logical properties of peptides derived from casein reflected more specific differences in chemical constitution.

SUMMARY

1. Columns of Sephadex G-25 have been used for the preliminary fractionation of tryptic digests of casein.

2. Fractions from Sephadex columns have been examined for their ability to stimulate the growth of a strain of *Streptococcus equisimilis*. Maximum stimulatory activity appeared to be localized in a fairly narrow region of the total peptide material.

3. Further fractionation of peptides derived from casein has been achieved by subjecting fractions from columns of Sephadex to two-dimensional separations involving the successive use of paper chromatography and high-voltage electrophoresis. Over one hundred spots due to peptides have been detected.

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The Colorimetric Reaction between Vitamin A₂ Aldehyde and Antimony Trichloride

By P. A. PLACK

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

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The occurrence of vitamin A₁ aldehyde in the eggs of some marine teleosts has recently been reported (Plack, Kon & Thompson, 1959). To extend this study to the eggs of freshwater teleosts, a method for the determination of vitamin A₂ aldehyde was required, since preliminary experiments had shown that the A₂ form was present together with vitamin A₁ aldehyde in salmon eggs (Plack, 1958). Cama *et al.* (1952) and Farrar, Hamlet, Henbest & Jones (1952) have prepared crystalline vitamin A₂ aldehyde and, for the colorimetric reaction with antimony trichloride, gave $\lambda_{\max}$ 730–740 m μ fading to 705 m μ , and 730 m μ fading to 705 m μ , respectively. Wald (1938–39) had previously found this $\lambda_{\max}$ to be 705 m μ , and Wilt (1959), with extracts from natural materials, found $\lambda_{\max}$ to be between 700 and 705 m μ . The study of the colorimetric reaction between vitamin A₂ aldehyde and antimony trichloride reported here shows how these differences of $\lambda_{\max}$ may have arisen and, though not exhaustive, provides the basis for a quantitative test.

EXPERIMENTAL

Preparation of vitamin A₁ aldehyde. Vitamin A₁ aldehyde was prepared from the crystalline alcohol (Roche Products Ltd.) as described by Plack *et al.* (1959). For the preparation of vitamin A₂ aldehyde, the A₁ aldehyde was purified by chromatography but not crystallized.

Preparation of vitamin A₂ aldehyde. Vitamin A₁ aldehyde (2.38 g.) was converted into vitamin A₂ aldehyde as described by Henbest, Jones & Owen (1955). The crude preparation in light petroleum (b.p. 40–60°), was adsorbed on a 20 cm. $\times$ 4 cm. column of 300 g. of aluminium oxide (British Drug Houses Ltd., for chromatographic adsorption analysis) weakened by the addition of 30 ml. of water, and the column was developed with 2.5 l. of 2% (v/v) of freshly-redistilled diethyl ether in light petroleum (b.p. 40–60°). A yellow diffuse band was eluted first, followed by a strong orange band, but neither contained vitamin A₂ aldehyde. The main orange band was eluted more slowly and was collected in three fractions. From their absorption curves, fraction 1 contained much vitamin A₁ aldehyde and was rejected, but fractions 2 and 3 contained reasonably pure vitamin A₂ aldehyde and were combined; the solvent was removed under reduced pressure, and the residue dissolved in 30 ml. of light petroleum (b.p. 40–60°). Spontaneous crystallization occurred overnight at –20°, appar-

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ently from one nucleus, and the yield was 0.695 g. Two recrystallizations from light petroleum were carried out, and the last traces of solvent were removed under high vacuum (0.01 mm. Hg), to give a final yield of 0.488 g. The crystals of aldehyde were sealed in 16 evacuated small glass ampoules.

The crystals were monoclinic, with m.p. 66–68° (Kofler apparatus), unchanged by further recrystallization. Both Cama *et al.* (1952) and Farrar *et al.* (1952) reported melting points of 77–78°, although one preparation (Cama *et al.*) had m.p. 61°. With the exception of $\lambda_{\max}$ in chloroform, values for the absorption characteristics of my preparation (Table 1) were in good agreement with those given by Farrar *et al.* (1952) and in fair agreement with the values obtained by Cama *et al.* (1952).

A sample of crystals of m.p. 77–79° was recently received (a gift from F. Hoffmann-La Roche and Co. Ltd., Basle, Switzerland); with the Kofler apparatus its m.p. was found to be 75–77°. A solution in light petroleum of the crystals of m.p. 66–68° was seeded with the higher-melting-point material and gave crystals of m.p. 75–77°. A solution of these last crystals was cooled to –20° and seeded with material of m.p. 66–68°, when two types of crystal formation were produced: a few clusters with a purple tinge, m.p. 75–77°, and a larger quantity of orange-red clusters, m.p. 66–68°.

