CCXX. VITAMIN A AND CAROTENOIDS IN THE LIVER OF MAMMALS, BIRDS, REPTILES AND MAN, WITH PARTICULAR REGARD TO THE INTENSITY OF THE ULTRAVIOLET ABSORPTION AND THE CARR-PRICE REACTION OF VITAMIN A

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THE amount of vitamin A in the liver has always attracted considerable interest, partly because of the industrial production of vitamin A preparations from the liver of lower vertebrates (fish) and certain mammals (whales), partly because of the physiological interest attached to this organ. By far the greatest amount of the body's vitamin A reserves are stored in it, and in this organ carotene is converted into vitamin A.

Using the SbCl_3 method of assay, large series of estimations of the amount of vitamin A in the human liver [Wolff, 1932; 1936; 1938; Moore, 1937] and the liver of common domestic and laboratory animals [e.g. Sen & Sharma, 1936; Holmes *et al.* 1936] have already been carried out. In the case of wild animals, however, only meagre data are available. Nevertheless, such investigations may be expected to furnish items of considerable interest, as the relation between the various kinds of food and environment on the one hand and the vitamin A content of the liver on the other, may yield important information on the physiology of the vitamin. The possible presence of the so-called vitamin A_2 and other vitamin A factors in certain species might cast some light on the role these substances play in the metabolism of vitamin A and the carotenoids in these animals.

In addition to these strictly biological problems, the quantitative estimation of vitamin A in liver tissue still lacks perfection.

Most investigators have used the SbCl_3 method when working on the liver of warm-blooded animals. This method is not commonly regarded as being quite accurate for determining the absolute quantity of vitamin A, as the conversion factor for expressing the strength of the reaction in terms of international units (I.U.) of vitamin A is liable to wide variations. While thus the factor $E_{\text{cm.}}^{1\%} = 1$ corresponding to 1600 I.U. per g. has been internationally accepted for the absorption at 328 $\text{m}\mu$, there is no similar international factor for the Carr-Price reaction [Hume & Chick, 1935; Wolff, 1938]. It is, therefore, necessary to determine the conversion factor of the Carr-Price reaction for each source of vitamin A if the results are to be expressed in I.U. The factor is to be determined by comparing the Carr-Price values and the results of biological or spectrographic estimations of vitamin A.

We have not succeeded in finding in the literature a conversion factor for the Carr-Price reaction of mammalian and bird livers; spectrographic determination of these sources of vitamin A appears to have been carried out in a few

instances only. Thus Capper [1930] and Capper *et al.* [1931] have made spectrographic estimations of the unsaponifiable matter of rat and fowl livers respectively. The curves obtained showed a maximum at 328 $m\mu$ and, on the whole, they resembled the curves obtained for fish liver oils. These authors, however, did not employ the extinctions as a quantitative measure of vitamin A; the spectrographic determination was used only as proof of its presence.

As far as we know, quantitative spectrographic analysis of the vitamin A content of the liver of warm-blooded wild animals and man has not been attempted. Some of the latest and most extensive work has been reported by Gillam [1938] and Karrer *et al.* [1932], who examined, respectively, 21 and 34 different vertebrates; however, these authors give their results only in terms of the Carr-Price reaction.

In order to find a conversion factor for the Carr-Price reaction of livers we have deemed it necessary to determine the Carr-Price reaction as well as the ultraviolet absorption of the liver extracts in a large number of mammals and birds representing as many zoological groups as possible; stress has accordingly been placed on the comparative study of the two methods. Also, the carotenoids have been examined chromatographically, as up to the present a few species only have been studied satisfactorily by this method [Zechmeister & Tuzson, 1935]. In the biological discussion of the results of our analyses, it will be seen that it is not possible to draw general conclusions regarding the normal vitamin A or carotenoid (carotene) content of livers from individuals of any one species, though, apparently, individual variations are smaller than variations from one species to another.

MATERIAL

Most of the animal livers examined were obtained from the Zoological Museum of the University; the livers of domestic animals were purchased at a butcher's; livers of game were obtained through acquaintances and those of cats and dogs from the Royal Veterinary School; human livers came from the Medico-Legal Institute of the University and the Pathological Department of the Bispebjerg Hospital.

The liver specimens were saponified immediately on receipt, or were stored in the refrigerator not more than 12 hr. In several cases, 2 days elapsed from the death of the animal to saponification, as some animals were killed far from Copenhagen, e.g. in Jutland. With very few exceptions, the interval was never more than 72 hr.

EXPERIMENTAL METHODS

(1) *Samples*

Small livers (under 100 g.) were examined *in toto*; in the case of larger ones pieces from different parts of the organ were taken to ensure an average sample. This procedure was adopted as rather marked differences in the concentration of vitamin A in different parts of the same liver were observed; Moore [1937] states that the concentration in all the hepatic lobes is practically the same, although he does not give details. Sen & Sharma [1936] found higher values generally in the central parts of the liver than in the peripheral; the difference, however, did not amount to more than 10%. Lindqvist [1938], in a series of 22 duplicate analyses on different samples of the same lobe, found a standard deviation of 3.78 on a single analysis, while differences between the right and

left human lobes amounted to 75 %. These workers all used the technique of Davies [1933] for extraction and measurement of the Carr-Price reaction (the two first used the Lovibond tintometer, the last-named the Pulfrich photometer).

