determined in subsidiary manometers. These, together with the readings of the main manometer at the times of sampling, enable the O_2 uptake, and the production of respiratory CO_2 and acid to be independently calculated for each interval between sampling. In this way changes with time in the rates of O_2 uptake, total CO_2 production and of R.Q. can be obtained.

4. Two forms of the apparatus have been designed and are described. The theory of the method is developed and a simplified way of computing the results is given.

The instruments were made by Messrs W. G. Flaig and Sons, Waterloo Road, London, N.W. 2.

REFERENCES

- Dickens, F. & Simer, F. (1930). *Biochem. J.* **24**, 905.
 Dickens, F. & Simer, F. (1931). *Biochem. J.* **25**, 973.
 Dixon, M. & Keilin, D. (1933). *Biochem. J.* **27**, 86.
 Laser, H. & Rothschild, Lord (1939). *Proc. Roy. Soc. B*, **126**, 539.
 Sumerson, W. H. (1939). *J. biol. Chem.* **131**, 579.
 Warburg, O. (1924). *Biochem. Z.* **152**, 51.
 Warburg, O. (1925). *Biochem. Z.* **164**, 481.

Carotenoids, Vitamin A and 7-Dehydrosteroid in the Frog (*Rana temporaria*)

BY R. A. MORTON AND D. G. ROSEN

Department of Biochemistry, University of Liverpool

(Received 2 March 1949)

Understanding of the biochemistry of carotenoids is rudimentary except as to the part played in the animal economy by a few of them as precursors of vitamin A. In a wider sense the animal biochemistry is complicated by species differences. Thus some animals absorb and retain so little of either the hydrocarbons or their hydroxylated derivatives as to suggest that they are not needed, whilst other species absorb relatively large quantities of different carotenoids selectively and retain them distributed over various organs. For the carotenes it could be argued that they are only absorbed as such when the capacity of enzyme systems in the gut to transform them is exceeded, and that the pigments, of no special value to the body, are slowly disposed of. Such an argument, however, neglects the regular occurrence of carotenoids in eyes, yellow bone marrow and corpora lutea and is of little value in respect to the assimilation and metabolism of hydroxylated carotenoids like lutein and zeaxanthin.

As a subject for detailed study the frog has several special advantages; carotenoids (including both β -carotene and lutein) are widely distributed in the body; the species can be studied through the life cycle, as embryos, as tadpoles and as immature and mature frogs; there is a marked seasonal cycle in both sexes and the reproductive phase entails a heavy drain on body reserves, especially in the female. Moreover, as an amphibian, the frog provides a link between work on mammals and fishes. There are, therefore, numerous factors potentially capable of correlation with data on the distribution of caro-

tenoids so as to lead to hypotheses concerning the functions of these substances.

For 10–11 weeks after using up the residual yolk the tadpole is mainly vegetarian, but when metamorphosis is complete, frogs become mainly carnivorous. Adult frogs build up large fat reserves in early summer, and from June to August the testes or ovaries enlarge considerably. The testes then shrink and during the next few months vary little in size until the spermatozoa become fully ripe; the ovaries go on developing, but after spawning in March the shrunken oviducts are orange in contrast to their earlier paleness. The corpora adiposa of Amphibia are associated with the gonads; in the frog they vary from strands of orange tissue to large cream-coloured lobes. After surgical removal of one fat body the ovary on the operated side remains small compared with the other. According to March (1937) sexually mature frogs have a body length (snout-cloaca) of about 4.8 cm. and weigh about 10 g. Applied with caution, body length is a guide to age.

FAT SOLUBLE SUBSTANCES IN THE FROG

Earlier work on carotenoids and vitamin A. Lönnberg (1929) reported the presence of a 'carotene-like pigment' in the skin (*Rana temporaria* and *R. esculenta*). Dietel (1933) noted the presence of lipochrome in skin, ovaries and liver, but thought it consisted wholly of carotene. Manunta (1934) found 'xanthophyll' and much carotene in the skin of *R. esculenta*. Rand (1935) observed vitamin A to be present in frog and toad livers with lipochrome pigments detectable in skin, liver, ovaries and eggs, oviducts, testes, kidneys,

lungs, spleen and fat bodies. He did not discriminate between free 'xanthophyll' and esterified 'xanthophylls' so that his analyses are incomplete. He used too few frogs for his work to be more than exploratory. Brunner & Stein (1935), using phase partition and chromatography, concluded that, in *R. esculenta*, β -carotene and lutein (free or esterified) were the only carotenoid pigments present. In the fat bodies, liver and skin, the 'xanthophyll' was all ester form, but free lutein also occurred in the ovaries. Zechmeister & Tuzson (1936), starting from 491 frog livers, prepared crystalline specimens of liver carotenoids. The skin, ovaries, fat bodies and livers of fourteen females (*R. esculenta*) were quantitatively analysed by chromatographic and spectroscopic methods. Three fractions were separated: (a) epiphasic pigment, (b) free hypophasic pigment, and (c) esterified hypophasic pigment, the latter always predominating; (a) consisted of α - and β -carotene, the β -isomeric preponderating; (b) was a mixture of lutein and zeaxanthin. Many unidentified zones were observed during the chromatography, but it is not clear to what extent the substances responsible were metabolic products or *in vitro* artefacts. Other workers include Bartz & Schmitt (1936), van Eekelen (1934), Wald (1935), Ackermann (1938), Gillam (1938) and Lederer & Rathmann (1938).

Origin of ingested carotenoids. Relatively little is known about the carotenoids of the various species of insects, snails and worms which form the diet of frogs, although β -carotene and lutein probably preponderate. Judging from the contents of the alimentary tracts of frogs (in which chlorophyll is always present) a large proportion of the ingested carotenoid is in the form of plant material unabsorbed from the digestive tracts of insects eaten. From the point of view of carotenoid intake the frog is indirectly vegetarian to the extent that it makes use of plant pigments which have not previously entered the body proper of animals.

GENERAL PLAN OF THE PRESENT WORK

Neither the design of the experiment as a whole nor the interpretation of data is easy. If it had been possible to work on 600 frogs individually rather than in batches of twenty, the plan could have been different and the data obtained could have been treated statistically. Many of the analyses, however, would have been impossible on organs from single frogs. The choice lay between making a general survey with its attendant limitations and greatly restricting the scope of the work. On balance, taking into account the paucity of information in the field, it seemed better to secure the analytical data which time permitted on samples convenient to deal with, than to select particular variables for study on larger numbers of frogs.

EXPERIMENTAL

Animals

The frogs were obtained from a commercial supplier (Norwich district) and delivered at monthly intervals. Each

batch consisted of about twenty males and twenty females. The sexes were segregated on arrival. The frogs were weighed, killed by pithing, and dissected. The various organs, livers, kidneys, fat bodies, etc. from twenty animals of the same sex were combined and weighed. Each sample was then analysed for total fat and the fat in turn analysed quantitatively for as many of the following as seemed appropriate in each case: (a) vitamin A (free and esterified), (b) total carotenoids, (c) carotene, (d) 'xanthophyll', (e) free 'xanthophyll', mono-ester and di-ester. Within a batch, the work on one sex was completed before beginning on the other.

Batches of frogs were obtained from late March to December in 1946 and 1947 and some in 1948. The only part of the annual cycle which was not studied was the period of actual hibernation. Ova were obtained at all stages of maturity and tadpoles at all stages between the fertilized egg and the young frog. At various periods one of us (D.G.R.) collected animals from Hillside, near Southport, Lancashire, for additional tests. These included the tadpoles and young frogs (*R. temporaria*) and tadpoles of the natterjack toad (*Bufo calamita*).

Chemical methods

Outline. Specimens of tissue were ground with sand and anhydrous Na_2SO_4 and extracted with ether. The solvent was removed and the lipid made up to volume with light petroleum. Measured volumes were taken for the determination of vitamin A, carotenoids and fat. Chromatographic methods were used to separate the components and the quantitative determinations were based on data obtained with the Beckman photoelectric spectrophotometer. Exposure of extracts to light was avoided so far as possible. The extracts were not subjected to heat treatment at any stage, except in removing solvent in the fat determination.

