found for different preparations of antimony trichloride, the characteristics of a particular preparation should be determined with pure vitamin A₂ aldehyde for accurate work, but for determinations of the aldehyde in natural materials, the mean value of 4200 can be used. The variations in $\lambda_{\max}$ (739–743 m μ , mean 741 m μ) are not significant since, with the broad maximum of the absorption curve, values of extinction over this range would be essentially the same, within the experimental error of the test.

SUMMARY

1. Crystalline vitamin A₂ aldehyde with m.p. 66–68° has been prepared from vitamin A₁ aldehyde.

2. With purified antimony trichloride, 14 % (w/v) in chloroform containing 2 % (v/v) acetic anhydride, the wavelength of maximum absorption of the green–blue colour produced with vitamin A₂ aldehyde increased with concentration of the aldehyde and decreased with time.

3. In the absence of acetic anhydride, the wavelength of maximum absorption of the colour product was steady at 741 m μ , the colour developed remained at full intensity for about 15 sec., the maximum extinction varied linearly with concentration of aldehyde and $E_{1\%}^{1\text{cm}}$ (741 m μ) was 4200 (range 4000–4400).

4. Some observations on antimony pentachloride and glycerol dichlorohydrin (1:3-dichloropropan-2-ol) as colorimetric reagents for vitamin A₂ aldehyde are presented.

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A Comparative Survey of the Distribution of Vitamin A Aldehyde in Eggs

BY P. A. PLACK AND S. K. KON

Unit for Biochemical Research bearing on Fisheries' Problems, National Institute for Research in Dairying, Shinfield, Reading*

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We have recently reported the occurrence of vitamin A₁ aldehyde in the ripe eggs of several marine teleosts (Plack, Kon & Thompson, 1959) and in hen's eggs (Plack, 1960). Pollard & Bieri (1959) confirmed generally our early findings, but found vitamin A₂ aldehyde in the eggs of American 'herring' from Chesapeake Bay, and Winterstein & Hegedüs (1960) confirmed our finding with hen's eggs. With cod, the aldehyde is found only in eggs from ripening ovaries (Plack, Woodhead & Woodhead, 1961) and from our experience it is so with other fish.

Some preliminary experiments with ripe eggs from salmon suggested that vitamin A₂ aldehyde

was present (Plack, 1958), but a study of the eggs of freshwater and anadromous fish was held up for lack of a suitable method of determining vitamin A₁ and A₂ aldehydes in the presence of each other. The two aldehydes can be determined by a simple extension of the colorimetric reactions of the two compounds with antimony trichloride, but this method could only be applied accurately when the anomalous behaviour of vitamin A₂ aldehyde in the colorimetric reaction had been clarified by Plack (1961).

With the method now available, a comparative survey of the occurrence of vitamin A₁ and A₂ aldehydes in eggs was made. The aldehydes were found in all the ripe vertebrate eggs so far examined, but not in eggs from five invertebrates.

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MATERIALS AND METHODS

Chemicals

Solvents. Solvents, of A.R. quality whenever available, were used as supplied by May and Baker Ltd., except that diethyl ether, 'peroxide free', of which recent batches were found to be contaminated with a substance that combines with vitamin A aldehydes, was kept over NaOH pellets overnight and carefully redistilled before use, the middle fractions only being collected. The contaminant is removed by this procedure. Hexane of spectroscopic grade was obtained from Hopkin and Williams Ltd.

Antimony trichloride. For the determination of vitamin A₁ and A₂ aldehydes, A.R. SbCl₃ crystals were purified as described by Plack (1961) and dissolved in CHCl₃ to give a 14% (w/w) solution. For the determination of vitamin A₁ and A₂ esters and alcohols, a solution of SbCl₃ in CHCl₃, for vitamin A tests, supplied by British Drug Houses Ltd. was used.

Vitamin A compounds. Vitamin A₁ aldehyde was prepared as described by Plack *et al.* (1959) and vitamin A₂ aldehyde as described by Plack (1961). Vitamin A₁ alcohol was purchased from Roche Products Ltd. and vitamin A₂ alcohol, m.p. 63–65°, was a gift from F. Hoffmann–La Roche and Co. Ltd., Basle, Switzerland.

Collection and storage of biological materials

Invertebrates. Egg cases of the cockroach, *Periplaneta americana* L., were provided by the D.S.I.R. Pest Infestation Laboratory, Slough, Bucks., and those of the African migratory locust, *Locusta migratoria migratorioides* R. & F., by the Anti-Locust Research Centre, London, S.W. 7. The lipids were extracted from these two samples on arrival. From the lobster, *Homarus vulgaris* Milne Edwards, eggs, which were developing but had not reached the 'eyed' stage, were collected for us by Mr G. Davies, Fisheries Experiment Station, Conway. Dr L. R. Fisher collected

ovaries from the scallop, *Pecten maximus* (L.) and ovaries from the sea urchin, *Echinus esculentus* L., were obtained from the Marine Station, Millport; these two samples and the lobster eggs were stored in deep-freeze at –25°.

Cephalochordate. Specimens of *Amphioxus lanceolatus* (Pallas) were obtained from the Laboratory of the Marine Biological Association of the United Kingdom, Plymouth, and ovaries were removed and stored in deep-freeze until sufficient material was available.

Vertebrates. (a) Lampreys. Specimens of the lampern, *Lampetra fluviatilis* (L.) and the sea lamprey, *Petromyzon marinus* L., were purchased from Mr P. J. Gaskins, New Haw Farm, Tirley. Ovaries from 30 lampern were bulked and analysed as one sample, and ovaries from 8 sea lampreys were similarly treated. Both samples were stored in deep-freeze before analysis.