(2) *Saponification and extraction*

Saponification and extraction were performed by a modification of the technique of Davies [1933]. Small pieces of liver (each under 5 g.) were prepared and weighed together. 4 ml. 5 % alcoholic KOH were used per g. liver and the mass was heated $\frac{1}{2}$ hr. on a boiling water bath under a reflux condenser. An addition of about $\frac{1}{3}$ volume light petroleum¹ to the mixture gave prompt boiling and accelerated the solution of the tissue. After saponification, an equal volume of distilled water was added. The extraction was performed 3–5 times until the extract was colourless, using about $\frac{1}{2}$ volume of peroxide-free ether. The combined extracts were washed 3–5 times with a liberal amount of distilled water and dried by suction through a 3–5 cm. layer of anhydrous Na_2SO_4 . The extract was then evaporated to dryness in an atmosphere of N_2 ; the remainder was dissolved in light petroleum and transferred to a graduated flask (5–50 ml., according to the amount of liver). A certain amount (usually half) of the solution was evaporated, the residue dissolved in absolute alcohol for spectrographic determination and the remainder used for measuring the Carr-Price reaction, the yellow value (total carotenoid content) and chromatography.

(3) *Spectrographic determination*

Spectrographic measurements were made with a Hilger E_3 quartz spectrograph with Echelon cell outfit following the method of Twyman [1933]. This equipment allows for rapid exposures and wide extinction range.

In making the spectrograms, three values of density were chosen so that the 30 pairs of spectra constituted a series of variations in which the logarithms of the extinctions calculated for 1 cm. layer of thickness varied for each step between the limits +0.07 and +1.08.

Absolute ethanol was used as a solvent throughout, the strongest solution possible being used for the first exposures, and when dilutions were necessary the dilutions 1 : 10 and 1 : 100 were used. By adopting this procedure, the parts of the curve represented by two consecutive plates lie in continuance of each other and the logarithms of the extinction values of the dilution 1 : 10 are comparable with those of the stock solution simply by addition of the figure 1, and so on. The match points were marked and the readings taken by two observers independently of each other. Thus a very ample and very exact representation of all parts of the extinction curve was obtained in the range of about 200–500 $\text{m}\mu$ if the original concentration was strong enough.

The graphs were plotted with the wave-length along the abscissa (1 cm. $\sim$ 20 $\text{m}\mu$) and the logarithm of the extinctions for 1 cm. thickness of liquid as ordinate (1 cm. $\sim \log \epsilon = 0.20$). The extinction curves should become uniform by this arrangement, i.e. only the height and not the shape of the curve depends on the concentration.

The extinction at 328 $\text{m}\mu$ and 450 $\text{m}\mu$ at the given concentration was read off on the curves, and the values $E_{1\text{ cm.}}^{1\%}$, 328 $\text{m}\mu$ and $\frac{\epsilon_{328\text{ m}\mu}}{\epsilon_{450\text{ m}\mu}}$ may then be calculated. Unfortunately, lack of space prevents us from giving illustrations of any of the graphs.

¹ The light petroleum had a B.P. of 65–70°.

(4) *Measurement of the Carr-Price reactions*

These measurements were made on a Zeiss Pulfrich photometer with a $\frac{1}{2}$ cm. cell. The stock solution was progressively diluted until the extinction lay between 0.15 and 0.50. 0.2 ml. of the solution was used for the reaction; 2 ml. Carr-Price reagent were added prior to which 1 drop (never more than 2 drops) of acetic anhydride was placed in the cell (details of the technique are given by Willstaedt & Jensen [1937]). The readings were taken as soon as possible after addition of the reagent, i.e. after about 5 sec.; 2–5 samples were measured, the cells interchanged, and corresponding readings taken on the other side. The mean value of the measurements has been given as the result.

The extinction coefficient of the blue reaction was calculated as follows¹: $B_{1\text{ cm.}}^{1\%} = \frac{E_n}{s \times c} \times E_n$ denotes the mean value of n measurements (half from each side of the photometer) of a 0.2 ml. solution + 2 ml. reagent; s denotes the layer of thickness expressed in cm., i.e. $\frac{1}{2}$; c is the concentration expressed in g. per 100 ml. of the solution in the cell (the original solution + the reagent), and therefore 1/11 of the concentration of the original solution.

The value of the blue colour readings with the various filters is designated by S 59, S 61 and S 66.6 (see Tables).

(5) *Analysis of the carotenoids*

As the amount of carotenoid in the liver is generally very small, the quantitative estimation was limited to the determination of the so-called "yellow value" (total carotenoid) and to the quantitative estimation by microchromatography.

The yellow value was measured on the Pulfrich photometer with the filter S 43, in several cases also with the filters S 45 and S 47. The values are given in the Tables as $C_{1\text{ cm.}}^{1\%}$, the number of the filter being stated. In some cases in which the material was insufficient for exact measurement only the upper limit has been given.

Chromatographic measurements were carried out by the technique of Willstaedt & With [1938], i.e. Al_2O_3 purissimum "Merck" and columns 2–5 mm. wide and 5–7 cm. in height; development was performed with benzene-petroleum (b.p. 65–70°) 1 : 1 or 2 : 1 or pure benzene.

From the appearance of the microchromatograms it could generally be judged with certainty if any considerable amount of carotene were present; amounts of carotene below 10–15 %, as well as the nature of the other bands, could merely be conjectured. In many cases, clear yellow bands were found at the top of the column; these were very likely due to phytoanthin, which was rarely present in any considerable amount. In most cases brownish or brownish-yellow bands, presumably due to oxidation products of the carotenoids, were found lower down. The carotene appeared as a rose-coloured zone partly or entirely passing into the filtrate; this zone in concentrations above 30 % was well marked. Occasionally a yellow zone (? α -carotene) might be distinguished below. Other zones, from red to rose-coloured, might appear below the oxidation products, sometimes separated from these by colourless bands; they were never diffuse like the carotene zone. The exact nature of these zones could not be determined by micro-analysis.

¹ This method of calculation is allowable as the Carr-Price reaction read in a spectrograph or a spectrophotometer follows Beer's law [Nottevarp & Weedon, 1936; Dann & Evelyn, 1938].

By painting the column with Carr-Price reagent [Zechmeister *et al.* 1936] the presence of vitamin A might be detected. If carotene were present in measurable amount, the blue colour stopped before the carotene zone, while the other zones, especially those of oxidation products, as a rule were coloured intensely blue.