Material for analysis. The frogs, which had been kept in tanks, supplied with water but no food, were roughly dried with blotting paper before weighing and dissecting. After weighing, the frogs were killed and dissected as quickly as possible. Before the viscera were weighed, adhering coelomic and other fluids and clotted blood were removed; the weighings were made without delay.

Solvents. Diethyl ether was always freshly distilled over a little reduced Fe before use. Light petroleum (b.p. 40–60°) must be practically free from 'polar' impurities; the commercial product is variable and must either be rigorously purified or selected for chromatography. A consignment of good quality petroleum was reserved for this study. The acetone was of ordinary reagent quality and the CHCl_3 of commercial B.P. quality. Ethanol and cyclohexane were specially purified for spectroscopy.

Total fat. The portion of the ethereal extract which was soluble in light petroleum was weighed after dehydration with ethanol and recorded as 'total fat'.

Extraction. The tissue was quickly ground, a little at a time, in a mortar with about five times its weight of anhydrous Na_2SO_4 to which fine acid-washed silver sand was added. Tadpoles were treated similarly without dissection. The well-ground material was transferred to a beaker and covered with ether. After standing 1–2 hr. in the dark, the yellow extract was decanted through a sintered glass filter (G4) under suction. The solid residue was then transferred to the filter cup and washed with successive portions of ether until the filtrate became colourless. The solid was then returned to the beaker and left to stand under ether for

0.5–1 hr. and filtered. A third extraction, during which the ether was boiled for a short time, yielded no more colour but removed residual vitamin A. In some cases the finely ground tissues were allowed to stand overnight under ether in the dark in a corked flask. The solution was decanted on the filter and the residue washed with ether until no more pigment came through. Skin, muscle, lungs, oviducts, oesophagus, stomach and intestines were extracted overnight, other tissues as quickly as possible. In all cases, the ether was removed by distillation under reduced pressure at room temperature. The residue was dissolved in light petroleum and made up to volume. A small amount of anhydrous Na_2SO_4 was added if the solution was not perfectly clear. A measured portion of the extract was used for direct estimation of vitamin A, the light petroleum being removed and replaced by a suitable volume of cyclohexane.

Chromatographic separation

Commercial bone meal was 'defatted' by means of boiling acetone. The fraction which then passed through a 120 British Standards Specification sieve was used for chromatography. When such material is stirred with acetone, the smallest particles do not settle readily, and if the liquid is decanted the 'fines' can be removed. This is an advantage as otherwise acetone eluates may be cloudy. Alumina (Savory and Moore) which had been in stock in the laboratory for some years and had become rather weakly adsorbent proved well suited to the separation of carotenoids. Type 'O' alumina (Peter Spence and Co.) could be reproducibly weakened by stirring in definite proportions of water.

A chromatographic tube of 18 mm. internal diameter was used throughout this work. A column of bone meal 3 cm. in height sufficed for the separation of vitamin A ester from the free vitamin and a 4 cm. column for carotenoid separation. The adsorbent was poured dry into the tube, light petroleum was added and the bone meal stirred with a long thin metal rod to liberate trapped air bubbles. Strong suction was applied, but from then onwards the column was not allowed to run 'dry'. The initial adsorption of solutes was effected slowly under gravity alone. Development and elution were more rapid at 120–150 drops/min. under suction. Small volumes of eluate were aimed at and colourless intermediate fractions obtained in carotenoid analyses were discarded. Vitamin A esters were eluted completely by 100 ml. of light petroleum and the free vitamin by means of a little acetone. Solvents were removed under reduced pressure and the residues made up to suitable volume in cyclohexane.

Carotene (in some cases contaminated with esterified 'xanthophyll') was carried through the bone-meal column by not more than 25 ml. of light petroleum. A measured portion of this eluate was put through a similar adsorption tube containing a 4 cm. column of alumina; here 30 ml. of light petroleum carried down all the carotene and the esterified 'xanthophyll' was washed through with acetone (10–20 ml.). The free 'xanthophyll' retained on the bone-meal column was eluted with acetone. Throughout this paper 'xanthophyll' refers to a fraction consisting mainly of lutein, but possibly containing some zeaxanthin and a little kryptoxanthin.

For a more complete separation of fractions containing little vitamin A a fresh portion of extract was adsorbed on alumina, the carotenes eluted by means of light petroleum,

the 'xanthophyll' di-esters by 1% of acetone in light petroleum (v/v) and the mono-esters by 8% of acetone in light petroleum (v/v). In some cases the 'xanthophyll' fraction from bone meal was eluted with acetone, the solvent completely removed and replaced by light petroleum and the solution chromatographed on alumina. When necessary, di-esters and mono-esters can be selectively eluted from bone meal by means of 2% (v/v) and 8% (v/v) CHCl_3 in light petroleum, respectively. The final fractions were made up to volumes suitable for spectroscopic examination. In replacing one solvent by another heat treatment was avoided. The visible spectra of the three 'xanthophyll' fractions are reproduced in Fig. 1. If the extracts were saponified before chromatography, the 'xanthophyll' fraction was substantially homogeneous.

Fig. 1. Visible absorption spectra in acetone of the three forms of 'xanthophyll': (1) free xanthophyll; (2) xanthophyll di-ester; (3) xanthophyll mono-ester.

Spectroscopic determinations

Vitamin A. Vitamin A was determined by the intensity of absorption at 328 $\text{m}\mu$. and the gross $E_{1\text{cm}}^{1\%}$ values were corrected for irrelevant absorption by the method of Morton & Stubbs (1946). The separation of esterified vitamin A from the free alcohol was carried out by the method of Glover, Goodwin & Morton (1947) using bone meal as chromatographic adsorbent. As a rule the amount of free vitamin A was small and was best determined by the difference between the total (corrected) and ester (corrected).

Carotenoids. The spectra of nearly all the fractions agreed very closely with those of the corresponding purified carotenoids from other sources when the criteria adopted were (a) wavelengths of maximum absorption in specified solvents, and (b) relative intensities of absorption at maxima and minima (Fig. 2). However, certain carotenoid fractions, from intestines and eyes, for example, exhibited irrelevant absorption. This was particularly noticeable in the lipids from frog eyes (Fig. 3). The colour is due mainly to 'xanthophyll' and the curve may be corrected for irrelevant

Fig. 2. Comparison of visible absorption spectra of pure carotenoids and frog carotenoid extracts: —, lutein in ethanol; $\times \times \times$, frog xanthophyll in ethanol; ---, β -carotene in *n*-hexane;, frog carotene in *n*-hexane.

Fig. 3. Visible absorption spectra of frog-eye extracts: —, whole extract (light petroleum 40-60°); ---, total 'xanthophyll' (acetone);, corrected 'xanthophyll'; -.-.-, irrelevant absorption.

absorption by the general method of Morton & Stubbs (1946) on the basis of the extent of distortion of the mean 'xanthophyll' curve (see Fig. 2) of extracts of other organs. The relative absorptions at the three wavelengths 448.5, 463 and 476 $m\mu$, are $E = 1$, 0.724, 0.881 respectively, and $E_{448.5} m\mu$. (corr.) = $E_1 \times 2.216 - E_2 \times 4.688 + E_3 \times 2.472$, where E_1 , E_2 and E_3 are observed values at the three wavelengths, respectively.

It was sometimes necessary to examine solutions so dilute that the photoelectric photometer was perforce used outside the recommended range of extinctions. The instrument was therefore calibrated over the range $E = 0.01$ to $E = 1.0$ using carotene in light petroleum and 'xanthophyll' esters in acetone or light petroleum. When readings below $E = 0.2$ were obtained with frog extracts, corrections derived from the calibration curves were applied. In the cases of mono-esterified and free 'xanthophyll' fractions in acetone similar calibration demonstrated that no such correction was necessary. The data in Table 1 were used as a basis for calculations.