The vitamin A₂ aldehyde crystals of m.p. 66–68° were used in the experiments reported here, but the quantitative aspects of the reaction were checked with crystals of m.p. 75–77°.

A solution of the aldehyde in hexane was isomerized with I₂ (see Fisher, Kon & Plack, 1957). The extinction was about 5% lower, and $\lambda_{\max}$ about 1 m μ lower, after isomerization, suggesting that the original aldehyde was all-*trans* rather than a *cis*-isomer. Solutions in CHCl₃ of the aldehyde were stable for many weeks when kept in brown-glass containers in a dark cupboard at room temperature.

Antimony trichloride. Two preparations were used. The solution of SbCl₃ in CHCl₃ supplied by British Drug Houses Ltd. for vitamin A tests, containing about 14% (w/w) of SbCl₃, will be referred to as SbCl₃ solution (commercial). Solutions prepared by distilling solid A.R. SbCl₃ (British Drug Houses Ltd.) and collecting the middle fractions in a flask containing a weighed quantity of CHCl₃ (used as supplied by May and Baker Ltd.) will be referred to as purified SbCl₃ solution. Initially, the CHCl₃ was purified by washing with water, to remove ethanol and decompose any COCl₂, and drying over anhydrous Na₂SO₄. Solutions of SbCl₃ in this purified CHCl₃ frequently gave an oily deposit, which disappeared on heating but returned on cooling. Such a deposit was not produced when CHCl₃, as supplied, was used. These solutions prepared in the laboratory were usually diluted to contain 14% (w/w) of SbCl₃, but other concentrations were also tested. Acetic anhy-

dride, A.R., when required, was added to the solutions at the rate of 2% (v/v).

For the reaction with vitamin A₂ aldehyde, 2 ml. of the reagent was rapidly pipetted into a glass cell of 1 cm. light-path containing 0.5 ml. of a solution of the aldehyde in CHCl₃, and the cell was quickly transferred to the spectrophotometer for measurement.

Antimony pentachloride. The diphasic system of Brügge-mann, Kraus & Tiews (1952) was used, and the reagent was prepared as described by them from solid SbCl₃, K₂Cr₂O₇, and conc. HCl. One drop (about 0.05 ml.) was added to 3 ml. of the aldehyde solution in dry, ethanol-free CHCl₃ in a stoppered flask, the flask was shaken gently for 1 min. and the contents poured into a spectrophotometer cell for measurement. Most of the aqueous phase remained in the flask.

Solutions of commercial SbCl₃ (British Drug Houses Ltd.) 15, 1.5 and 0.15% (v/v) in CHCl₃ were also used, 1 drop together with 1 drop of acetic anhydride being added to 2 ml. of the aldehyde solution in CHCl₃.

Glycerol dichlorohydrin. 1:3-Dichloropropan-2-ol, for vitamin A determination, was obtained from Kodak Ltd. and used as supplied, since preliminary tests only were carried out. To 2 ml. of the reagent in a spectrophotometer cell, 0.5 ml. of the aldehyde solution in CHCl₃ was added, the two solutions were mixed with a Polythene rod and the cell was transferred to the instrument.

Spectrophotometers. Most measurements of light-absorption were carried out with an Optica CF4 double-beam recording spectrophotometer, equipped with a red-sensitive DuMont photomultiplier. This instrument was used in one of three ways: (a) to give a plot of percentage transmission against time after addition of reagent for one particular wavelength; (b) to give percentage transmission against wavelength; (c), a combination of (a) and (b), with the chart running continuously to give a time scale, and the region of maximum absorption scanned in rapid succession, so that changes of $\lambda_{\max}$ with time could be followed. Readings in terms of percentage transmissions were converted into extinction values, and in some later experiments extinction values were plotted directly.

After $\lambda_{\max}$ had been determined with the recording spectrophotometer, more exact readings of extinction were obtained with fresh samples in a Beckman model DU quartz spectrophotometer, or in the manual version of the Optica CF4 spectrophotometer.