In some chromatograms of livers rich in vitamin A, no distinct colour zones appeared; instead there was a cloudy discoloration of the whole column with an intense dark blue Carr-Price reaction. However, this occurred only in livers rich in vitamin A that exhibited a low yellow value.

Comparison of the yellow values found with S 43, S 45 and S 47 often gave a valuable hint of the nature of the carotenoids present, as the values are of the same order, more or less, for carotene, xanthophyll, zeaxanthin and kryptoxanthin, while for lycopin the extinction value with S 47 is considerably higher than with S 43, and the oxidation products often show great differences in the extinction values with the filters mentioned.

(6) Investigation of the loss due to time

Davies [1933] was not able to demonstrate any loss after keeping his samples for 3 days at room temperature. We also have investigated the problem, as will appear below. After mincing and weighing portions of 10 g. (± 0.1 g.), 2 portions were analysed immediately, 2 after standing 2 days in the dark in a closed vessel at room temperature, 2 portions after standing 4 and 2 after standing 8 days. The extinctions of the extracts of 10 g. liver made up to 5 ml. are given below. Microchromatography revealed that practically all the carotene was β -carotene. It will be seen that under these conditions the loss of vitamin A and of carotene does not exceed 10% in 8 days. As these experimental conditions closely resemble the condition of the vitamin A in the liver of the dead animal, it may be concluded that no significant loss has occurred due to keeping.

	Before	2 days	4 days	8 days
Carotene (S 43; $\frac{1}{2}$ cm.)	0.310	0.319	0.297	0.295
	0.323	0.314	0.292	0.288
Vitamin A (S 61; $\frac{1}{2}$ cm.; 0.2 ml. sol. + 2.0 ml. reag.)	0.224	0.228	0.221	0.186
	0.208	0.207	0.204	0.206

Explanation of the Tables

In the Tables the results have been schematically arranged for mammals (Table I), birds (Table II), reptiles (Table III) and the human subject (Table IV).

Column 1 gives the systematic zoological order, column 2 the name of the species. Columns 3, 4 and 5 give the extinctions " $B_{1\text{cm}}^{1\%}$ " ($E_{1\text{cm}}^{1\%}$ for the Carr-Price reaction) with the three filters used. Column 6 gives the extinction " $E_{1\text{cm}}^{1\%}$ " at 328 m μ of vitamin A. To give some idea of the form of the extinction curve, the absorption maxima are set up in column 14. Figures without any parentheses denote a clear maximum represented by a peak on the curve, single parentheses denote an inflexion on the curve and double parentheses signify that the curve at the given wave-length has a slanting direction without any sign of a maximum or inflexion. Similarly, we have tried to give briefly a rough idea of possible variations in the Carr-Price reaction extinction curve in columns 8 and 9 ($B_{1\text{cm}}^{1\%}$ S 61 = 1); in column 8 is given the extinction with filter S 59 divided by the extinction with S 61, and in column 9 the extinction value with the filter S 66.6 is divided by the extinction with S 61. Column 7 gives the relations $\frac{e_{328\text{m}\mu}}{e_{450\text{m}\mu}}$

between the extinctions at 328 $m\mu$ (the vitamin A absorption maximum) and 450 $m\mu$ (the absorption maximum of the carotenoids), as read off on the spectrographic curves. The relation has been termed larger than ($>$) a certain value if the extinction at 450 $m\mu$ has been less than the smallest extinction which can be read at the greatest concentration in which an exposure has been possible. Column 10 gives the relation " $B_{1\text{ cm.}}^{1\%}$ S 61: $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ " between the extinction coefficient of the Carr-Price reaction at its maximum (S 61) and the extinction coefficient of vitamin A at 328 $m\mu$; this ratio is of great importance in determining the utility of the Carr-Price reaction as a quantitative method of assay of vitamin A. In the remaining columns, 11 and 12, the carotenoid extinction is given per g. in 100 ml. in 1 cm. layer " $C_{1\text{ cm.}}^{1\%}$ " with the filters S 43 and S 47. Column 13 shows the results of the microchromatographic estimation of carotene.

The results obtained with human livers are in the main presented in the same way; columns 1-3 furnish data such as protocol number, sex, age and autopsy findings. The other columns are identical; the column dealing with spectral maxima has been omitted, as all the livers showed a clear maximum at 328 $m\mu$.

Owing to prevailing circumstances, all the determinations were not carried out on some livers.

The results have not been converted into micrograms carotene or to I.U. of vitamin A, the extinction coefficient $E_{1\text{ cm.}}^{1\%}$ having been preferred as a basis for the quantitative estimations.

With the help of the Tables it is easy to convert the absorption at 328 $m\mu$ into I.U. per g. liver, the value $E_{1\text{ cm.}}^{1\%}$ having simply to be multiplied by 1600; however, this obtains only when the curve has a clear maximum at 328 $m\mu$ or thereabout. The conversion of the carotenoid value into micrograms may be performed by using the current value of the extinction coefficient $E_{1\text{ cm.}}^{1\%}$ of carotene with the given filters; this conversion factor ranges from 1800 to 2500, according to different investigators. The following values for β -carotene were reported by Willstaedt & Jensen [1939, 2]: $C_{1\text{ cm.}}^{1\%}$ S 43 = 1963, $C_{1\text{ cm.}}^{1\%}$ S 45 = 2352, $C_{1\text{ cm.}}^{1\%}$ S 47 = 2242.

Physico-chemical discussion

From the commencement of this work, the main interest has been centred on the relation between the extinction coefficients of the Carr-Price reaction and of the ultraviolet absorption, because, as mentioned in the introduction, the value of the Carr-Price reaction as a quantitative method of assay of vitamin A depends on it. The computed mean values are practically the same in the series (column 10) of values found in the mammals, birds and human subjects examined (2.60-2.75), and the standard deviation is fairly small (coefficient of variation about 15%). Thus this interesting relation is found not only in different individuals of the same species, but in all warm-blooded animals, in spite of the great variation in the quality of their food.