(b) Marine elasmobranchs. Ovaries from marine elasmobranchs were collected for us by Dr B. B. Rae, Marine Laboratory, Aberdeen, and stored in deep-freeze. Table 1 gives information about the ovaries analysed, and in all samples eggs of various degrees of ripeness were present.

(c) Marine teleosts. Deep-frozen herring were purchased locally. Plaice ovaries were collected by Mr A. B. Bowers, Marine Biology Station, Port Erin, Isle of Man, and ovaries of the other marine teleosts by Dr B. B. Rae; all were stored in deep-freeze. Table 2 gives particulars of the material analysed.

(d) Freshwater teleosts and salmon. The ovaries of the carp (*Cyprinus carpio* L.) and the inactive ovaries of the pike (*Esox lucius* L.) were collected in September 1958 from the Neusiedler See in Burgenland, Austria, by one of us (S. K. K.), and a sample of ripe pike eggs stripped from the fish was sent to us frozen from the same locality by Dr P. Schubert. Salmon (*Salmo salar* L.) eggs were supplied by Dr G. Howells, Freshwater Fisheries Laboratory, London, and eggs or ovaries from char [*Salvelinus alpinus willughbii* (Günther)], perch (*Perca fluviatilis* L.), pike (*Esox lucius* L.) and trout (*Salmo trutta* L.) were collected by Mr

Table 1. *Particulars of the elasmobranch ovaries analysed*

Species	Date caught	Length of fish (cm.)	Weight of ovary (g.)	No. and size of eggs
Black-mouth dogfish, <i>Pristiurus melanostomus</i> (Rafin.)	21 June 1959	63	5.5	7 eggs 0.5–0.7 cm. diameter and smaller eggs
Rabbit fish, <i>Chimaera monstrosa</i> L.*	23 June 1959	—	54.5	5 eggs 2.0 cm. diameter and smaller eggs
	24 June 1959	—	113	About 12 eggs 1.0–2.0 cm. diameter and smaller eggs
Spur dog, <i>Squalus acanthias</i> L.	26 Sept. 1958	92	194†	7 eggs 3.5–4.0 cm. diameter and smaller eggs
	26 Sept. 1958	89	190†	6 eggs 3.4–4.2 cm. diameter and smaller eggs
	19 June 1959	—	214	Probably 6–7 large eggs and smaller eggs, rather disintegrated
Thornback ray, <i>Raia clavata</i> L.	25 June 1959	71	40.1	5 eggs 1.0–3.0 cm. diameter and smaller eggs
	26 Sept. 1959	71, 72	233‡	About 50 eggs 1.3–2.0 cm. diameter and smaller eggs

* Subclass Holocephali.

† Does not include weight of embryos, which were discarded.

‡ Combined weight of ovaries from two fish.

Table 2. *Particulars of the samples of ovaries from marine teleosts*

Species	Date caught	No. of pairs of ovaries in sample	Weight of sample (g.)
<i>Coelorhynchus coelorhynchus</i> (Risso)	23 June 1959	2	32.1
	24 June 1959	2	20.6
Grey gurnard, <i>Trigla gurnardus</i> L.	26 June 1959	3	36.8
Herring, <i>Clupea harengus</i> L.	Oct.-Nov. 1960	1	40.3
		1	39.5
		1	27.3
		1	32.4
John Dory, <i>Zeus faber</i> L. (spent)	29 June 1959	1	8.3
	(ripe) 26 June 1959	1	55.7
Plaice, <i>Pleuronectes platessa</i> L.	2 Feb. 1959	1	156
	12 Feb. 1959	4	219
	12 Feb. 1959	4	301
Red gurnard, <i>Trigla cuculus</i> L.	25 June 1959	1	62.6
	26 June 1959	2	119

G. J. Thompson, Freshwater Biological Association, Windermere. All these eggs or ovaries were stored in deep-freeze and, with the exception of the pike ovaries from Austria, all were ripe.

(e) Reptiles. Adders, *Vipera berus* (L.), killed in the New Forest were collected by Mr G. N. Gould and stored at 0° until sent to Shinfield. Ovaries were then dissected out and stored in deep-freeze. Eggs 0.5–1.5 cm. long from adders caught in May 1960 were analysed. Four eggs from a European water tortoise, *Emys orbicularis* (L.), and two eggs from a South American terrapin (species not known) were obtained for us from the Bristol Zoo by Miss V. M. Kon and six terrapin eggs [from a mixed tank containing *Pelusios subniger* (Lacépède), *Geomyda punctularia* (Daudin) and *Pseudemys scripta* (Schoepff)] were received from Dr H. G. Ververs, Zoological Society of London. These eggs were stored in deep-freeze. Fresh turtle eggs [probably from *Chelonia mydas* (L.)] were the gift of Mr A. C. Lusty of William Lusty Ltd. They were flown from Mombasa and stored in a refrigerator for 2 days before analysis.

(f) Amphibian. Six female common frogs, *Rana temporaria* L., were purchased from L. Haig and Co. Ltd., Newdigate, Surrey, at the end of February 1961. They were pithed and the eggs removed, mixed and used immediately.

(g) Bird. Eggs from the domestic hen, *Gallus gallus* L. (Rhode Island Red × Light Sussex) were obtained from one of the Institute's breeding stocks. The diet contained 200 µg. of added vitamin A/100 g. and 5% (w/w) of dried grass, but was deficient in vitamin B₁₂.

Extraction of lipids

The sample was made as homogeneous as possible. For this purpose ovary tissue was blended if sufficient was available, otherwise it was cut into small pieces and mixed. Egg cases of insects were cut in half before extraction. Egg shells of the water tortoise, terrapins and turtle were removed and the whites and yolks extracted together. The yolks only of hen's eggs were extracted.

