465	45.71	
3	73	80	73	75.3	2927	0.1142	20.33	$x_{av} = \frac{256.61}{1679} = 0.1528$
4	110	107	102.5	106.5	4139	0.1653	109.10	
				257.3	10000		256.61	

 $D = 0.547 \text{ cm.}^2/24 \text{ hours; } D_{20} = 0.569 \text{ cm.}^2/24 \text{ hours.}$

III.

Diffusion time: 3.042 days; temp. 19.5°–19.9°; average 19.7°.

	A	B	C	Average	0/000	x	$p \cdot x$	$x_{av} = \frac{221.42}{1679} = 0.1319$
1	48	46	46	46.7	1253	0.1327	70.20	
2	73	73	75	73.7	1978	0.1285	40.09	
3	108	107	112	109.0	2926	0.1141	20.31	
4	142.5	148	139	143.2	3843	0.1376	90.82	
				372.6	10000		221.42	

 $D = 0.592 \text{ cm.}^2/24 \text{ hours}$; $D_{20} = 0.594 \text{ cm.}^2/24 \text{ hours}$.*Carotene; first series.*

I.

Diffusion time: 2.930 days; temp. 19.3°–16.8° (mostly 17.8°–16.8°) average 17.3°.

	A	B	C	Average	0/000	x
1	39.5	40.5	—	40	851	0.1802
2	83	79.5	—	81	1724	0.1798
3	162	160.5	—	161	3426	—
4	193.5	182.5	—	188	4000	0.1518
				470	10001	

Section 1 + 2 = 2575; section 3 + 4 = 7426; $x = 0.1803$; $D = 0.450 \text{ cm.}^2/24 \text{ hours}$; $D_{20} = 0.467 \text{ cm.}^2/24 \text{ hours}$.

II.

Diffusion time: 2.920 days; temp. 18.2°–17.6°; average 17.9°.

	A	B	C	Average	0/000	x	$p \cdot x$	$x_{av} = \frac{316.63}{1679} = 0.1886$
1	—	8.6	9.0	8.8	962	0.1647	87.13	
2	—	14.2	13.9	14.05	1536	0.2228	69.51	
3	—	28.8	30.0	29.4	3215	0.2288	40.73	
4	—	38.4	40.0	39.2	4287	0.1807	119.26	
				91.45	10000		316.63	

 $D = 0.431 \text{ cm.}^2/24 \text{ hours}$; $D_{20} = 0.444 \text{ cm.}^2/24 \text{ hours}$.*Carotene; second series.*

I.

Diffusion time: 2.816 days; temp. 17.2°–16.5°; average 16.9°.

	A	B	C	Average	0/000	x	$p \cdot x$	$x_{av} = \frac{327.69}{1679} = 0.1952$
1	36	36	35.5	36	754	0.1935	102.36	
2	80	78	77.5	78.5	1644	0.1973	61.56	
3	148.5	147.5	148.5	148	3100	0.1614	28.73	
4	215.5	214	215	215	4502	0.2046	135.04	
				477.5	10000		327.69	

 $D = 0.432 \text{ cm.}^2/24 \text{ hours}$; $D_{20} = 0.450 \text{ cm.}^2/24 \text{ hours}$.

II.

	A	B	C	Average	0/000	x	$p \cdot x$	$x_{av} = \frac{303.04}{1679} = 0.1805$
1	28	27	27.5	27.5	872	0.1773	93.79	
2	53.5	53.5	53.5	53.5	1698	0.1854	57.84	
3	100	97.5	98.6	99.0	3143	0.1806	32.15	
4	135.5	136.5	132	135.0	4287	0.1807	119.26	
				315.0	10000		303.04	

 $D = 0.439 \text{ cm.}^2/24 \text{ hours}$; $D_{20} = 0.457 \text{ cm.}^2/24 \text{ hours}$.

Note. In Exp. 1 of the first series referring to carotene the concentration of section 3 was found very much too high and correspondingly the concentration of section 4 too low. Probably convection took place here.