The animals that did not show a definite maximum on the absorption curve at 328 $m\mu$ have been omitted from the results, as the absorption at this wavelength in these cases was due not only to vitamin A,¹ but also to other causes such as the presence of carotenoid.

¹ The considerable discrepancy in the relation $B_{1\text{ cm.}}^{1\%}$ S 61: $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$ found in the white-nosed grivet must be regarded as being due to the technical error of omitting to record the dilution 1:10 of the solution employed in the Carr-Price reaction. We have, therefore, placed the value found in parentheses, and in our calculations reckoned with 10 times the amount.

Table I. *Mammals*

1	2	3-5		6	7	8-9		10	11-12		13	14
Order	Species	$B_{1\text{cm}}^{1\%}$		$E_{1\text{cm}}^{1\%}$ 328 mμ	$e_{328\text{ m}\mu}$ $e_{460\text{ m}\mu}$	$B_{1\text{cm}}^{1\%}$ S 61 = 1	$B_{1\text{cm}}^{1\%}$ S 66-6	$B_{1\text{cm}}^{1\%}$ S 61 $E_{1\text{cm}}^{1\%}$ S 328 mμ	$C_{1\text{cm}}^{1\%}$ S 43	$C_{1\text{cm}}^{1\%}$ S 47	Carotene (micro- chromato- graphic estimation)	Spectra maxima (mμ)
Cetacea	Grampus (<i>Grampus griseus</i>)	I. 0.0418	No reaction	—	—	—	—	—	—	—	0	—
	Mole (<i>Talpa europaea</i>)	II. 0.065	0.125	—	—	0.882	0.403	—	0.0012	0.0011	0	—
Insectivora						0.762	—	—	<0.002	—	0	—
Chiroptera	Bat (<i>Vespertilio</i> sp.)	—	1-16	0.385	>8.13	—	—	3.02	0.014	0.009	0	325
Rodentia	House-mouse (<i>Mus musculus</i>)	—	No reaction	—	—	—	—	—	<0.005	—	0	—
	Squirrel (<i>Sciurus vulgaris</i>)	0.161	<0.023	0.114	9.55	0.817	0.386	(1.74)	<0.002	—	0	320 (450)
	Hare* (<i>Lepus timidus</i>)	I. 0.495	0.703	0.236	251	0.704	0.124	2.97	0.0022	0.0012	0	325 (450)
		II. 0.238	0.329	0.046	—	0.723	0.140	—	0.0016	0.0013	—	—
	Guinea-pig (<i>Cavia porcellus</i>)	I. 0.0036	0.0044	0.0038	2.00	0.817	0.208	(1.15)	0.0042	0.0039	0	(328) 450
		II. —	—	0.0024	3.72	—	—	—	—	—	—	(260) (328) (390) (450)
Carnivora	Capibara (<i>Hydrochoerus capivarus</i>)	0.176	0.247	0.097	>28	0.713	0.142	2.56	<0.0004	—	0	325
	Cat (<i>Felis catus</i>)	I. 0.496	0.727	0.106	74.1	0.682	0.146	2.25	0.0048	0.0040	About 40%	328 (450)
		II. 0.440	0.686	0.120	64.4	0.641	0.175	2.89	0.0038	0.0036	—	326 (450)
	Dog (<i>Canis familiaris</i>)	I. 0.334	0.449	0.153	89.1	0.744	0.236	2.94	0.0018	0.0012	0	324 (450)
		II. 0.0775	0.109	0.033	>20.9	0.711	0.303	2.84	0.0012	0.0010	Trace	324
		III. —	0.060	—	—	—	—	—	0.0032	0.0027	—	—
		IV. —	0.172	—	—	—	—	—	0.0022	0.0016	—	—
	Badger* (<i>Meles meles</i>)	I. 0.0606	0.0786	0.0133	3.72	0.771	0.169	2.55*	0.008	—	About 60%	283 322 450
	Fox (<i>Canis vulpes</i>)	I. 1.37	1.78	0.352	51.3	0.770	0.198	2.77	0.0153	0.0146	About 33%	325 (450)
		II. 2.81	3.90	0.569	166	0.720	0.146	2.53	0.0120	0.0109	About 40%	325 (450)
		III. —	0.450	—	—	—	—	—	0.0112	0.0102	About 60%	—
	Ermine (<i>Mustela erminea</i>)	I. 0.0735	0.102	0.0092	>5.37	0.721	0.090	(1.91)	<0.0005	—	0	300 (328)
		II. 0.121	0.139	0.046	>5.50	0.871	0.331	(1.34)	<0.002	—	0	(300) (328)
	Fitchet (<i>Mustela putorius</i>)	I. 1.05	1.50	0.328	64.6	0.700	0.219	2.57	0.013	0.013	About 30%	326 (450)
		II. 1.82	2.46	0.582	83.2	0.740	0.144	3.08	0.009	0.008	About 60%	325 (450)
		III. 1.83	2.78	0.824	331	0.658	0.297	2.54	0.007	0.003	0	325 (450)
	Otter (<i>Lutra lutra</i>)	I. 0.122	0.172	0.039	>22.9	0.709	0.237	2.77	<0.0002	—	0	326 (450)
	Swine (<i>Sus scrofa domestica</i>)	I. 0.844	1.08	0.360	69.2	0.781	0.333	2.94	0.0055	0.0054	About 80%	325 450 (475)
	Horse (<i>Equus caballus</i>)	I. 0.570	0.817	0.167	6.61	0.698	0.204	2.76	—	—	—	325 450
	Cow† (<i>Bos taurus</i>)	II. 0.0758	0.0969	0.0168	281	0.782	0.173	2.42	—	—	—	325 450
	Sheep (<i>Ovis aries</i>)	I. 1.77	2.47	0.540	74.1	0.717	0.219	2.58	—	—	—	325 (450)
	Roedter (<i>Capreolus capreolus</i>)	I. 1.40	2.29	0.265	200	0.611	0.116	2.55	0.0074	0.0065	About 30%	324 (450)
	Whitenosed grivet (<i>Cerco- pithecus nictitans</i>)	0.0234	0.0275	0.108	>22.4	0.851	0.349	(0.255)	0.003	—	0	322