Method A. A portion of the sample, usually 30 g., was freeze-dried in a centrifugal freeze-drier of the type described by Record & Taylor (1958). The dried sample was weighed, ground to a powder, transferred to a Soxhlet

thimble and extracted with 150 ml. of diethyl ether for 4 hr. The residue from the thimble was then transferred to a Waring Blendor, blended with 200 ml. of light petroleum (b.p. 40–60°), and 60 ml. of ethanol and 30 ml. of water were added. Blending was continued for 2 min. and the light-petroleum layer poured off. The aqueous residue was extracted twice more with 200 ml. lots of light petroleum and the three extracts were combined. Any solid matter in the diethyl ether and light-petroleum extracts was allowed to settle and the supernatant extracts were evaporated to dryness in clean flasks on a steam bath, the last portions of solvent being removed under reduced pressure. The residues were weighed and dissolved in hexane, and the solutions made to suitable volumes (usually 12 ml.) with hexane.

Method B. A portion of the sample, usually 30 g., was homogenized in lots of about 10 g., each with 7.5 ml. of water, in a Potter-Elvehjem homogenizer with a nylon pestle. The homogenates, together with one washing of the homogenizer with 7.5 ml. of water, were transferred to a Waring Blendor, blended with 200 ml. of light petroleum (b.p. 40–60°); 60 ml. of ethanol was then added, and the whole was blended for a further 2 min. The procedure of method A was then followed. When the sample contained much connective tissue, homogenization was not possible and a portion of the sample was treated directly in the Waring Blendor.

Determination of dry-matter content

Dry matter was determined by drying about 5 g. of the sample to constant weight in an oven at 105°.

Determination of vitamin A₁ and A₂ aldehydes

Separation of the aldehydes from vitamin A esters and alcohol. The method of separation described by Plack *et al.* (1959) was applied to the light-petroleum extract obtained by method A, or to the whole extract obtained by method B. Usually one-third of the extract was used, but not more than 0.5 g. of lipid was put on the column. Aluminium oxide, for chromatographic adsorption analysis (British Drug Houses Ltd.), was later substituted for alumina from Peter Spence and Sons Ltd. in the separation and, after

drying at 150°, was weakened by the addition of 12% of its weight of water. Vitamin A₁ and A₂ aldehydes were not separated by this method.

The diethyl ether extracts obtained from frog's and hen's eggs and sea-urchin ovaries by method A were saponified by a method similar to that described by Winterstein & Hegedüs (1960) before separation of the aldehydes. To each 1 g. of lipid, 50 ml. of diethyl ether and 5 ml. of 10% (w/v) KOH in methanol were added, and the mixture was left at room temperature overnight in an atmosphere of N₂. The mixture was then washed with 3 × 20 ml. of water, the remaining diethyl ether solution was dried over anhydrous Na₂SO₄ and evaporated to dryness, and the residue dissolved in hexane. The recovery of vitamin A₁ aldehyde after this mild saponification was about 80%, so that the content of aldehyde in the diethyl ether extracts could not be determined accurately.

Portions of the diethyl ether extract from the rabbit fish, thornback ray, grey gurnard, red gurnard and adder were tested for aldehyde by the chromatographic method without this mild saponification.

Carr-Price test. The two aldehydes were determined by the Carr-Price colorimetric reaction. An Optica CF4 double-beam recording spectrophotometer fitted with a red-sensitive DuMont photomultiplier was used to measure the intensity of the blue colour while a stream of N₂ was passed through the cell compartments. The spectrophotometer was set up at 665 mμ with CHCl₃ in the control cell and 0.5 ml. of CHCl₃ and 2 ml. of purified SbCl₃ solution in CHCl₃ in the stoppered experimental cell, and the base-line over the range 600–840 mμ was recorded. The instrument was reset to 665 mμ. To the sample, 0.5 ml., in the experimental cell was added 2 ml. of purified SbCl₃ solution from a bulb pipette and the cell was transferred to the instrument. The chart drive was switched on 20 sec. after addition of the SbCl₃ solution had begun, and the chart kept running at a speed of 2 in./min. The percentage transmission, or extinction, was recorded at 665 mμ for 10 sec., then at 740 mμ for 10 sec. and then alternately at 665 and 740 mμ until the maximum extinctions at the two wavelengths had been reached, usually between 20 and 80 sec. after mixing. A single value for the extinction at 840 mμ was then recorded, and with the wavelength drive on to give a scanning speed of 1.25 mμ/sec. a curve of the light-absorption of the blue colour product was drawn. The curve is not a true absorption curve, since the blue colour is fading during the scanning, but it does give the wavelengths of absorption maxima and of inflexions with reasonable accuracy, as they are scanned in a few seconds. The wavelengths of 665 and 740 mμ chosen are both 1 mμ below the maxima for vitamin A₁ and A₂ aldehyde in this test (Plack, 1961) but were used for convenience in changing the spectrophotometer rapidly from one wavelength to the other. At 840 mμ there should be no significant absorption of light due to the colour products of vitamin A₁ and A₂ aldehydes.

The procedure of reading the extinctions of the colour product alternately at 665 and 740 mμ could be carried out on a Beckman DU spectrophotometer although the curve could not be traced. Readings were then obtained at intervals of 15–20 sec., so that 30–40 sec. elapsed between successive readings at one wavelength. During this time the maximum extinction may have been missed, but the error involved was small.