In calculating the diffusion constant the sections 1 and 2 and 3 and 4 were added together and the value of x calculated by interpolating for the sum of these concentrations in the tables of Kawalki.

Discussion of the results.

The following table contains the summary of results referring to the diffusion constants:

Vitamin A.

	First series	Second series
I	$D_{20}=0.572$	$D_{20}=0.606$
II	$D_{20}=0.552$	$D_{20}=0.569$
III	$D_{20}=0.570$	$D_{20}=0.594$
Average	0.565	0.590
Average $D_{20}=0.578$ cm. ² /24 hours.		

Carotene.

	First series	Second series
I	$D_{20}=0.467$	$D_{20}=0.450$
II	$D_{20}=0.444$	$D_{20}=0.457$
Average	0.456	0.454
Average $D_{20}=0.455$ cm. ² /24 hours.		

The diffusion constant of vitamin A is found to be considerably higher than that of carotene, the difference exceeding appreciably the probable error of the experiments.

The results of parallel estimations of the vitamin experiments do not agree amongst each other as accurately as do other diffusion experiments done with the same apparatus. Probably the method of analysis must be considered as the principal source of inexactitude; as already stated the probable error of one determination amounts to 10 %. The accuracy of the observations was however enhanced by their number, considering that every value of the diffusion constant as recorded above is based on the average of 36 analyses. The accuracy of the analytical method for carotene exceeds that for vitamin A; this is perhaps the reason why the values found for carotene are more consistent than those for vitamin A.

Other sources of errors are the following.

1. Fluctuations of temperature; these were rather great for diffusion experiments. Xylene is not an advantageous solvent for these determinations. It has a small specific heat, a high dilatation coefficient and a low viscosity (drawbacks which however it shares with most other organic solvents). The occurrence of convection currents is very much favoured by these properties.

2. Evaporation of the xylene while filling and emptying the diffusion cylinders.

3. In the first series some glycerol was found at the bottom of the diffusion cylinder, after the experiments were finished.

4. Oxidation of the substance investigated.

Though the influence of these four sources of error upon the ultimate result is perhaps inferior to that involved by inadequacy of the analyses they doubtless vitiate the results to some extent. We estimate the probable error in the final figures above recorded (provided that there are no intrinsic causes due to systematic errors), for vitamin A 4 %, for carotene 2 %.

The molecular weight of vitamin A.

As to the molecular weight of vitamin A we may state the following. For the relation between the diffusion constant and the molecular weight, an empirical formula has been found:

$$D \sqrt{M} = c,$$

in which D is the diffusion constant, M the molecular weight and c a constant, dependent on the nature of the solvent and the temperature. This formula was experimentally tested by Öholm [1909] and found to be fairly satisfactory.

By comparing the diffusion constants found above with the molecular weights we obtain the following result:

$$\frac{\sqrt{M_{\text{car}}}}{\sqrt{M_{\text{vit}}}} = \frac{D_{\text{vit}}}{D_{\text{car}}} = \frac{0.578}{0.455} = 1.27.$$

If we now substitute in this formula the known value for the molecular weight of carotene we obtain:

$$M_{\text{vit}} = \frac{M_{\text{car}}}{1.27^2} = \frac{536}{1.61} = 333.$$

According to these calculations the molecular weight of vitamin A should be equal to 333, a figure which may be accepted only with reserve considering the uncertainty about the determination of the diffusion constant of vitamin A, and the error in M being twice as much as the error in D .

In addition we emphasise that the formula used above is not founded on the exact kinetic theory of diffusion; according to this the diffusion constant is connected more closely with the size, *i.e.* the diameter of the particles, than with their weight.

SUMMARY.

The diffusion constants of carotene and vitamin A in xylene were determined and found to be 0.455 cm.²/24 hours, and 0.578 cm.²/24 hours, respectively at 20°. The difference found exceeds considerably the probable errors of measurement. The ratio of the molecular weights of the two substances has been calculated and from this a molecular weight of about 330 has been deduced for vitamin A. The value obtained causes the assumption of a simple chemical relation between vitamin A and carotene to appear improbable.

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