RESULTS

Reaction with antimony trichloride

Commercial solutions and solutions of purified antimony trichloride. When antimony trichloride

Table 1. Absorption characteristics of the preparation of vitamin A₂ aldehyde (m.p. 66–68°) compared with those of preparations obtained by other workers

Solvent	Plack, this paper		Cama <i>et al.</i> (1952)		Farrar <i>et al.</i> (1952)	
	$\lambda_{\max}$ (m μ)	$E_{1\text{cm.}}^{1\%}$	$\lambda_{\max}$ (m μ)	$E_{1\text{cm.}}^{1\%}$	$\lambda_{\max}$ (m μ)	$E_{1\text{cm.}}^{1\%}$ *
Chloroform	410	1280	407	1360	407	1295
Ethanol	400	1370	397	1410	400	1350
Hexane	387	1440	385†	1490†	388‡	1460‡

* Calculated from values for ϵ .

† In light petroleum (b.p. 40–60°).

‡ In pentane.

Table 2. Colorimetric reaction between chloroform solutions of vitamin A₂ aldehyde and purified antimony trichloride (14%): variations in $\lambda_{\max}$ with time after mixing and with concentration of aldehyde in the presence or the absence of acetic anhydride

Vitamin A ₂ aldehyde concentration in reaction mixture (μg./ml.)																				
2.83			2.27			1.70			1.13			0.566			0.283			0.141		
Time (min. sec.)	λ _{max} (mμ)	Time (min. sec.)	λ _{max} (mμ)	Time (min. sec.)	λ _{max} (mμ)	Time (min. sec.)	λ _{max} (mμ)	Time (min. sec.)	λ _{max} (mμ)	Time (min. sec.)	λ _{max} (mμ)	Time (min. sec.)	λ _{max} (mμ)	Time (min. sec.)	λ _{max} (mμ)	Time (min. sec.)	λ _{max} (mμ)	Time (min. sec.)	λ _{max} (mμ)	
With acetic anhydride																				
0 47	732	0 48	732	0 47	730	0 47	728	0 49	724	0 46	708	0 49	724	0 46	708	0 49	724	0 46	708	
1 33	731	1 35	730	1 34	727	1 28	722	1 28	710	1 43	704	1 28	710	1 43	704	1 28	710	1 43	704	
2 16	730	2 24	731	2 20	724	2 10	715	2 21	705	2 27	702	2 21	705	2 27	702	2 21	705	2 27	702	
3 3	728	3 13	728	2 55	719	2 55	711	3 8	704	3 4	702	3 8	704	3 4	702	3 8	704	3 4	702	
3 35	728	4 0	727	3 39	715	3 41	709	3 43	701	4 0	700	3 43	701	4 0	700	3 43	701	4 0	700	
4 17	725	4 36	726	4 23	713	4 27	707	4 30	701	4 46	700	4 30	701	4 46	700	4 30	701	4 46	700	
5 3	726	—	—	5 19	711	5 4	706	5 17	702	—	—	5 17	702	—	—	5 17	702	—	—	
Without acetic anhydride																				
0 56	742	0 54	742	0 57	741	0 53	740	0 51	741	0 55	740	0 51	741	0 55	740	0 51	741	0 55	740	
1 30	741	1 39	742	1 34	741	2 10	740	1 38	741	1 43	741	1 38	741	1 43	741	1 38	741	1 43	741	
2 5	742	2 14	742	2 11	741	3 0	741	2 24	742	2 27	740	2 24	742	2 27	740	2 24	742	2 27	740	
2 39	742	2 47	741	2 57	741	3 47	741	3 9	740	3 14	740	3 9	740	3 14	740	3 9	740	3 14	740	
3 26	742	3 21	742	3 45	741	4 35	741	3 57	741	3 59	740	3 57	741	3 59	740	3 57	741	3 59	740	
4 0	742	3 56	742	4 24	741	5 20	741	4 43	740	4 43	739	4 43	740	4 43	739	4 43	740	4 43	739	
4 35	741	4 39	741	4 57	741	—	—	—	—	—	—	—	—	—	—	—	—	—	—	

solution (commercial) with acetic anhydride was used as the reagent, solutions of vitamin A₂ aldehyde gave a green-blue colour, the $\lambda_{\max}$ of which varied with the concentration of aldehyde. In one instance, a reaction mixture containing 2.05 $\mu\text{g.}$ of aldehyde/ml. had $\lambda_{\max}$ 733 m μ , and one containing 0.205 $\mu\text{g./ml.}$ had $\lambda_{\max}$ 705 m μ . Similar variations were found with all batches of antimony trichloride solution (commercial), but different batches gave different values of $\lambda_{\max}$ for the same solution of aldehyde. They also required different times to reach $E_{\max}$, the variation being from 30 sec. to 2 min. 30 sec., and gave different rates of fall of $\lambda_{\max}$ with time. The colour reactions of these various batches of antimony trichloride solution (commercial) with vitamin A₁ alcohol were perfectly normal.