Calculation of results. The method is based on the simple additive effect of the colour product of vitamin A₁ and A₂ aldehydes. At 740 mμ, the extinction of the colour product of vitamin A₁ aldehyde was 0.01 $E_{665\text{ m}\mu}^{1\text{ cm}}$; at 665 mμ, the extinction of the colour product of vitamin A₂ aldehyde was 0.38 $E_{740\text{ m}\mu}^{1\text{ cm}}$. In the absence of acetic anhydride $E_{665\text{ m}\mu}^{1\text{ cm}}$ (666 mμ) = 4150 was found for the colour product of vitamin A₁ aldehyde, and $E_{740\text{ m}\mu}^{1\text{ cm}}$ (741 mμ) = 4200 for the colour product of vitamin A₂ aldehyde was found by Plack (1961). At 665 and 740 mμ, the two extinction coefficients did not differ significantly from the values given above for 666 and 741 mμ.

If the curve of the colour product was of the normal form, i.e. peaks or inflexions at about 665 and 740 mμ, with the curve falling off to low extinctions on each side, the extinctions at 665 mμ due to vitamin A₁ aldehyde (x) and at 740 mμ due to vitamin A₂ aldehyde (y) were calculated from the simultaneous equations:

$$x + 0.38y = E_{665\text{ m}\mu} - E_{840\text{ m}\mu},$$

$$0.01x + y = E_{740\text{ m}\mu} - E_{840\text{ m}\mu},$$

the maximum extinctions found at 665 and 740 mμ being corrected for the small extinction at 840 mμ, which was regarded as due to general background absorption. The concentrations of vitamin A₁ and A₂ aldehydes were then calculated from the values of the specific extinction coefficients given above.

Mixtures containing as little as 5% of vitamin A₁ aldehyde and 95% of vitamin A₂ aldehyde showed the presence of the former by an inflexion at about 683 mμ on the curve of the Carr-Price colour product. Mixtures of 95% A₁ and 5% A₂ aldehydes did not show a definite inflexion of the curve in the region of 740 mμ, but when the proportion of vitamin A₂ aldehyde reached 10% an inflexion was found.

Proof of the presence of vitamin A₂ aldehyde. The sample of pike eggs from Austria was chosen for further tests, since it contained a high proportion of vitamin A₂ aldehyde and there was sufficient material available. Method B was used to extract 60 g. of eggs, the extract in hexane was divided into six parts and the aldehyde separated from each by gradient elution as described above. Those fractions with $E_{370\text{ m}\mu} > 0.015$ were put together, the solvent was removed, and 10 ml. of spectroscopic-grade hexane was added for the determination of λ_{max} in the ultraviolet. The hexane was then removed under reduced pressure and 8 ml. of ethanol were added to the mixture. Not all the lipid dissolved, and the supernatant liquid was poured off from the residue. A few milligrams of NaBH₄ were added to the solution, which was then shaken. After about 5 min., when the yellow colour had disappeared, the solution was transferred to a separating funnel and 20 ml. of light petroleum and 8 ml. of water were added to extract the vitamin A₂ alcohol formed by reduction together with the other lipids. Two further extractions with 20 ml. quantities of light petroleum followed, the combined extracts were washed with 3 × 20 ml. of water and dried over anhydrous Na₂SO₄, the solvent was removed under reduced pressure, and about 3 ml. of hexane was added. Chromatography on alumina followed (see above), and the eluate obtained with 8% (v/v) ethanol in hexane was dried under reduced pressure, and the residue dissolved in 8 ml. of ethanol. λ_{max} in the ultraviolet and in the Carr-Price test (in CHCl₃ solution) were determined with the recording spectrophotometer.

To 1.5 ml. of this ethanol solution, 1.5 ml. of a 0.04*N* solution of HCl in ethanol was added and consecutive absorption curves were traced with the recording spectrophotometer to follow the formation of ethoxyanhydrovitamin A₁ (Henbest, Jones, Owen & Thaller, 1955).

Determination of vitamin A₁ and A₂ esters and alcohols

Purification by chromatography and saponification. The separation of vitamin A esters and alcohol in the two extracts obtained by method A and in the extract obtained by method B was carried out by the chromatographic method of Thompson, Ganguly & Kon (1949) with the modification of Kon, McGillivray & Thompson (1955). In general one-third of the total extract was used for this separation, but not more than 0.5 g. of lipid was put on the column. The two fractions were saponified and extracted as described by Plack (1956) except that aqueous 60% (w/w) KOH solution was used. Each fraction was subjected to a further chromatography, usually as described by Thompson *et al.* (1949), and the eluates containing vitamin A, now in the alcohol form, were dried and CHCl₃, 1.5 ml. or more, was added for the Carr-Price test.

With the frog's and hen's eggs, the final chromatography after saponification was replaced by a gradient elution chromatography to separate vitamin A alcohol from xanthophylls. The apparatus described by Plack *et al.* (1959) was used, with 20% (v/v) diethyl ether in light petroleum in the bottle and diethyl ether in the separating funnel. The non-saponifiable residue in hexane was put on the column and elution begun. The first 40 ml. of eluate contained β-carotene, if present, and was discarded; the next 100 ml. contained vitamin A alcohol, and the residue after removal of solvent was taken up in CHCl₃; the xanthophylls remained on the column.

In all these procedures, vitamins A₁ and A₂ remained together.

Carr-Price test. The determination of vitamin A₁ and A₂ alcohols by the Carr-Price reaction was similar to that already described for vitamin A₁ and A₂ aldehydes but, with the alcohols, the maximum intensity of the blue colour is reached a few seconds after addition of the reagent, and the recording spectrophotometer could not be used. The direct-reading instrument described by Thompson (1949) was therefore used. To 0.5 ml. of the CHCl₃ solution of vitamin A in the cell 1 drop of acetic anhydride was added followed by 2 ml. of the commercial solution of

SbCl₅ in CHCl₃. The maximum intensity of the blue colour was read first at 620 mμ and afterwards with another 0.5 ml. of the CHCl₃ solution, at 693 mμ. No correction for possible background absorption could be made with this instrument (see above).