Table 1. *Spectrophotometric data on carotenoids, used in the analytical work*

Substance	$\lambda_{\max}$ ($m\mu$)	$E_{1\text{ cm.}}^1$	Solvent	Remarks
β -Carotene	449.5	2550	Light petroleum	(a)
Lutein	446.5	2550	Ethanol	(b)
Vitamin A	328	1700	CycloHexane	(a)
Frog 'xanthophyll'	448.5	2640	Acetone	(c)
Frog 'xanthophyll' di-esters	448	2550	Acetone	(c)
Frog 'xanthophyll' mono-esters	448	2600	Acetone	(c)

(a) Determinations in this laboratory; (b) Zscheile, White, Beadle & Roach (1942); (c) values determined in the course of the present work relative to $E_{1\text{ cm.}}^1$ 2550 for pure free lutein.

If, in determining the 'total carotenoid' per frog in a given type of tissue, x frogs were used to provide an extract in y ml. and the solution had to be diluted by a factor z so as to bring $E_{\max}$ for a 1 cm. layer to a convenient figure; then

$$\frac{Ez}{100x/y} = \frac{Eyz}{100x} = Q.$$

The use of Q avoids the difficulty that 'total carotenoid' is a mixture of components in which neither $\lambda_{\max}$ nor $E_{1\text{ cm.}}^1$ is necessarily exactly the same. Since, however, $E_{1\text{ cm.}}^1$ at the visible maximum is of the order 2500, i.e. E is about 2.5 for a 1 cm. layer of solution of concentration 1 mg./100 ml., $Eyz/250x$ is the weight of solute per frog in mg., so that roughly $0.4Q = \text{mg. total carotenoid/frog}$.

In this investigation the number of analyses was necessarily very large and the preparation of material time-consuming. In choosing methods the aim was the greatest accuracy compatible with speed. It was found to be essential to complete the tests on livers, kidneys and eyes, so far as vitamin A was concerned, in one working day; standing overnight might have caused a loss of as much as 40% of the vitamin. The intact organs could, however, be kept at 0° without appreciable loss (Table 2). The method of decomposing the tissue by boiling with ethanolic KOH and testing the non-saponifiable extract was tried side by side

with the procedure already described, and was found to be attended by significant losses or isomerization of carotenoids (Table 3).

Table 2. *Stability of carotenoids in frog tissues stored at 0° after dissection*

Tissue	Period of storage (days)	Total carotenoid $E_{1\text{cm.}}^{1\%}$ *	Total vitamin A $E_{1\text{cm.}}^{1\%}$ (corr.)*
Liver	0	0.0976	0.117
	1	0.0998	0.115
	3	0.0968	0.113
Kidney	0	Not tested	0.543
	1	Not tested	0.557
Muscle	0	0.00487	Nil
	3	0.00482	Nil

* Calculated on wet wt. of tissue.

Table 3. *Comparison of analyses for carotenoids and vitamin A carried out on (a) ether-extractable lipid and (b) non-saponifiable ether extract after treating tissue with ethanolic KOH*

Tissue	Total carotenoid $E_{1\text{cm.}}^{1\%}$		Vitamin A $E_{1\text{cm.}}^{1\%}$ (corr.)*	
	(a)	(b)	(a)	(b)
Cock liver	0.008	0.0072	Not measured	
Horse liver	0.0192	0.0179	0.248	0.263
Hare liver	Negligibly small		0.034	0.035
Frog skin	0.031	0.030	Negligibly small	
Frog liver	0.080	0.069	0.105	0.107
Frog liver	0.227	0.175	0.278	0.243
Frog testes	0.053	0.046	Negligibly small	

* Calculated by the procedure of Morton & Stubbs (1946).

Samples of frog carotenoid fractions with accompanying lipids were used to ascertain the magnitude of manipulative losses in the chromatographic procedures. Over the relevant range, recoveries of 97–99% were obtained. Similar tests with free and combined vitamin A provided equally good recoveries.

The magnitude of the correction for irrelevant absorption at 328 $m\mu$. in tests on liver extracts is illustrated in Fig. 4, and on extracts from eyes and kidneys in Fig. 5. The full lines in Fig. 4 show irrelevant absorption at its greatest in the present series (in all there were three such cases); the broken-line curves illustrate the normal case in which the irrelevant contribution is about 20% (thirty cases). The carotenoid contribution amounted at 328 $m\mu$. to only 3–4% of the total absorption. The need for correction at 328 $m\mu$. is much more obvious in the extracts from eyes and kidneys, but the validity of corrected estimates for vitamin A content was confirmed using the SbCl_3 colour test.

When the ultraviolet absorption of an extract suggested the absence of vitamin A, the colour test was used to provide confirmation.

RESULTS AND DISCUSSION

The experimental findings are recorded in tables and figures.

Lipochromes and vitamin A in tadpoles and young frogs

Presence of chlorophyll. The absorption spectra of chromatographic fractions containing 'xanthophylls' show some chlorophyll to be present also.

Fig. 4. Ultraviolet absorption spectra of liver extracts illustrating the magnitude of the correction: ----, typical spectrum; —, atypical spectrum. In each set the top curve is observed, the middle curve corrected and the lowest curve represents irrelevant absorption.

Fig. 5. Typical ultraviolet absorption spectra of eye (----) and kidney (—) extracts, showing gross, corrected and irrelevant absorption.

Thus, tadpole extracts exhibit maxima at 662-664, 615 and 432-434 $m\mu$.; $E_{662\ m\mu}/E_{615\ m\mu} = 4.2$. Harris & Zscheile (1943) give for chlorophyll-*a* λ_{max} 661.5, 615.5 and 431.5 $m\mu$.; $E_{661.5\ m\mu}/E_{615.5\ m\mu} = 6.3$. The low value for the ratio of the intensities of the two chlorophyll bands (Figs. 6 and 7) suggests the presence of chlorophyll-*b*. By applying the method of Comar & Zscheile (1942) to a

Fig. 6. Visible absorption spectra of frog tadpole 'xanthophyll' in acetone: —, 'xanthophyll' from chromatogram; ---, computed chlorophyll contribution; -.-, xanthophyll contribution.

fresh ether extract of tadpoles, chlorophyll-*a* was found to preponderate over chlorophyll-*b* by a factor of 7.5 to 1. The observed absorption in the region 400-500 $m\mu$. is therefore a summation of contributions from 'xanthophyll' and 'chlorophyll'. The absorption in the region 600-700 $m\mu$. is due solely to the latter, and from the absorption curve for pure chlorophyll-*a*, the curve from the red region can be continued through to the violet using the following relative *E* values:

λ	662	400	412	420	430
<i>E</i>	0.976	0.7	0.913	0.875	1.218
λ	432	440	450	460 $m\mu$.	
<i>E</i>	1.225	0.595	0.074	0.018	

The 'xanthophyll' contribution in tadpole extracts is then obtained by difference (Fig. 6). In two samples, the 'chlorophyll' and 'xanthophyll' were separated by chromatography on weakened alumina and the validity of the difference method confirmed.

In extracts of natterjack tadpoles the chlorophyll contribution was computed in a similar manner, but an additional maximum near 420 $m\mu$. due to the presence of an artefact was observed when the

'xanthophyll' contribution was obtained by difference. This substance, which showed a maximum at 412 $m\mu$., was probably a degradation product of chlorophyll; it was easily removed by adsorption on alumina and the anomalous 'xanthophyll' absorption was corrected by subtraction (Fig. 7). Carotenoids are decomposed by exposure to light in the presence of chlorophyll and oxygen (Pepkowitz, 1944); it is necessary, therefore, to protect the

Fig. 7. Visible absorption spectra of natterjack tadpole 'xanthophyll' in acetone: —, 'xanthophyll' from chromatogram; ---, computed chlorophyll contribution; -.-, absorption of 'xanthophyll' plus artefact; x-x-x, artefact contribution; 'xanthophyll' contribution.

extracts as much as possible. Vitamin A in traces is very difficult to detect in such mixtures, and the only way in which its presence was demonstrated in natterjack tadpoles was by the antimony trichloride colour test applied to extracts from the dissected eyes.

Occurrence of carotenoids. The number of tadpoles used was ample for the methods applied, and the data obtained are shown in Tables 4-6. The tadpoles were grouped in accordance with the stages of development A-H which can be fitted into an approximate time scale (see Fig. 8).