Table II. *Birds*

1 Order	2 Species	3-5 $B_{10}^{1/2}$		6 $E_{328}^{1/2}$ 328 m μ	7 $\frac{e_{328} \text{ m}\mu}{e_{459} \text{ m}\mu}$	8-9 $B_{10}^{1/2}$ S 61 = 1		10 $B_{10}^{1/2}$ S 61 $E_{10}^{1/2}$ 328 m μ	11-12 $C_{10}^{1/2}$		13 Carotene (micro- chromato- graphic estimation)	14 Spectral maxima (m μ)
		S 59	S 61			S 59	S 66-6		S 43	S 47		
Colymbiformes	Polar loon (<i>Colymbus arcticus</i>)	—	2-275	—	—	—	—	—	0-009	0-005	0	—
	Cormorant (<i>Phalacrocorax carbo</i>)	0-174	0-251	0-092	0-107	0-693	0-367	2-35	0-0197	0-0194	0	326 (450) (470)
Steganopodes	Gannet (<i>Sula bassana</i>)	4-56	6-65	1-34	2-15	0-686	0-202	3-10	0-0058	0-0044	0	326 (450)
Lamellirostrres	Velvet-duck (<i>Oidemia fusca</i>)	0-196	0-249	0-049	0-0928	0-787	0-197	2-69	0-014	0-013	0	(290) 328 450
	Garganey (<i>Anas querquedula</i>)	0-202	0-213	0-059	0-126	0-948	0-277	(1-69)	0-127	0-135	About 50 %	280 (328) 455
	Wild duck (<i>Anas boschas</i>)	0-171	0-261	0-054	0-0886	0-655	0-207	2-94	0-013	0-013	Trace	328 455
	Teal (<i>Anas crecca</i>)	0-289	0-370	0-065	0-196	0-781	0-176	1-89	0-011	0-012	Trace	285 324 (450)
Raptatores	Merlin (<i>Falco aesalon</i>)	0-137	0-177	0-025	0-0895	0-774	0-141	1-98	<0-0008	—	0	320
	Kestrel (<i>Falco tinnunculus</i>)	2-71	3-75	0-674	1-31	0-723	0-180	2-86	0-018	0-014	0	325 (450)
	Shaggy-footed buzzard (<i>Buteo lapogus</i>)	6-16	9-12	1-68	3-21	0-675	0-184	2-85	0-009	0-006	0	328 ((450))
	Buzzard (<i>Buteo buteo</i>)	I. 2-13 II. —	3-06 2-51	0-415 —	0-953 —	0-696 —	0-136 —	3-21 —	0-003 0-023	— 0-017	0 15-20 %	326 —
Galliformes	Curassow (<i>Craz fasciolatus</i>)	0-0228	0-0250	0-0069	0-0226	0-83	0-396	(1-11)	0-031	0-027	About 10 %	(328)) 450
	Eared pheasant (<i>Crossoptilon auritum</i>)	—	0-219	—	—	—	—	—	0-0043	0-0039	Trace	—
	Black grouse (<i>Tetrao tetrix</i>)	3-52	4-39	1-03	1-61	0-802	0-235	2-73	0-019	0-019	0	324 (450)
	Hen (<i>Gallus domesticus</i>)	I. 0-121 *II. — III. — IV. —	0-169 0-550 0-123 6-85	0-037 — — —	0-0682 0-230 0-063 3-55	3-02 — — —	0-716 — — —	0-219 — — —	— 0-016 0-011 0-002	— — — —	— — — —	326 445 — — —

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* II, III and IV are medians of groups each of 5 hens.

Table III. *Reptiles*

1	2	3-5		6	7	8-9		10	11-12		13	14	
		$B_{1\text{cm}}^{\circ/\text{cm}}$				$B_{1\text{cm}}^{\circ/\text{cm}}$ S 61 = 1			$C_{1\text{cm}}^{\circ/\text{cm}}$				
Order	Species	S 59	S 61	$E_{1\text{cm}}^{\circ/\text{cm}}$ 328 m μ	$e_{328\text{ m}\mu}$	S 59	S 66-6	$E_{1\text{cm}}^{\circ/\text{cm}}$ 328 m μ	$B_{1\text{cm}}^{\circ/\text{cm}}$ S 61	S 43	S 47	Carotene (micro-chromato-graphic estimation)	Spectral maxima (m μ)
Ophidae	Viper (<i>Vipera berus</i>)	I. —	0.0331	—	—	—	—	—	—	<0.0005	—	0	—
		II. —	0.016	—	—	—	—	—	—	—	—	0	—
		III. —	0.016	—	—	—	—	—	—	—	—	0	—
Varanus	Monitor (<i>Varanus comodensis</i>)	1.60	2.15	0.822	159	0.744	0.152	2.62	0.023	0.017	10-20 %	328	