Under these conditions, a value of $E_{1\text{cm}}^{1\%}$ (620 mμ) = 5000 was found for vitamin A₁ alcohol (see Fisher, Kon & Thompson, 1956) and the ratio $E_{620\text{ m}\mu}/E_{693\text{ m}\mu}$ was 0.01. For vitamin A₂ alcohol, a value of $E_{1\text{cm}}^{1\%}$ (693 mμ) = 3520 was found, and the ratio $E_{620\text{ m}\mu}/E_{693\text{ m}\mu}$ was 0.42. With mixtures of the pure compounds, the extinctions due to the two components at 620 and 693 mμ were found to be additive, and x , the extinction at 620 mμ due to vitamin A₁ alcohol, and y , the extinction at 693 mμ due to vitamin A₂ alcohol, could be calculated from the equations:

$$x + 0.42y = E_{620\text{ m}\mu},$$

$$0.01x + y = E_{693\text{ m}\mu}.$$

The concentrations of the two components were then calculated from the specific extinction coefficients given above. In the results, the concentrations of vitamin A₁ and A₂ esters and alcohols in the extracts have been added together, the results being expressed in terms of vitamin A₁ alcohol; the small difference in molecular weight between vitamin A₁ and A₂ alcohols has been ignored.

Estimation of carotenoids

A rough estimate of the total carotenoids present in a sample was obtained by diluting a portion of the extract with an appropriate volume of hexane, centrifuging to clear if necessary, and determining the absorption curve over the range 380–540 mμ with the recording spectrophotometer. A value for $E_{1\text{cm}}^{1\%}$ of 2500 was used to calculate the quantity of carotenoids present from the extinction of the highest peak. Some extracts were yellow in colour but either did not show the characteristic peaks of the carotenoid pigments, or showed them only as inflexions on a curve, the extinction of which decreased steadily with increase in wavelength. The small amounts of carotenoids in such extracts, if present, are reported as not measurable.

RESULTS

Table 3 shows the results of the determination of total vitamin A₁ and A₂ aldehydes and the percentage of vitamin A₂ aldehyde in solutions of

Table 3. *Total vitamin A₁ and A₂ aldehydes and percentage of vitamin A₂ aldehyde determined in mixtures of known composition*

Determinations were by the Carr-Price test (see Methods).

Composition of mixture		Total vitamin A aldehyde found (as percentage of theoretical)	Percentage of vitamin A ₂ aldehyde found (as percentage of theoretical)
Vitamin A ₁ aldehyde (μg./ml.)	Vitamin A ₂ aldehyde (μg./ml.)		
4.64	0.51	101.0	97.0
4.12	1.02	101.4	97.9
3.09	2.05	101.0	100.0
2.06	3.07	99.2	100.8
1.03	4.10	99.9	100.4
0.51	4.61	101.9	101.0

known composition containing mixtures of the pure compounds. Table 4 shows similar results for vitamin A₁ and A₂ alcohols. The two methods gave satisfactory results with the pure substances.

Ovaries of the sea urchin were extracted by method A and eggs or ovaries of the cockroach, locust, lobster and escallop by method B. No vitamin A was found in the eggs or ovaries of these animals and the cockroach eggs contained no carotenoids, but eggs of the locust and lobster, and ovaries of the escallop and sea urchin, contained

60.1, 498, 64.5 and 18.6 µg. of carotenoids/g. dry matter respectively. Ovaries of *Amphioxus lanceolatus*, a cephalochordate, were extracted by method B and contained 0.23 µg. of vitamin A aldehyde and 0.26 µg. of total vitamin A esters and alcohol/g. wet wt., the vitamin A₂ forms not being determined.

Tables 5-8 give the results of the other analyses. All the ripe eggs, or ovaries containing ripe eggs, of the vertebrates contained vitamin A aldehyde and the ester and alcohol forms of vitamin A.

Table 4. Total vitamin A₁ and A₂ alcohols and percentage of vitamin A₂ alcohol determined in mixtures of known composition

Determinations were by the Carr-Price test (see Methods).

Composition of mixture		Total vitamin A alcohol found (as percentage of theoretical)	Percentage of vitamin A ₂ alcohol found (as percentage of theoretical)
Vitamin A ₁ alcohol (µg./ml.)	Vitamin A ₂ alcohol (µg./ml.)		
9.00	1.00	98.6	101.0
8.00	2.00	100.2	99.0
6.00	4.00	97.9	102.0
4.00	6.00	98.7	101.3
2.00	8.00	99.1	100.6
1.00	9.00	97.5	101.1

Table 5. Mean concentrations of vitamin A and carotenoids in the eggs of lampreys and marine elasmobranchs

On a dry-matter basis. Numbers of samples analysed are shown in parentheses. All samples were ovaries, and were extracted by method A. N.M., Not measurable.

Species	Vitamin A aldehyde		Vitamin A esters + alcohol		Carotenoids (µg./g.)
	Total (µg./g.)	Percentage of A ₂	Total (µg./g.)	Percentage of A ₂	
Lampern (1)	0.37	21	22.0	22	7.3
Sea lamprey (1)	0.08	—	2.10*	49*	N.M.
Black-mouthed dogfish (1)	1.31	10	2.24	11	N.M.
Rabbit fish (2)	1.02	8	1.29*	17*	N.M.
Spur dog (3)	2.02	10	8.84	14	N.M.
Thornback ray (2)	0.80	21	0.88*	51*	5.0

* See p. 567.

Table 6. Mean concentrations of vitamin A and carotenoids in the eggs of marine teleosts

On a dry-matter basis. Numbers of samples analysed are shown in parentheses. All samples were ovaries. N.M., Not measurable.