Comment. The chlorophyll is almost certainly all present in the digestive tract. The tadpoles were kept for 3 days in captivity after collection, but although they were active and faecal excretion copious, it was found that those collected at stage A had to be kept for 9 days before chlorophyll disappeared. In tadpoles caught at different ages the chlorophyll content is considerable in stages B, C and D, less in E, very low in F and negligible in G. This suggests a decrease in food intake which is borne out by the weights and the carotenoid analyses (Table 4).

Table 4. *Carotenoids in tadpoles of Rana temporaria at various stages of development*

(Chlorophyll was present in A-F, not detected in G and H.)

Stage	Date and number used (in brackets)	Average wt. (g.)	Carotene/tadpole ($\mu\text{g.}$)	'Xanthophyll'/tadpole ($\mu\text{g.}$)	Ratio 'xanthophyll'/carotene
A, young tadpoles, 10-20 mm.	2. v. 47 (312)	0.049	0.012	0.11	9.7
B, tadpoles, 20-30 mm.	2. v. 47 (236)	0.12	0.033	0.30	9.1
C, tadpoles, 30-40 mm., rear leg just seen	20. v. 47 (75)	0.32	0.31	1.78	5.6
D, rear legs less than 4 mm.	4. vi. 47 (82)	0.22	0.27	0.93	3.4
E, rear legs greater than 4 mm.	4. vi. 47 (52)	0.25	0.40	1.21	3.0
F, fore limbs formed, some free	4. vi. 47 (73)	0.22	0.25	0.92	3.7
G, all four limbs well formed, long tails persisting	10. vi. 47 (88)	0.19	0.23	0.72	3.1
H, small frogs, tails almost resorbed	10. vi. 47 (93)	0.15	0.25	0.76	2.8

Table 5. *Rate of disappearance of carotene and 'xanthophyll' during metamorphosis of tadpoles*

	Average amounts of pigments ($\mu\text{g.}/\text{tadpole}$)		Loss (%)
	Stages C, D and E*	Stages F, G and H†	
Carotene	0.33	0.25	24
'Xanthophyll'	1.31	0.80	38

* Feeding freely. † Fasting.

Table 6. *Rate of disappearance of carotene and 'xanthophyll' from young tadpoles caught at stage A and kept in captivity without food*

	Pigments expressed as ($\mu\text{g.}/\text{tadpole}$)		Loss (%) (approx.)
	Tested 3 days after capture	Tested 9 days after capture*	
Carotene	0.0116	0.0093	20
'Xanthophyll'	0.112	0.087	22

* Chlorophyll absent.

The amount of carotenoid in the original eggs is less than one quarter of that present in active tadpoles. Now the ratio of 'xanthophylls' to carotenes in the plant world, although variable, is only very rarely outside the range 3-10. In young tadpoles the ratio is initially about 9 and falls to about 3 at the time of metamorphosis and then falls further in young frogs. Up to maximum growth 'xanthophyll' storage increases more rapidly than carotene storage. The abrupt fall in 'xanthophyll' level when the rear legs first appeared obviously needs confirmation, but the very small concomitant drop in carotene affords a hint that it is not illusory. The fact that little or no food is eaten during metamorphosis allows the disappearance of carotenoids to be followed. The data given in Fig. 8 and Tables 5 and 6 suggest that the main process does not discriminate sharply between carotene and 'xanthophyll'. The change in the 'xanthophyll'/carotene ratio recorded in Table 4 is not then simply a matter of selective

'utilization' as measured by rate of disappearance. If, during the period of metamorphosis, carotene had been used for the synthesis of vitamin A, the hydrocarbon might have been expected to disappear more quickly than the 'xanthophyll' (mainly lutein in the tadpole). There is no doubt that vitamin A is formed in the tadpole. Natterjack tadpoles each contained about 0.027 $\mu\text{g.}$ in the eyes and newly metamorphosed frogs about 0.1 $\mu\text{g.}$

Fig. 8. Carotenoids in the developing tadpole: (a) total carotenoid; (b) total 'xanthophyll'; (c) carotene. The stages A-H are specified in Table 4.

It is now clear that the main, if not the sole site of conversion of carotene to vitamin A in mammals is the absorptive portion of the gut, and it seems very probable (although obviously difficult to prove) that the same holds true for the tadpole. It is very unlikely that the 'xanthophyll'/carotene ratio in the kind of vegetation ingested is ever as high as 9; the appearance of this ratio in the early stages of development (A and B) suggests a selective utilization of carotene in the gut wall. On this hypothesis, as development proceeds and food intake increases, the 'carotenase' enzyme system becomes unable to cope with more than a fraction of the carotene presented to it. The residue will follow the normal pathway of lipid absorption, and the 'xanthophyll'/carotene

ratio will fall until it reaches a figure much nearer to that of the diet. The carnivorous young frogs will continue to convert a proportion of ingested carotene and to absorb carotene and 'xanthophyll' non-selectively.

It is possible that in the future the hatching of frog spawn and the growth of tadpoles in captivity on artificial diets may be of service if either carotene or 'xanthophyll', or both, can be excluded.

Lipochromes and vitamin A in adult frogs

Vitamin A. There does not appear to be any pre-formed vitamin A in the diet of the frog. The vitamin formed *in vivo* from provitamin is found in the liver (about 85 %) and in the eyes and kidneys in approximately equal amounts (Tables 9 and 11). In each site it occurs mainly as ester. The amount of vitamin A in kidney (about 30 µg./g.) is very much larger than that found in mammalian kidneys.

Carotenoid distribution. Carotene as such is less efficiently absorbed than the hydroxylated carotenoids since the 'xanthophyll'/carotene ratios are approx. 3:1 in the diet, 2:1 in the faeces and, in the body, 4.7:1 for females and 5.7:1 for males (October 1947).

In contrast with the restricted distribution of vitamin A, carotenoids occur in all the organs studied (Table 9). They also appear to occur in nerve tissue and in glands such as the thymus. If the amounts of carotenoids at different sites are expressed in terms of that present in the skin (chosen for comparison because of its relative constancy), the results in Table 7 give an idea of the approximate distribution over the body. In males, the skin, liver and muscle are important; in the female the gonads dominate the situation (Table 7).

Table 7. *Relative amounts of total carotenoid in frog organs*

(These are mostly seasonal averages, without distinction between the sexes.)

Skin*	1
Ovaries and mature eggs	2.65
Liver	0.7
Muscle (total leg)†	0.17
Oviducts	0.12
Immature ovaries	0.1
Stomach	0.05
Testes	0.035
Small intestine, fat bodies, kidneys	0.23-0.28
Oesophagus	0.16
Tongue	0.09
Pancreas, large intestine, lungs, eyes, heart	0.004-0.008
Seminal vesicles, spleen	Traces

* Skin carotenoid is taken as unity, because the absolute amount is large and is less influenced by sex and season than other tissues rich in carotenoids.

† Carotenoids of leg muscle/ventral and side muscle/arm muscle = 36/21.

Table 8. *Weights of frog organs expressed as percentage of body weight*

Organ	Minimum	Maximum
Testes	0.32	3.66 (Aug.)
Ovaries	0.925 (Apr.)	36.0 (Mar.)
Oviducts	1.47 (Apr.)	17.5 (Dec.)
Livers (male)	{ 1.4 (Apr.) 1.3 (Apr.)	3.6 (Nov.-Dec.) 2.3 (Oct.-Dec.)
Fat bodies (male)	{ Nearly 0 (Apr.) Nearly 0 (Apr.)	8 (Aug.-Sept.) 7 (Aug.-Sept.)
Skin	Approx. 10	
Leg muscle	Approx. 15	
Kidneys	0.3-0.5	
Pancreas	0.06-0.13	
Stomach	Approx. 1.0	
Lungs (male)	{ 0.42 0.25	
Tongue	0.8	
Eyes	Approx. 1.0	
Heart	Approx. 0.25	

Although the left fat body is larger than the right one, the carotenoid content per unit weight is the same. The adrenals are somewhat richer in carotenoids than the kidneys. In all tissues α -carotene seems to be practically absent and the 'xanthophyll' is nearly all lutein. Very small amounts of other carotenoids were sometimes found, but there was too little for characterization. A few toads (*Bufo vulgaris* and *B. calamita*) have been studied in the course of the present work and no significant differences were observed from experience on frogs.