Table IV. *Human livers*

1	2 Sex and age	3 Diagnosis (post-mortem examination)	4-6 $B_{1\text{cm}}^{\circ/\text{cm}}$		7 $E_{1\text{cm}}^{\circ/\text{cm}}$	8 $e_{328\text{ m}\mu}$ $e_{450\text{ m}\mu}$		9-10 $B_{1\text{cm}}^{\circ/\text{cm}}$ S 61 = 1		11 $B_{1\text{cm}}^{\circ/\text{cm}}$ S 61 $E_{1\text{cm}}^{\circ/\text{cm}}$ 328 m μ		12-14 $C_{1\text{cm}}^{\circ/\text{cm}}$		15 Carotene (micro- chromato- graphic estimation)
			S 59	S 61	S 66-6	$e_{328\text{ m}\mu}$	$e_{450\text{ m}\mu}$	S 59	S 66-6	$E_{1\text{cm}}^{\circ/\text{cm}}$	$E_{1\text{cm}}^{\circ/\text{cm}}$ 328 m μ	S 43	S 47	
I.	♂ 70	Pneumonia	0.107	0.131	0.040	0.0546	6.17	0.817	0.305	2.40	—	—	—	—
II.	♂ 77	Aneurism	0.278	0.370	0.066	0.159	11.5	0.751	0.178	2.32	0.0151	0.0149	0.0105	About 50 %
III.	♀ 79	Pneumonia. Mb. cordis	0.0716	0.0980	0.0191	0.0359	3.89	0.731	0.195	2.73	0.0113	0.0105	0.0005	About 50 %
IV.	♂ 57	Cirrhosis hepatis. Osteo- malacia. Steatorrhoea	0.0179	0.0226	0.0047	0.0126	>4.47	0.792	0.208	(1.79)	0.0006	0.0005	0.0005	Trace
V.	♀ 59	Embolism	0.762	1.06	0.228	0.302	21.4	0.719	0.215	3.52	0.009	0.0081	0.0081	About 30 %
1.	♀ 45	Suicide	0.336	0.482	0.090	0.185	12.0	0.697	0.187	2.60	0.021	0.019	0.019	About 70 %
2.	♂ 60	Mb. cordis	0.269	0.388	0.073	0.138	38.9	0.693	0.188	2.82	0.0045	0.0042	0.0042	About 40 %
3.	♂ 59	Mb. cordis	0.218	0.316	0.046	0.120	7.41	0.691	0.145	2.62	0.0149	0.0146	0.0146	About 50 %
								M: 0.736	0.203	2.75				
								σ : 0.050	0.022	0.406				

The presence of greater amounts of carotenoids appears in the ratio $e_{328\text{ m}\mu} : e_{450\text{ m}\mu}$ (column 7) which is particularly small in these cases, i.e. the garganey, the currassow and the guinea-pigs.

It seems that the absorption curves showing a maximum neither at 328 m μ nor at 450 m μ are due to peculiarities of the species; they have been found in the two ermines, the golden plover, the dotterel plover and partly also in the squirrel, in which the curve shows a maximum at 320–330 m μ , but is conspicuously flat. One of the human livers (no. IV) is also an exception; this must be ascribed to a pathological condition, as this person suffered from a severe hepatic disorder with fatty diarrhoea; this furnishes a plausible explanation of the peculiarities of the curve and the low concentration of vitamin A and carotene (cf. Table IV).

The relation between the ultraviolet absorption at 328 m μ of vitamin A and the absorption of carotenoids in the maximum wave-length (near 450 m μ) (see column 7 in the Tables) varies considerably; this must be attributed to the great variation in the amount of carotenoid in the food of the different species, as the amount of carotenoid in the liver depends in the first instance on the carotenoid content of the food; this is converted into vitamin A in the liver, metabolized or stored in small quantities. The carotenoids found in the liver are, therefore, as stated above, for the most part non-characteristic metabolic products, and the amounts found are not necessarily considerable even though the animal receives a rich supply of carotenoids with the food. The concentration in the liver depends on two factors, i.e. the amount ingested and the metabolic processes in the liver. Considerable amounts have been found only in a few cases, namely in the garganey, the currassow and the guinea-pig; the extinctions due to carotenoids in these cases were of the same magnitude as the ultraviolet absorption of vitamin A. In the other livers examined, the carotenoid concentration has been so small in comparison with the concentration of vitamin A that the ultraviolet absorption due to the former has caused no error in the spectrographic measurement of the latter.

As stated above, the curves obtained for vitamin A absorption in the ultraviolet range are, with the exceptions thus mentioned, practically identical in all cases.

The absorptions of the Carr-Price reactions also show comparatively slight variation, as the extinction with S 59 divided by the extinction S 61 shows a mean value of 0.73–0.76 with a coefficient of variation of 5–10%. However, there is a considerably larger variation in the relation between the extinctions with S 66.6 and S 61, namely, a mean variation of 0.20–0.22, and a coefficient of variation between 20 and 40% (in the human material only 10%)—a natural consequence of the fact that the filter S 66.6 corresponds to that part of the absorption curve which is far from the maximum, and in which, therefore, measuring takes place in a strongly sloping part of the curve.

The form of the spectral absorption curve and the relation of the Carr-Price reaction extinctions with S 61 and S 66.6 furnish information as to the presence of vitamin A₂ and possibly other vitamin A factors, e.g. the factors found by Pritchard *et al.* [1937] in mammalian livers and by Willstaedt & Jensen [1939, 1] in whale liver oil. As the vitamin A₂ absorption maxima lie on the 340–350 and 280–290 m μ ranges, and the factors of Pritchard *et al.* [1937] as well as the factors of Willstaedt & Jensen [1939, 1] at 290 m μ , the presence of these factors in considerable amount would result in maxima or inflexions at these wavelengths, or at least give a flattened curve. These phenomena have not been observed by us.