Species	Method of extraction	Vitamin A aldehyde		Vitamin A esters + alcohol		Carotenoids (µg./g.)
		Total (µg./g.)	Percentage of A ₂	Total (µg./g.)	Percentage of A ₂	
<i>Coelorrhynchus coelorrhynchus</i> (2)	A	14.10	4	4.66	33	19.3
Grey gurnard (1)	A	2.95	14	2.10	23	13.3
Herring (4)	B	3.73	13	1.33*	50*	N.M.
John Dory (spent) (1)	B	0	—	1.66*	52*	18.4
(ripe) (1)	B	3.07	9	1.03*	—	24.2
Plaice (3)	B	5.30	16	0.47*	—	—
Red gurnard (2)	A, B	3.46	18	0.95*	46*	9.4

* See p. 567.

Amongst the lampreys and marine elasmobranch fish (Table 5) the concentration of vitamin A aldehyde in the ovaries was about equal to, or less than, the concentration of esters + alcohol; vitamin A₁ was predominant, and small amounts of carotenoids were present. In the eggs of marine teleosts (Table 6), vitamin A₁ was again predominant, but the concentration of aldehyde was equal to or greater than the concentration of esters + alcohol, and substantial amounts of carotenoids were usually present. With the exception of those of the carp and the char, the eggs of the freshwater teleosts and the anadromous salmon (Table 7) contained predominantly vitamin A₂, and in general the concentration of aldehyde was equal to or slightly greater than the concentration of esters + alcohol; with the exception of the perch, substantial amounts of carotenoids were present. Eggs of the amphibian, the reptiles and the bird (Table 8) contained predominantly vitamin A₁, the concentration of the aldehyde form was about equal to or less than that of the esters + alcohol, and sub-

stantial amounts of carotenoids were present in the eggs of all animals except those of the adder. With the latter some 24 % of the total aldehyde was present in the diethyl ether extract.

The high values for the proportion of vitamin A₂ esters and alcohols in the eggs of certain marine elasmobranchs and teleosts are possibly erroneous, owing to the accumulation of experimental errors and the small concentrations of esters and alcohols present (see Discussion).

Evidence for the presence of vitamin A₁ and A₂ aldehydes was usually obtained by their chromatographic behaviour and the $\lambda_{\max}$ of their colour products in the Carr-Price test. With the extract of ripe pike eggs from Austria, further experiments were carried out to establish the presence of vitamin A₂ aldehyde. The vitamin A aldehydes were separated from the esters and alcohols and $\lambda_{\max}$ in hexane was 389 m μ ; in the Carr-Price test $\lambda_{\max}$ was 743 m μ . Reduction with sodium borohydride gave $\lambda_{\max}$ in ethanol 355 m μ , and in the Carr-Price test 691 m μ , both close to the values for

Table 7. *Mean concentrations of vitamin A and carotenoids in the eggs of freshwater teleosts and the salmon*

On a dry-matter basis. Numbers of samples analysed are shown in parentheses. N.M., Not measurable.

Species	Ovaries (O) or eggs (E) analysed	Method of extraction	Vitamin A aldehyde		Vitamin A esters + alcohol		Carotenoids ($\mu\text{g./g.}$)
			Total ($\mu\text{g./g.}$)	Percentage of A ₂	Total ($\mu\text{g./g.}$)	Percentage of A ₂	
Carp (1)	O	B	6.25	34	7.45	52	75.2
Char (3)	E	A	3.47	37	1.83	54	18.1
Perch (6)	4O, 2E	B	5.97	97	3.38	88	N.M.
Pike (Austria) (1)	E	B	4.94	96	4.14	98	10.3
(2) (inactive ovaries)	O	B	0	—	1.70	94	11.0
Pike (England) (6)	4O, 2E	B	3.78	90	2.40	86	8.8
Salmon (4)	E	A	3.75	72	2.05	64	16.9
Trout (5)	E	2A, 3B	2.89	80	3.66	73	24.2

Table 8. *Mean weight and vitamin A and carotenoid content of the eggs of an amphibian, some reptiles and a bird*

Numbers of samples analysed are shown in parentheses. All samples were extracted by method A. N.M., Not measurable.

Species	Weight of egg (g.)	Vitamin A aldehyde		Vitamin A esters + alcohol		Carotenoids ($\mu\text{g./egg}$)
		Total ($\mu\text{g./egg}$)	Percentage of A ₂	Total ($\mu\text{g./egg}$)	Percentage of A ₂	
Amphibian						
Frog (1)	—	1.72*	11	2.74*	27	35.0*
Reptile						
Adder (1)	—	1.00†	4	0.77†	14	N.M.
European water tortoise (1)	7.7	3.25	13	6.19	14	20.6
South American terrapin (2)	30.8	8.71	18	8.59	34	280
Terrapin (1)	7.5	2.96	14	5.32	11	47.5
Turtle (8)	46.6	6.80	4	12.1	—	91.6
Bird						
Domestic hen (2)	69.7	24.3	4	131	10	885

* $\mu\text{g./g.}$ dry matter.

† $\mu\text{g./g.}$ wet wt.

vitamin A₂ alcohol given by Farrar, Hamlet, Henbest & Jones (1952). Treatment of the reduced product with ethanolic hydrogen chloride gave a substance with λ_{max} . 353, 370 and 391 m μ in ethanol, corresponding to ethoxyanhydrovitamin A₁ (Henbest *et al.* 1955). Vitamin A₂ aldehyde was therefore the predominant form in the extract.