One of the implications of the distribution data is that the carotenoids are not merely distributed with the fat; thus for females in October 1947 the concentrations of carotenoids in fat varied from about 0.01 mg./g. for fat bodies to 8.4 mg./g. for skin, with liver 2.7 mg./g. and ovaries 1.1 mg./g. (Table 15). The external appearance of frogs and toads owes little or nothing to the lipochromes present in the skin. The outer layers are rich in melanins and in pigments soluble in dilute ammonia.

Lederer & Rathmann (1938) found small proportions of vitamin A₂ in *R. esculenta* liver fat, but in the present work with *R. temporaria* no A₂ was observed, confirming the finding of Gillam (1938).

Ultraviolet absorption spectra of lipid fractions from frog organs

The absorption spectra of liver extracts have already been referred to (Fig. 4). Observations on extracts from other organs are recorded. Selective absorption of undetermined origin in the region 280-300 mµ. was recorded in lipids from the eyes of frogs and toad (Fig. 5) and also rather erratically in frog kidneys. With one exception (November 1946) the testes extracts showed continuous end-absorption over the range 250-400 mµ. The aberrant sample showed an inflexion near 265 mµ.

Extracts from ovaries were more interesting. Material from the mature ovaries of frogs in the pre-hibernation period exhibited the well-resolved absorption shown in Fig. 9. The characteristic curve

Table 9. *Distribution of fat, carotenoid and vitamin A in the organs of sixty male frogs, divided into three main groups*

Group	Mean body length (cm.)	Mean weight (g.)
A	6.6	22.8
B	5.6	14.2
C	5.1	11.0

In table below means were not measured; a dash (—) means 'not measured'.

Organ	Wt. as % body wt.	Wt. of fat (mg./frog)	Total carotenoid $Q \times 10^{2*}$	Carotene (μ g./frog)	'Xanthophyll' (μ g./frog)	Vitamin A (μ g./frog)
Fat bodies						
A	0.058	5.6	0.33	0.158	1.40	0
B	0.061	5.4	0.08	—	—	0
C	0.030	2.5	0.02	—	—	0
Pancreas						
A	0.117	—	0.11	—	—	0
B	0.105	—	0.06	—	—	0
C	0.120	—	0.057	—	—	0
Kidneys						
A	0.35	—	0.29	—	—	5.3
B	0.35	—	0.235	—	—	2.4
C	0.35	—	0.18	—	—	1.6
Testes						
A	1.20	4.1	0.33	0.59	0.77	0
B	1.24	2.7	0.22	0.38	0.56	0
C	1.23	1.7	0.17	0.32	0.40	0
Eyes						
A	0.86	—	—	—	—	3.0
B	1.02	—	—	—	—	2.3
C	1.30	—	—	—	—	2.1
Liver						
A	2.31	3.6	4.46	3.4	13.0	39.6
B	2.31	2.9	3.84	3.3	11.5	20.6
C	1.92	2.2	2.32	1.25	7.2	12.1
Tongue						
A	0.63	—	0.11	—	—	0
B	0.74	—	0.082	—	—	0
C	0.72	—	0.078	—	—	0
Stomach						
A	1.09	—	0.48	0.52	1.24	0
B	1.12	—	0.44	0.49	1.16	0
C	1.27	—	0.34	0.35	0.90	0
Skin						
A	11.6	16.2	12.5	7.25	43.6	0
B	11.1	12.6	8.5	5.02	27.8	0
C	11.1	9.7	6.9	2.19	24.4	0
Leg muscle						
A	17.3	18.3	3.42	1.04	10.6	0
B	15.6	10.6	2.04	0.97	6.9	0
C	14.5	6.8	1.54	0.69	5.0	0

* See p. 615 of text.

with maxima at 292.5, 281.5, 271 and 261 m μ . was reproduced in the non-saponifiable fraction. The spectrum is qualitatively indistinguishable from that shown by provitamins D, e.g. 7-dehydrocholesterol and ergosterol. Without using a large amount of material it is not possible to decide which of the several substances known to exhibit this spectrum occurs in the frog ovaries. Applying a

correction procedure based on the method of Morton & Stubbs (1946) using reference data obtained on highly purified ergosterol (which is spectroscopically almost identical with 7-dehydrocholesterol) the corrected $E_{1\text{cm}}^{1\%}$ 281.5 m μ . value was 0.117, corresponding with a concentration of 1 in 2430 in the ovaries [$E_{1\text{cm}}^{1\%}$ (ergosterol) = 285]. A calculation based on the work of Boyd (1938) indicates

that mature ovaries contain about 0.8% of cholesterol. It thus appears that the ovarian steroids contain approx. 0.5% of 7-dehydrosteroid.

Fig. 9. Ultraviolet absorption spectra of the extract of mature frog ova in ethanol: (a) gross absorption; (b) corrected curve; (c) irrelevant absorption.

Fig. 10. Ultraviolet absorption spectra of frog ova extracts: A, mature ova just prior to oviposition (ethanol); B, December ova immature through deprivation (ethanol); C, immature ova from frogs which yielded mature ova (curve A) (in ethanol); and D, immature ova from spring frogs 1947 (cyclohexane).

The characteristic spectrum only appears when the ovaries are mature, but it persists through the period of hibernation and is seen (Fig. 10A) just before oviposition occurs. There is some loss of definition and possibly a decrease in intensity, but it is difficult to say how much of the apparent loss is fictitious, since the protein layer and water are

accumulated at this time. Curve C (Fig. 10) shows the absorption spectrum of an extract of immature ovaries obtained from the same frogs as the ripe ova in March 1948. Curve D refers to immature ovaries obtained in the spring of 1947 and B to ovaries from frogs which had fasted for a long time (p. 625). The weak selective absorption at 330 mμ. (Fig. 9) is not due to vitamin A or carotenoids.

The data on extracts from other organs show no well-defined spectra, but there are numerous indications of the presence of traces of absorbing constituents. Much larger amounts of material would have to be used in following up these observations.

The occurrence of the ergosterol-like bands in mature ovaries is surprising. It suggests that the hypothesis of a role for 7-dehydrosteroids other than that of a provitamin D is worthy of study.

Seasonal variations in carotenoids and vitamin A

A considerable body of metrical data accumulated during the study of some 600 frogs, the main features of which are summarized in Tables 8 and 10. The quantitative picture which emerges from a study extending over only two seasons is in some respects rather tentative, but it is likely to be broadly correct. In 1946 the liver fat for both sexes showed a 'xanthophyll'/carotene ratio (X/C) of 1 in the autumn, but a year later X/C was about 3 (Table 12). The

Table 10. Size and weight of frogs used at different dates in the present work

(The average body lengths were computed from the data of March (1937), except those marked with an asterisk.)

Date of dissection	Number		Average body wt. (g.)	Average body length (cm.)
	Males	Females		
14. viii. 46	—	18	27.5	6.9
19. viii. 46	18	—	20.4	6.5
9. ix. 46	20	—	30.5	7.3
11. ix. 46	—	19	25.6	6.9
17. x. 46	18	—	26.8	6.9
21. x. 46	—	18	26.7	6.5
6. xi. 46	20	—	29.3	7.1
12. xi. 46	—	20	34.5	6.9
9. xii. 46	19	—	25.4	6.7
16. xii. 46	—	21	31.1	6.7
1. iv. 47	—	13	29.5	6.6
7. iv. 47	18	—	22.7	6.2
13. iv. 47	—	14	14.5	6.5
12. v. 47	20	—	20.3	6.5
28. v. 47	—	19	16.3	6.3
8. vii. 47	14	—	25.3	7.1
22. vii. 47	—	19	19.9	6.7
3. x. 47	17	—	13.1	5.5*
8. x. 47	—	17	16.8	5.9*
30. x. 47	20	—	22.8	6.6*
30. x. 47	20	—	14.2	5.6*
30. x. 47	20	—	11.0	5.1*

weather remained mild until early December in 1946 and the frog fat bodies were much larger in October and November than a year later. Apart from complications arising from the fact that no two years have the same weather conditions,

the age (size) variable must be distinguished from the variable of an annual cycle in the frog at all ages after puberty.