As classical vitamin A has its maximum in the Carr-Price reaction at 610–620 $m\mu$, while vitamin A₂ shows strong absorption bands at 690–700 $m\mu$, and the factor of Pritchard *et al.* [1937] shows two bands at 490 and 590 $m\mu$ and that of Willstaedt & Jensen [1939, 1] a band near 570 $m\mu$, the presence of the first substance should be revealed by an increase of the ratio $B_{1\text{ cm}}^{1\%}$ S 66.6 : $B_{1\text{ cm}}^{1\%}$ S 61 and the latter by an increase in the ratio $B_{1\text{ cm}}^{1\%}$ S 59 : $B_{1\text{ cm}}^{1\%}$ S 61. This we have not observed. True, one ermine showed a maximum at 300 $m\mu$ and the other had a inflexion here, while the maximum at 328 $m\mu$ was absent; presumably this was due neither to the factor of Pritchard *et al.* nor to the factor of Willstaedt & Jensen, as the Carr-Price reactions were not characteristic of these substances. It has not been possible to determine the nature of these variations as the samples at our disposal were insufficient for fractionating and purifying possible new vitamin A factors. Thus other factors have not been demonstrated even in the otter, in which Gillam [1938] has been able to find vitamin A₂. It may be mentioned that the otter examined by us was a wild specimen shot by a stream in Mid-Jutland; it had, therefore, presumably fed mainly on fresh-water fish.

BIOLOGICAL DISCUSSION

The great differences in the vitamin A contents of the livers of different species are shown in the Tables. These differences are often considerable in species closely related both zoologically and with regard to the nature of their food. The variations between different individuals of the same species are unfortunately insufficiently illustrated by the present material; as far as can be seen, however, the variation from individual to individual in the same species, even though considerable at times, is nevertheless of a different magnitude from the variation from one species to another. Some examples will be given. The gannet and the cormorant are closely related zoologically and both feed on marine fish (the gannet, however, only feeds on surface fish as it is a plunger) which they devour in great quantities. This makes the dissimilarity in the vitamin A concentrations in the liver very striking, as both the specimens were full-grown and were shot in Danish waters in the autumn of 1938. The gannet liver contained about 3500 I.U. per g., while the cormorant contained only about 300. In the polar loom which subsists on nearly the same food, a value of about 1200 I.U. per g. was found.

In the duck group the liver has a fairly constant amount ranging from 150 to 300 I.U. per g., although the garganey, as will be mentioned presently, shows wide deviation.

The kestrel and the merlin differ greatly, although closely related systematically and in their food consumption. The amounts of vitamin A in the two buzzards are high and almost identical, while the shaggy-footed buzzard shows the highest value found in the wild birds examined.

The grouse liver contains about the same amount as those of gannet and polar loom, which shows that birds living on land may take as much vitamin A with their food as marine birds.

The four samples of hen livers are of particular interest, as the animals were of the same kind but had been fed in different ways. Sample I was bought in a shop; sample II is from 5 hens that received 48 % food without vitamin A + 42 % yellow maize from birth till they were killed at 8 weeks of age; sample III is from 5 hens which had received food without vitamin A with an addition of 6 mg. carotene (as carotene oil) per kg. food, and sample IV is from 5 hens that received the vitamin A-free food with the addition of 0.5 % whale liver oil

(containing 190,000 I.U. per g.). Further details of these investigations are given in papers by With [1938] and by With & Wanscher [1939]. As expected, group IV shows a very high concentration of vitamin A in the liver; it is remarkable that the vitamin A in all four cases showed identical physical and chemical properties although it was derived from different sources.

The gulls show fairly high values. Of the passerine birds, the lark has a considerable amount of vitamin A in the liver. The Amazon parrot shows the lowest value found (5–10 I.U. per g.); however, this bird succumbed spontaneously in the Zoo. The golden plover and the currawong were also relatively poor in vitamin A.

The few analyses of reptiles have a certain interest. Of the three lots of vipers examined, the first was a mature specimen killed just before hibernation, the second (a full-grown snake) and the third (two immature snakes) were killed in February, i.e. several months after commencement of the winter sleep. There was no appreciable diminution of vitamin A value in the hibernating reptiles, as the higher value found in the first specimen may be due to the larger size (age) of the animal. Thus probably no considerable amounts of vitamin A are consumed during reptilian hibernation.

General inanition appears to present similarities in this respect, judging from an observation of a specimen of *Varanus comodensis*. This animal had been brought to Denmark from the Comodo Island by Dr Paul Fejos. During the voyage and stay in the Zoo it took practically no nourishment; nevertheless, after its death a very considerable layer of body fat was present and a large amount of vitamin A was found in the liver. As the adipose tissue was extremely poor in vitamin A and carotenoids ($B_{1\text{cm}}^{1\%}$ S 61 = 0.00023; $E_{1\text{cm}}^{1\%}$ S 43 = 0.00032), by far the largest store of vitamin A was contained in the liver. The large store of vitamin A even after about 6 months of inanition is remarkable.

The mammals have shown the same results as the birds. Exceptions are, however, the foxes; one of the specimens (the largest) showed values about 10 times greater than the others.

The whale liver examined was, strangely enough, almost devoid of vitamin A. This animal had been lying for a couple of weeks on the shore, but the temperature was low (winter), a condition which is deemed unfavourable for the destruction of the vitamin. It may thus be assumed that this kind of whale (the grampus) stores insignificant amounts of vitamin A in the liver.

Vitamin A in the mole and the bat—two animals which subsist mainly on insects and worms—has the same physico-chemical properties as vitamin A in the livers of the other animals. Judging from the amount in the bat's liver, insects are rich sources of vitamin A.

The rodents examined have only small amounts in the liver. The capibara examined was a specimen destined for the Zoological Gardens, but it died on the voyage; the body was, however, kept in cold storage until we examined it.

The lack of vitamin A₂ in the otter liver examined has been mentioned above, also the peculiar absorption curves given by the extract of the ermine liver; the concentration in this animal's liver was fairly low. The highest concentration of vitamin A was found in the largest of the foxes (about 2000 I.U. per g. liver); this is about one-third of the highest value in the wild birds.

The domesticated beasts of prey (dog and cat) nearly all have a considerable amount of vitamin A in the liver, although the amount is notably less than in the wild species (fox, fitchet).

The pig (omnivorous) has a rather low value (about 100 I.U. per g.), which is not surprising, as pigs in this country are generally given food poor in vitamin A.