DISCUSSION

Methods of extraction

The two-stage method of extraction, in which the sample is freeze-dried, first extracted with diethyl ether and the residue extracted with light petroleum in the presence of ethanol and water, was introduced to cope with the large proportion of lipid present in the yolk of hen's eggs, which interferes with the determination of vitamin A aldehyde (Plack, 1960). The diethyl ether extraction removed 75–80% of the lipid of these eggs but little or no aldehyde, which was extracted with the remainder of the lipid by light petroleum in the presence of ethanol and water. This method has been used with other eggs known to contain much lipid, or known to give anomalous results in the determination of vitamin A aldehyde. Such anomalous behaviour, for instance a rapidly rising extinction at 665 m μ , was not found after a preliminary freeze-drying and Soxhlet extraction with diethyl ether had removed some of the lipid. We have tested chromatographically the diethyl ether extracts from two samples of marine teleost ovaries and two of elasmobranch ovaries, which contained only small quantities of fat, and could find no definite proof of the presence of aldehyde; application of the mild-saponification method to the diethyl ether extract from frog's and hen's eggs revealed no aldehyde, and a trace, respectively. With the eggs of the adder, and herring ovaries (P. A. Plack, unpublished observations), some aldehyde was present in the diethyl ether extracts, and was probably the 'free', as opposed to the combined, aldehyde reported previously (Plack *et al.* 1959). This 'free' aldehyde is possibly an artifact produced under certain storage conditions. The adders were kept for some days at 0° before the ovaries were removed and frozen, and the herring, obtained from commercial sources, probably had the same treatment.

The combined aldehyde, left behind in the solid residue after diethyl ether extraction, was mostly broken down by the extraction with light petroleum in the presence of ethanol and water, so that the light-petroleum extract contained largely 'free' aldehyde and a small amount of combined aldehyde, from which 'free' aldehyde was later liberated by chromatography through water-weakened alumina.

The second extraction method involves breaking the small fish eggs in a Potter–Elvehjem homogenizer and extracting with light petroleum in the presence of ethanol. By this means all the aldehyde and the lipids are extracted together. With this second method the total vitamin A was divided into four parts, vitamin A₁ and A₂ esters and alcohols, whereas, after the two-stage extraction, vitamin A₁ and A₂ esters and alcohols were determined separately in the diethyl ether and light-petroleum extracts, so that eight separate values had to be added to give the total for esters and alcohol.

We have previously reported (Plack *et al.* 1959) that although chloroform–methanol mixtures extract more lipid from herring eggs, the yield of aldehyde is similar to that obtained by method B.

From the experience gained in the course of this study, we would suggest that for the extraction of eggs the two-stage method A should be used, but the diethyl ether extract should be tested for aldehyde, after a mild saponification, in addition to the light-petroleum extract, and portions of the two extracts should be combined, saponified and chromatographed for the determination together of the ester and alcohol forms of vitamin A.

Determination of vitamin A₁ and A₂ aldehydes

The behaviour of vitamin A₂ aldehyde in the Carr–Price test has been described by Plack (1961), and the method described here is based on his findings. Tests of the method with solutions containing mixtures of pure vitamin A₁ and A₂ aldehydes have given satisfactory results (Table 3); but at lower concentrations in extracts from natural materials, in which the magnitude of the extinction at 840 m μ may be similar to that of the extinctions at 665 and 740 m μ from which it is subtracted, the determination of the two forms of aldehyde is likely to be less precise.

A similar method for the determination of vitamin A₁ and A₂ aldehydes in frog's eyes has been described by Wilt (1959), but under his conditions vitamin A₂ aldehyde showed a maximum extinction at 705 m μ in the Carr–Price test. The equations used in the two methods are therefore not comparable.

Chloroform solutions of crystalline vitamin A₁ and A₂ aldehydes are stable for many weeks when kept in amber flasks in a dark cupboard at room temperature, and samples of an extract of pike's eggs, stored in hexane at 4° in the dark, gave similar values for the concentration of total vitamin A aldehyde over a period of 5 days, the lowest value being 97% of the highest (P. A. Plack, unpublished observations). It seems likely, therefore, that little loss of aldehyde would have occurred during the one or two days between extraction and determination.

Determination of vitamin A₁ and A₂ esters and alcohols

Many workers (e.g. Cama, Collins & Morton, 1951) have used the Carr-Price test to determine vitamin A₁ and A₂ alcohols in mixtures, and the method is described here merely to indicate the conditions and the factors used.

Schwietzer (1960) has recently crystallized vitamin A₂ alcohol and a sample of the all-*trans* form was given to us by F. Hoffmann-La Roche and Co. Ltd. In the Carr-Price test, $\lambda_{\max}$ was 693 m μ , in agreement with the findings of Farrar *et al.* (1952) and Cama & Morton (1953), but $E_{1\%}^{1\text{cm}}$ (693 m μ) was 3520, lower than the values of 4000 and 3870 reported by these authors. The difference is no doubt due in part to the different conditions under which the test was carried out. Cama & Morton (1953) gave a value of 0.409 for the ratio $E_{690\text{m}\mu}/E_{693\text{m}\mu}$, and the value used by us (0.42) is similar. Values for this ratio tended to vary slightly, but variations in the range 0.40–0.42 had no appreciable effect on the results calculated in terms of total vitamin A alcohol and percentage of vitamin A₂ alcohol.

The results for the total vitamin A esters and alcohol content of eggs are based on four or eight individual readings, as mentioned above. In general the concentration of total vitamin A esters and alcohol was low, so that these individual readings were low, and frequently below E 0.060, when the reaction mixture had no visible blue colour. At these low extinctions, errors due to extraneous absorption may be high and such errors from the four or eight separate readings are additive. Extinctions below 0.020, corresponding to about 0.03 $\mu\text{g./g.}$ wet wt., have been ignored. In addition, of the vitamin A compounds studied in this work, vitamin A₂ alcohol is the least stable. For these various reasons, the values given for concentration (or total amount) of vitamin A esters + alcohol and for the percentage of vitamin A₂ in these esters and alcohols are not as accurate as similar values for the aldehydes.