A group of sixty male frogs, captured near Norwich in October 1947, was divided into three groups. Table 9 summarizes the experimental data. The weights of organs expressed as percentage of body weight are reasonably constant apart from the cases of fat bodies and eyes. No simple expression can be found to cover the lipochrome or vitamin A variations, but both increase with increasing age.

An experiment with a further group of sixty male frogs caught in the spring of 1948 at Hillside showed that two groups of twenty not differing in average size (6.7 cm.) differed little in respect of skin carotenoid, liver carotenoid and liver vitamin A. A third group of the same size showed very much less liver vitamin A and slightly less liver carotenoid. The frogs in the third group, unlike the others, were spent (seminal vesicles empty, and testes shrunken), and, as will be seen, it is plausible to connect that fact with the decrease. Only rather gross signs of seasonal variations, reinforced by data on the average body length of the frogs employed (see Table 10), can therefore be regarded as significant at this stage in the problem.

The variations in carotenoid and vitamin A content of certain organs during the annual cycle are illustrated in Figs. 11–15. The results obtained on the first sixty male frogs in groups A, B, C (p. 620) have been taken into account in the discussion.

Vitamin A

(i) *Liver*. Males and females showed a summer maximum with a fall in early autumn and a rise in late autumn (Fig. 11). There may have been a real decrease prior to

Fig. 11. Seasonal variation in frog liver vitamin A: x-x-x, male; ---, female.

hibernation, but there was no sign of a drop during winter. Some females shed their eggs in the laboratory without pairing (April 1947) and showed low liver levels afterwards. Similarly, in 1948, the three sets of males already referred to showed respectively 36.1, 36.2 and 20.7 µg./liver, the low value being obtained in the 'spent' animals.

(ii) *Eyes*. Both sexes showed a late autumn maximum and a fall in vitamin A before hibernation. Males but not females appeared to exhibit a winter minimum (Fig. 12).

(iii) *Kidney*. Beyond the fact that kidney storage of vitamin A seems to be maximal in autumn, there is too little evidence for postulating a true annual cycle (Fig. 12). There is little evidence that hibernating frogs deplete their vitamin A reserves, but reproduction causes a heavy drain. The temperature coefficient of the enzyme system responsible for the conversion of provitamin A to vitamin A is probably more responsible for the summer rise in the liver reserve than the copious feeding.

Fig. 12. Seasonal variation in frog eye and frog kidney vitamin A: x, eye (male); ●, kidney (male); ▲, eye (female); ■, kidney (female).

Table 11 shows for frogs at different seasons the vitamin A contents of the three organs expressed as percentage of the total reserves. The seasonal variations suggest sex differences, and show clearly that the liver is always the main storage organ. So far as the data go, 75–88% of the vitamin A occurs as ester in all sites with 80% as a typical figure. A few discrepant observations were, however, made.

Table 11. Seasonal variations in the distribution of vitamin A in frogs of both sexes

Season	Sex	Average body length (cm.)	Vitamin (% of total)		
			Liver	Eyes	Kidneys
Spring	M.	6.2	87.0	6.9	6.1
	F.	6.5	88.4	7.2	4.5
Summer	M.	7.1	88.8	4.1	7.1
	F.	6.7	90.2	5.5	4.3
Autumn	M.	5.5	77.5	11.7	10.9
	F.	6.0	82.7	9.9	7.5

Total carotenoid

(i) *Liver*. No reduction was manifested during hibernation for either sex, in fact there may have been a small rise for males. In both males and females the decrease on mating was followed by a rise to a summer maximum. There was

then a gradual decrease continuing until December with nothing analogous to the autumnal rise in hepatic vitamin A (Fig. 13).

Fig. 13. Seasonal variation in frog liver total carotenoid: x-x, male; ●-●, female. (Q, see p. 615.)

Fig. 14. Seasonal variations in testes and fat body total carotenoid: x-x, testes; ●-●, female fat body; ■-■, male fat body. (Q, see p. 615.)

Fig. 15. Seasonal variation in ovarian and oviduct total carotenoid: x-x, ovaries; ●-●, oviducts. The ringed cross represents the total carotenoid content of mature ova just prior to oviposition. (Q, see p. 615.)

(ii) *Sex organs* (Figs. 14 and 15). The gonads and also the fat bodies showed marked seasonal variations in carotenoid content. The testes were very poor in lipochrome after mating and remained so until August when a sharp rise occurred; there was some fluctuation from September to

November, but no clear-cut secondary maximum was recorded. Transfer to the testes seems to occur during or just after hibernation. Immature ovaries contain very little carotenoid, but just prior to hibernation a thirtyfold rise had been attained. Whether or not there is a drain on ovarian carotenoids during the winter sleep cannot be stated, as contradictory results were obtained in the 1947 and 1948 seasons, but it could not be large. The oviducts showed a rise in late autumn, but there was no drop in winter (when the ducts are functioning actively). On spawning, the ducts atrophy and there is a sudden decrease to a low level which is maintained through the summer.

Male fat bodies showed a marked rise in November, whereas in females the levels were lower and fairly constant throughout the autumn. Hibernation losses occurred in both sexes and for females especially, marked depletion occurred on spawning, with recovery during summer feeding to lower levels for females than for males.

(iii) *Kidneys, skin and organs of the digestive tract.* No significant seasonal variations in kidney or skin carotenoids could be observed for either sex. Some variations occurred in the digestive tract. Males showed summer and autumn peaks in the stomach, but females only showed the autumn rise. In the small intestine there was a maximum in autumn for both sexes. During winter the intestinal carotenoids were maintained (males) or even rose a little (females).

(iv) *Leg muscle.* A temporary rise appeared to occur in the autumn.

(v) *Tongue, lungs, pancreas, eyes.* There was evidence of an autumn maximum, but the levels were fairly steady on the whole. In the male pancreas an increase is suggested during or immediately after hibernation, but in the female there is a fall on egg-laying. In the male eye the total 'xanthophyll' drops in early summer and then rises, but there is no clear-cut autumn maximum in the female. Carotene was found in traces ($<0.05 \mu\text{g./eye}$) but 'xanthophyll' preponderates in the eye.

Comment on seasonal variations

The foregoing results are not easy to interpret fully. A tendency for carotenoid content to rise after heavy feeding is quite definite, but some organs respond quickly and others much more slowly. Furthermore, there may be a seasonal change in storage sites. For example, during the early summer-feeding flush, the liver lipochrome increases markedly, yet the muscle content does not rise appreciably; during the autumn feeding this is reversed. However, it is not possible to distinguish between carotenoids carried with fat in lipid transfer and carotenoids mobilized in accordance with the cycle of specific utilization if such exists.

The gonadal cycle in the female results in a larger storage of carotenoids in the developing ovaries than in the whole of the rest of the body. Expulsion of ova is by far the largest cause of carotenoid loss, probably larger even than the total oxidative degradation. The cycle in the testes and oviducts is not responsive to dietary changes, but this is no more than a hint at functional significance. The sex difference in carotenoid storage in the fat bodies recalls the dissimilar gonadal sequence, for spermatogenesis is spread over

no more than six weeks, whereas ovarian development proceeds steadily until the autumn acceleration of growth.

Although muscle and kidney stores fluctuate relatively little it is noticeable that the absolute amount stored in muscle (especially for males) is quite appreciable. Muscle fat and carotenoid is probably mobilized readily. It is interesting that, although frogs feed soon after mating, carotenoid storage does not occur until late May. There is a similar lag in liver glycogen and blood sugar increment (Smith, 1947).

The 'xanthophyll'/carotene ratio (X/C)

The relative amounts of 'xanthophyll' and carotene stored differ markedly in the various organs. For frogs of different sizes the quotient varies as follows: testes 1.2-1.5, liver 3.5-5.8 and stomach 2.4-2.55, skin 5.5-11, muscle 7-10. There is, however, no uniform trend with increasing age in different organs. The data on seasonal changes are summarized in Table 12. It is quite possible that the picture is distorted by some local or other factor connected with the availability of particular kinds of food; in fact marked annual differences have been observed. Some of the grosser effects can, however, be interpreted. When carotene predominates as it

appears to do in female livers (August-December 1946), transfer of 'xanthophyll' from liver to gonads is the probable explanation, because the quantitative demand of female gonads for 'xanthophyll' is great. During spermatogenesis (when the total carotenoid is maximal) the X/C ratio for testes is doubled, but the reason is not clear.