There is considerable variation among the herbivorous animals: the horse and ox show values round about 500 I.U. per g. liver, while the calf (cow II) contains only about 100 I.U., presumably because young animals on the whole have lower reserves of vitamin A than older ones. The sheep (the specimen examined was a lamb) and the roe deer (full-grown animal) show considerably higher values (about 1600 I.U. per g. liver). These differences are not explicable by differences in the fodder, as the horse, cow and sheep receive most of their vitamin A requirements during spring and summer pasturing; the roe deer feeds on plants all the year round and vitamin A in its food is presumably also derived from green buds and leaves in the early summer.

However, the interesting biological problem regarding the variations in vitamin A reserves in the different species has been far from solved by the present material examined; an exhaustive investigation would require analyses of numerous individuals of each species.

The irregularities in the vitamin A contents of the liver have also been found to apply to the carotenoid contents.

Our investigations have led to conclusions which necessitate a revision on some points of the current view of the role of these substances in vitamin A metabolism in the various species. Thus it is stated by several investigators that birds do not store carotene but only phytoxanthin, and that carnivora are unable to store and metabolize carotene. It will appear from the present investigations that this is, at any rate, not the case in some species in the groups analysed. Furthermore, it may be mentioned that considerable quantities of carotene are found in the liver of hens shortly after a meal rich in carotene [With & Wanscher, 1939]; this is not necessarily opposed to the results of Palmer *et al.* [Palmer, 1922], in which it was shown that carotene was not stored in the liver of hens under normal conditions. It will be noted that among the birds, the garganey and the currawong have a high concentration of carotenoids in the liver, while the concentration of vitamin A is low. Only in the garganey does the main part of the carotenoids consist of carotene. The high concentration of carotenoid in these animals may be due to a preceding meal rich in carotenoids; though this supposition is rather unlikely, it can be refuted only by analysis of a great number of individuals of these species.

In the carnivora, large amounts of carotene are present in the livers of the badger, fox and fitchet (both the fitchets and all the three foxes examined), the values being of the same order as those found in the horse and the roe deer. Two of the dogs showed a rather high carotene value; these results are perhaps somewhat doubtful as the chromatogram did not give the beautiful β -carotene zone found in the fitchets and foxes.

At first sight, it appears almost absurd that the carotene content of the liver of a decidedly carnivorous animal should be the same as that of herbivora, but a detailed analysis of the carotene content of the food of the former group of animals explains this phenomenon. The fitchet exists mainly on frogs, and the fox similarly devours frogs as well as other lower species. Zechmeister & Tuzson [1936] have shown that the liver of the frog (*Rana esculenta*) contains a considerable amount of carotene, viz. about 10 mg. per kg., besides other carotenoids. Furthermore, carotene is found in moderate amounts in other organs, especially the skin. Even though the carotenoid content of the food of these carnivora is far less than that of the fodder of herbivorous animals, it appears likely that the former animals may consume considerable quantities of carotene, especially as frogs are probably not the only items of their diet containing ample amounts of carotene.

Besides, the fact that the liver of the garganey contains a concentration of carotene 10 times larger than that of the herbivora shows that the storing of carotene in the liver is not proportional to the amount of carotene ingested.

SUMMARY

The amounts of vitamin A and carotenoids in the liver have been determined in 33 different mammals belonging to 21 species, 41 birds of 36 species, 4 reptiles of 2 species and in 8 human specimens.

A detailed spectrographic analysis in the ultraviolet range, a detailed determination of the absorption of the Carr-Price reaction for three different spectral filters and an estimation of the carotenoids, including microchromatography, have been performed on the majority of the liver samples.

The technique employed in the treatment of the material, saponification, extraction, spectrography, measurement of the Carr-Price reaction and the carotenoids is described.

Most livers have yielded typical ultraviolet absorption curves similar to the curve of cod liver oils. Some of the curves are atypical.

Tables I-IV show the extinction coefficient $E_{1\text{ cm.}}^{1\%}$ (1 g. liver in 100 ml.; 1 cm. layer thickness) of vitamin A at 328 $m\mu$ (column 6), the Carr-Price reaction with the filters S 59, S 61 and S 66-6 in the Pulfrich photometer (columns 3-5) and certain ratios of the extinction values (column 10), a brief description of the absorption curves (columns 8, 9 and 14) and the zoological classification of the species examined (columns 1-2).

It was found that the relation between the extinction of the Carr-Price reaction with S 61 in the Pulfrich photometer and vitamin A absorption at 328 $m\mu$ ($B_{1\text{ cm.}}^{1\%}$ S 61 : $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu$) (column 10) had a fairly constant value of 2.60-2.75, with a coefficient of variation of 10-15 %. (However this only obtains for livers showing a typical absorption curve in the ultraviolet.)

The conversion of the extinctions measured with S 61 in the Carr-Price reaction into I.U. per g. liver must, therefore, be calculated from the formula $B_{1\text{ cm.}}^{1\%}$ S 61 = 2.60-2.75, corresponding to about 1600 I.U. per g. (or if the amount of vitamin A is expressed in micrograms by the formula $B_{1\text{ cm.}}^{1\%}$ = 4100-4400 for the Carr-Price reaction of the pure vitamin A with S 61).

In no instance has vitamin A₂ been demonstrated. The Carr-Price absorption curve has been fairly constant in the livers examined, as illustrated by the variation in the extinction ratios of the reaction with the three spectral filters.

The variation in the amount of vitamin A in the livers of the various species examined was very considerable; the same was the case with the amount of carotenoid (and carotene). There was no simple proportionality between the amount of vitamin A and carotene in the food ingested and the concentration in the liver. Carotene was found in considerable amount in the liver of certain beasts of prey and birds as well as in the herbivora but the majority of the livers examined contained almost no carotenoid.

Our results encourage further investigations on still larger series, comprising a greater number of species and, if possible, a greater number of individuals of each species.

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