Vitamin A aldehyde in eggs

The values reported here for the three forms of vitamin A₁ and A₂ and for the carotenoids should be regarded as giving only the order of magnitude of the concentrations for the species as a whole, since in many instances only one sample was analysed, and it is well known that the vitamin A content of hen's eggs varies with the vitamin A content of the hen's diet. P. A. Plack (unpublished observations) has likewise found this variation in the vitamin A aldehyde content of hen's eggs. Presumably such a relationship between vitamin A content of the diet and vitamin A content of the

egg applies to the other species, and it may explain why our first range of values for the vitamin A aldehyde content of herring ovaries (Plack *et al.* 1959; values on a wet weight basis) is higher than that found by subsequent investigators (Braekkan, Myklestad & Njaa, 1960) and higher than the value reported here.

Examined by our methods, eggs or ovaries of the five invertebrates studied contained no vitamin A, whereas those of the cephalochordate *Amphioxus lanceolatus* and ripe eggs or ovaries of all the vertebrates studied contained vitamin A aldehyde, esters and alcohol. In the two instances when unripe eggs were tested, inactive ovaries of the pike and spent ovaries of the John Dory, no aldehyde was found, thus confirming the previous finding with cod that eggs in an early stage of development do not contain vitamin A aldehyde, which appears to be laid down with the yolk (Plack *et al.* 1961). Of the vertebrates, we have analysed the eggs or ovaries from fish, an amphibian, reptiles and a bird, but not so far from the monotremes, or egg-laying mammals (e.g. duck-billed platypus and spiny anteater), or from other mammals, though collection of eggs from the monotremes is in progress.

Vitamin A has not so far been shown to be a growth factor for invertebrates, although the visual pigments of some of them do contain vitamin A aldehyde (see Fisher & Kon, 1959). In mammals, vitamin A not only takes part in the visual cycle but it is also an essential nutrient. Females deficient in vitamin A may give rise to abnormal offspring, and Hubbs & Stavenhagen (1958) have shown the same to be true of a fish, *Etheostoma lepidum* (Baird Girard). It might therefore be expected that megalecithal eggs, on which the embryos depend for some time for their food supply, should contain vitamin A, but it is not known why it should be partly in the form of vitamin A aldehyde. Winterstein & Hegedüs (1960) have recently demonstrated the presence of vitamin A aldehyde in the livers of several mammals and in some plants, and this form of vitamin A may well be of more widespread occurrence and of definite physiological importance, particularly in embryonic development.

SUMMARY

1. A method is described for the determination of vitamin A₁ and A₂ aldehydes in solutions containing mixtures of the two.

2. The eggs or ovaries of five invertebrates did not contain vitamin A aldehyde, but ovaries of a cephalochordate and ripe eggs or ovaries of two species of lamprey, four of marine elasmobranch fish, six of marine teleost fish, five of freshwater teleost, one anadromous teleost, an amphibian, five species of reptile and a bird all contained vitamin A

aldehyde. Vitamin A₁ aldehyde was the predominant form in all except the freshwater perch, pike, and trout and the anadromous salmon, which contained predominantly vitamin A₂ aldehyde.

3. Values are given for the content of total vitamin A₁ and A₂ esters and alcohols, the percentage of the A₂ form and the carotenoid content of the eggs or ovaries.

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The Assay of Insulin Solutions by Paper Chromatography

By E. L. FENTON

The Wellcome Research Laboratories (Biological Division), Langley Court, Beckenham, Kent

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The quantitative determination of insulin by paper chromatography has been the subject of numerous publications in recent years. Using the system butanol-acetic acid-water, in varying proportions, Light & Simpson (1956*a, b*) and Grodsky & Tarver (1956) separated insulin from crude pancreatic and liver proteins, Robinson & Fehr (1952) developed a method for the estimation of insulin and protamine in protamine zinc-insulin preparations, and Bouman & Homan (1958) determined insulin potency by radial chromatography. Fenton (1959) described a method for the chromatographic assay of crystalline insulin by using the solvent system butan-2-ol-1% acetic acid (1:1, v/v). This method has since been used in these and other Laboratories, and the procedure has been extended to the estimation of insulin in solutions of composition similar to insulin injection, B.P., for which the present method describes a four-point slope ratio assay. For reasons given in the Discussion it is unlikely that these methods *in vitro* will ever be used in place of the orthodox biological assays of insulin, but attention has once again been drawn

(Rückert & Schöne, 1960) to their value for production control and experimental purposes. These authors reported a simplified chromatographic procedure by which they obtained results in close agreement with those of biological assay, but they were unable quantitatively to assay residual insulin in solutions during thermal-decay tests.

EXPERIMENTAL

Materials and methods

Protein precipitant. This was a saturated aqueous solution of picric acid.

Acidified acetone reagent. Prepared by diluting 2 ml. of conc. HCl to 1 l. with acetone B.P.C. (Thos. Morson and Son Ltd.).

Developing solvent, eluent, volumetric vessels, and protein stain. These were used as described by Fenton (1959), but the following procedure must be adopted in preparing the protein stain. Bromocresol green (0.425 g.) (Hopkin and Williams Ltd.) was dissolved in 125 ml. of 0.1 N-NaOH by sprinkling the dye on the surface of the alkali and stirring. The solution was then added to 2 l. of 0.1 N-acetic acid and the mixture filtered. The reaction should be pH 3.6; the