Free and combined 'xanthophyll'

Seasonal effects. Table 13 shows the distribution of free 'xanthophyll', and mono- and di-esterified 'xanthophyll' as a percentage of the total. Although high accuracy cannot be claimed for the figures it is obvious that the organs fall into two classes in which the 'xanthophyll' is respectively mainly free and mainly combined. The significance of the data in Table 13 is not clear, because it implies a distribution of esterases for which no explanation can be offered.

Comparison of fat and carotenoid storage

Since lipid is the normal vehicle for carotenoids it is important to know whether the storage and mobilization of lipochrome is correlated with that of fat. Tables 14 and 15 show that it is not. (The ratio fat/carotenoid (C/F) is approximate, since the figures for total carotenoid are obtained by the roughly

Table 12. *Seasonal changes in the 'xanthophyll'/carotene ratio in frog organs*

Organs	Sex	1946						1947				
		July	Aug.	Sept.	Oct.	Nov.	Dec.	Apr.	Apr.	May	July	Oct.
Testes	—	—	2.5 (?)	1.95	1.06	0.87	1.06	0.85	—	0.88	1.92	1.42
Ovaries	—	7.2	6.3	7.2	7.2	6.7	7.4	7.7	11.6	9.6	11.2	8.2
Fat bodies	M.	—	—	7.7	5.7	5.0	4.9	6.7	—	16.0	10.9	7.9
	F.	—	—	—	5.0	4.4	—	—	7.2	5.1	9.2	—
Skin	M.	—	—	—	—	—	—	5.0	—	4.6	5.3	4.8
	F.	—	—	—	—	—	4.1	2.6	4.3	4.1	4.6	2.8
Liver	M.	1.12	0.72	1.97	1.59	1.19	1.46	1.51	—	1.57	1.74	3.0
	F.	1.02	0.51	0.78	0.88	0.71	0.95	0.81	1.58	1.38	1.74	2.8
Kidney	M.	—	—	—	—	—	—	3.6	—	—	5.0	4.5
	F.	—	—	—	—	—	—	2.9	—	2.7	9.3	2.2
Leg muscle	M.	—	—	—	—	—	—	—	—	—	10	7.5
	F.	—	—	—	—	—	—	—	—	—	8.3	8.7
Lungs	M.	—	—	—	—	—	—	3.0	—	—	—	—
	F.	—	—	—	—	—	—	—	—	—	3.9	9.0
Pancreas	M.	—	—	—	—	—	—	6.8	—	—	15.8	7.7
	F.	—	—	—	—	—	—	—	—	—	—	6.2
Stomach	M.	—	—	—	—	2.1	1.7	1.8	—	2.4	—	2.1
	F.	—	—	—	—	—	—	—	—	—	2.4	—
Intestine	M.	—	—	—	—	—	4.1	2.6	—	—	—	4.0
	F.	—	—	—	—	2.6	—	—	—	—	4.2	3.1
Large intestine	M.	—	—	—	—	—	—	1.9	—	—	—	0.9
	F.	—	—	—	—	—	—	—	—	—	2.3	1.0
Tongue	M.	—	—	—	—	—	—	—	—	—	3.8	1.1
	F.	—	—	—	—	—	—	—	—	—	1.8	2.2
Oesophagus	M.	—	—	—	—	—	—	—	—	—	—	2.4
	F.	—	—	—	—	—	—	—	—	—	4.9	—
Oviducts*	—	—	—	—	—	—	—	0.83	0.67	0.76	0.72	1.0

* Oviducts alone fail to show 'xanthophyll' predominating at any season of the year. X/C ratio was 0.88 in Jan. 1948.

Table 13. *Seasonal distribution of free 'xanthophyll' and xanthophyll mono- and di-esters in frog organs*

Organ	Season	Sex	Percentage of total 'xanthophyll' as		
			Di-ester	Mono-ester	Free
Skin	Summer	F.	68	15	27
Skin	Summer	M.	61	19	20
Skin	Autumn	M.	68	15	17
Skin	Spring	M.	74	13	13
Ovaries	Summer	—	52	33	15
Ovaries	Autumn	—	60	27	13
Ova	Spring	—	58	20	22
Liver	Autumn	M.	9	18	73
Muscle	Autumn	M.	12	8	80
Muscle	Autumn	F.	8	8	84
Muscle	Spring	F.	10	9	81
Oviduct	Autumn	—	11	22	67
Testes	Winter	—	7	23	70
Fat bodies	Winter	M.	11	34	55
Small intestine	Winter	M.	6	13	81

Table 14. *Total carotenoids and total fat of organs of male frogs of different sizes*(Mean body lengths: Group A, 6.6; B, 5.6; C, 5.1 cm. Total carotenoid calculated on basis of mean $E_{1\text{cm}}^{1\%}$ 450 m μ . 2500.)

Group ...	A			B			C		
	Fat (mg.)	Carotenoid ($\mu\text{g.}$)	C/F (mg./g.)	Fat (mg.)	Carotenoid ($\mu\text{g.}$)	C/F (mg./g.)	Fat (mg.)	Carotenoid ($\mu\text{g.}$)	C/F (mg./g.)
Testes	4.1	1.3	0.3	2.7	0.87	0.32	1.7	0.68	0.4
Liver	3.6	18	5.0	2.9	15.4	5.3	2.2	9.3	4.2
Skin	16.2	50	3.1	12.6	34	2.7	9.9	28	2.9
Muscle	18.3	13.7	0.75	10.6	8.2	0.77	6.8	6.2	0.9

Table 15. *Seasonal variations (1947) in ratio of total carotenoid/fat in frog organs*

(Ratios expressed as mg. total carotenoid/g. fat.)

Organ	Sex	April	May	July	October
Testes	—	—	0.63	0.44	0.42
Ovaries	—	3.4	1.3	1.12	0.68
Fat bodies	M.	—	0.49	0.01	0.09
	F.	—	0.02	0.01	0.12
Kidneys	F.	—	0.66	0.3	1.5
	M.	—	0.28	0.47	1.4
Liver	F.	3.3	3.6	1.6	3.6
	M.	3.2	1.6	2.7	1.1
Leg muscle	M.	—	—	—	1.8
	F.	—	—	0.34	1.2
Skin	M.	2.8	3.5	3.1	3.2
	F.	3.4	2.3	8.4	4.0
Oviducts	—	0.3	0.3	0.2	0.4

correct assumption of an average $E_{1\text{cm}}^{1\%}$ 450 m μ . of 2500 for the constituent pigments.) Variations with size are obviously less important than inter-organ variations. Seasonal variations (Table 15) are striking, but again there is no correlation between fat transfer (or metabolism) and the fate of carotenoids.

Vitamin A fed to frogs

One male and one female were each given 1 drop (approx. 3500 i.u.) of an ester concentrate daily for a month, with no

other food. Much of the vitamin was excreted, but the liver level rose threefold. Some vitamin appeared in the extracts from lungs, fat bodies and testes, but not in the skin or the ovaries. The experiment indicates poor assimilation, but this may have been due to the fact that the animals (like all frogs in captivity) refused to eat spontaneously. The absence of vitamin A from the ovaries is consistent with the observation that it is not detectable in newly spawned eggs.

Fasting frogs

The ability to do without food for long periods is a well-known characteristic of frogs. In an experiment in which twenty-five female frogs were kept without food from 18 July to 9 December 1947, the fat bodies had been exhausted and body weights had declined by some 35%. Ovarian development was greatly retarded. The decreases in weight of the viscera during inanition were in general agreement with previous observations (Ott, 1924). Most of the intestines contained faeces of liquid consistency with a 'xanthophyll'/carotene ratio of 4/1 as compared with the normal faecal value of 2. The vitamin A level of the liver had not fallen very much, but the eyes and the kidneys had been depleted; 97% of the total vitamin was found in the liver.

The results recorded in Tables 16 and 17 need little discussion. Perhaps the major point is that despite the great changes incidental to prolonged fasting the overall X/C quotient for the whole frog rises to 4.6 exactly as in normal animals (3.6-4.6) over the same period of the year. This seems to eliminate dietary influence and supports the idea of selective utilization of different carotenoids.

Table 16. *Fat, carotenoids and vitamin A in female frogs kept in captivity for 25 weeks*

(Average decrease in body weight 35%. Means not measured.)

Organ	Average wt. of organ as percentage of body wt.*	Fat (mg./frog)	Vitamin A (μ g./frog)	Carotenoid (μ g./frog)	Xanthophyll Carotene
Liver	0.99	1.29	24.7	7.4	1.97
Eye	1.34	0.72	0.62	—	—
Kidney	0.23	1.21	0.074	0.5	2.09
Skin	9.01	3.39	—	19.2	2.71
Ovaries	5.26	17.0	—	22.1	10.2
Immature ovaries	1.19	2.17	—	8.7	19.0
Oviducts	8.30	5.19	—	1.3	0.78
Immature oviducts	0.94	—	—	0.3	3.37
Tongue	0.83	—	—	0.15	4.92
Pancreas	0.025	—	—	0.15	11.0
Lungs	0.274	—	—	0.13	—
Leg muscle	9.47	3.3	—	4.8	7.25
Oesophagus	0.31	—	—	0.12	—
Stomach	0.75	—	—	0.4	1.78
Intestine	0.43	—	—	0.2	6.37
Large intestine	0.19	—	—	0.05	—

* Compare Table 9 column 2.

Table 17. *Distribution of 'xanthophyll' among the free form, mono- and di-esters in the organs of fasted female frogs*

Organ	Percentage of total 'xanthophyll' existing as		
	Di-ester	Mono-ester	Free form
Liver	8	18	74
Leg muscle	4	11	85
Skin	69	19	12
Oviducts	25	15	60
Ovaries	37	47	16
Immature ovaries	26	60	14

GENERAL COMMENTS

The present study perhaps makes timely a general comment on the place of distribution studies in the growth of ideas on the biological significance of particular compounds. It is a very uncertain place; the pattern of distribution is a resultant of superimposed patterns, of intake, assimilation, storage and utilization, of precursors and metabolites, and of enzyme capacities and 'detoxication' processes. Sometimes distribution studies seem merely to add new difficulties, but at other times they suggest fruitful lines of attack. A few examples may be given:

Vitamin A. The distribution data are consistent with storage in the liver and with localized function in the eyes, but the apparent absence of vitamin A from normal nerve tissue, bone and mucosa is surprising in the light of the pathology of avitaminosis A. Comparative biochemistry adds to the complexity, e.g. the basking shark is found to store no vitamin A in its liver and the tunny fish to store enormous amounts.

Cholesterol. The concentration of cholesterol in the adrenals pointed to functions, the nature of which is clarified by the effects of stress or injections of adrenocorticotrophic hormone. On the other hand, the broad picture of cholesterol distribution merely emphasizes the inadequacy of existing knowledge.

Provitamin D. The occurrence of 7-dehydro-cholesterol in skin fat is now seen to be significant, but its presence in ripe frog ovaries, in intestinal mucosa and in the sterols from worms is no easier to understand than the high concentration of ergosterol in yeast.

As was implied at the outset, this investigation was prompted by the idea that carotenoids may have functional significance in at least some animals. The advantage of the frog is that carotenoids are present at all stages in the life cycle; its disadvantage is that possible clues may be masked by the casual ecological variables and by shifting enzyme capacities. In the present state of carotenoid biochemistry the need is to find a bodily site or a developmental phase marked out by the distribution as possessing special interest.

Research in the field is halted by the lack of serviceable hypotheses. The present work suggests that: (a) beneath much that may be casual there are strong suggestions of significance; (b) in both males and females, the sexual cycle exhibits patterns of storage, transfer and disappearance of carotene and xanthophyll, consistent with independent and significant roles; (c) the whole method of approach is in essence exploratory and its aim is to provoke the experiments which turn surmises into certainties.

SUMMARY

1. The occurrence of carotenoids and vitamin A in frogs (*Rana temporaria*) and tadpoles has been studied and new metrical data have been obtained on size and weight.

2. Analytical methods for the accurate and speedy determination of carotenoids and vitamin A have been described and tested.

3. Whole tadpoles were studied at different stages of development and the changes in total 'xanthophyll' and carotene content recorded. The presence of chlorophyll is ascribed to green food undergoing digestion. The 'xanthophyll'/carotene quotient declines from about 9 to about 3 as development proceeds. Vitamin A was detectable in tadpole eyes.

4. The weights of frog organs expressed as percentage of body weight are recorded and maximum and minimum values given when variations are considerable. Much of the carotenoid actually ingested is contained in undigested food present in the alimentary tracts of insects or other prey. The frog does not ingest preformed vitamin A but converts carotene to the vitamin. About 85 % of the total vitamin occurs in the liver (mainly as ester); the remainder is normally about equally divided between kidneys and eyes. Frog kidneys contain a higher proportion of vitamin A than do mammalian kidneys. Most of the

vitamin A in the eye is ester, stored in the choroid. Carotenoids are dispersed over most organs but quite unevenly. The deposition of carotenoids in the ovaries is so large that a marked difference results between the sexes in carotenoid storage and utilization.

5. Mature ovaries contain in relatively high concentration a substance showing the four absorption maxima characteristic of 7-dehydrocholesterol and ergosterol.

6. Data have been obtained on the seasonal variation in different organs of some or all of the following: carotene, 'xanthophyll' (free, mono-ester, di-ester), vitamin A (free and esterified) and total fat. The results on normal and fasting frogs reveal: (a) the retention of liver reserves in hibernation and inanition; (b) well-defined seasonal variations in the carotenoid storage of different organs; (c) some indication of the continuance of carotenoid metabolism during hibernation; (d) the overwhelming quantitative importance of the ovarian requirements; (e) a drain on vitamin A reserves during reproduction; (f) the distinct patterns of storage and utilization of vitamin A, carotenes, 'xanthophylls' and fat in the frog.

We are indebted to the Medical Research Council and the Ministry of Food for financial assistance. One of us (D.G.R.) held an Evans, Lescher and Webb Studentship and later a Johnston Fellowship in the University of Liverpool.

REFERENCES

- Ackermann, J. (1938). *Bull. int. Acad. Cracovie*, B. II, 241.
 Bartz, J. P. & Schmitt, F. C. (1936). *Amer. J. Physiol.* **117**, 280.
 Boyd, E. M. (1938). *J. Physiol.* **91**, 394.
 Brunner, O. & Stein, R. (1935). *Biochem. Z.* **282**, 47.
 Comar, C. L. & Zscheile, F. P. (1942). *Plant Physiol.* **17**, 198.
 Dietel, F. G. (1933). *Klin. Wschr.* **12**, 601.
 Eekelen, M. van (1934). *Acta Brev. neerl. Physiol.* **4**, 65.
 Gillam, A. E. (1938). *Biochem. J.* **32**, 1496.
 Glover, J., Goodwin, T. W. & Morton, R. A. (1947). *Biochem. J.* **41**, 97.
 Harris, D. G. & Zscheile, F. P. (1943). *Bot. Gaz.* **104**, 515.
 Lederer, E. & Rathmann, F. H. (1938). *Biochem. J.* **32**, 1252.
 Lönnberg, E. (1929). *Ark. Zool.* **21**, B (3).
 Manunta, C. (1934). *Atti Soc. Nat. Mat. Modena*, **65**, 71.
 March, F. (1937). *Proc. zool. Soc. Lond.* **107**, 415.
 Morton, R. A. & Stubbs, A. L. (1946). *Analyst*, **71**, 348.
 Ott, M. D. (1924). *Amer. J. Anat.* **33**, 17.
 Pepkowitz, L. P. (1944). *J. biol. Chem.* **155**, 219.
 Rand, C. (1935). *Biochem. Z.* **281**, 200.
 Smith, C. L. (1947). Private communication.
 Wald, G. (1935). *J. gen. Physiol.* **19**, 781.
 Zechmeister, L. & Tuzson, P. (1936). *Hoppe-Seyl. Z.* **238**, 197.
 Zscheile, F. P., White, J. W. Jr., Beadle, B. W. & Roach, J. R. (1942). *Plant Physiol.* **17**, 331.