Antimony trichloride was therefore purified by distillation, and dissolved in chloroform. A 14% solution in the presence of acetic anhydride gave no improvement in the variations of $\lambda_{\max}$ (Table 2).

Effect of acetic anhydride. With a 14% solution of purified antimony trichloride, the effect of acetic anhydride was studied. In Table 2 are shown the values for $\lambda_{\max}$ for different concentrations of vitamin A₂ aldehyde at different times after the reagent and aldehyde solutions had been mixed. In one experiment, acetic anhydride was added to the reagent, in the other it was omitted. Only when acetic anhydride was absent were the values for $\lambda_{\max}$ constant (741 m μ ; range, in this and other experiments, 739–743 m μ) irrespective of time after mixing and concentration of vitamin A₂ aldehyde. This result was reproducible with all the batches of 14% purified antimony trichloride solution prepared.

The same experiments were done with vitamin A₁ aldehyde, and, both in the presence and absence of acetic anhydride, $\lambda_{\max}$ was constant with time and with concentration of aldehyde. The two values were slightly different, however, being 661 m μ (range 659–663 m μ) with added acetic anhydride and 666 m μ (range 665–667 m μ) in its absence.

A commercial solution of antimony trichloride, with no added acetic anhydride, still gave variations in $\lambda_{\max}$ of from 738 to 730 m μ with final concentrations of vitamin A₂ aldehyde of from 1.98 to 0.198 $\mu\text{g./ml.}$ Purification of the reagent and omission of acetic anhydride were therefore both necessary for stability of $\lambda_{\max}$.

Acetic anhydride had no effect on the ultra-violet curve of vitamin A₁ aldehyde or A₂ aldehyde.

Effect of concentration of antimony trichloride. Table 3 shows the effect of different concentrations of purified antimony trichloride with a single concentration of vitamin A₂ aldehyde in the absence of acetic anhydride. Values for $E_{1\text{cm}}^{1\%}$ were nearly constant over the range 10–16% of antimony tri-

Table 3. *Effect of concentration of purified antimony trichloride on the colorimetric reaction with vitamin A₂ aldehyde*

The reaction mixture contained 0.5 ml. of a solution of vitamin A₂ aldehyde in CHCl₃ (7.16 µg./ml.) and 2 ml. of a solution of purified SbCl₃ in CHCl₃, to give a final concentration of 1.43 µg. of aldehyde/ml. $E_{\max}$ (741 mµ) was measured with the manual Optica spectrophotometer and $E_{1\text{cm}}^{1\%}$ calculated from the mean of three measurements; the time factor and $\lambda_{\max}$ were determined with the Optica recording spectrophotometer.

Concn. of SbCl ₃ solution added (% w/w)	$E_{1\text{cm}}^{1\%}$ (741 mµ)	Time after mixing when $E_{\max}$ steady (sec.)	$\lambda_{\max}$ (mµ)
5	3500	20-25	738
10	4330	20-35	742
12	4400	20-35	742
14	4400	30-45	742
16	4300	40-75	743
20	4030	50-90	743
25	3770	20-60	745
30	3840	20-45	744

Fig. 1. Curves drawn by the recording spectrophotometer for the green-blue colour produced by the reaction of 0.5 ml. of a solution of vitamin A₂ aldehyde in chloroform (7.16 µg./ml.) and 2 ml. of a 14% solution of purified antimony trichloride in chloroform, showing the fading of the colour with time. The peak of curve (a) was traced 45 sec. after the reaction had begun, (b) 2 min. 7 sec., (c) 6 min. 22 sec., (d) 10 min. 35 sec. and (e) 14 min. 38 sec. Speed of scanning 8 mµ/sec.

chloride, but at concentrations above and below they were lower. Since the absorption maximum was broad, values for $E_{1\text{cm}}^{1\%}$ at $\lambda_{\max}$ would not be significantly different from those given at 741 mµ. $\lambda_{\max}$ tended to increase with concentration of antimony trichloride, but was reasonably constant over the range 10-20%. The period of stability of $E_{\max}$ varied with concentration of trichloride, but the maximum stability, at concentrations of 20 and 25% (approx. 40 sec., Table 3), was associated with values of $E_{1\text{cm}}^{1\%}$ below the maximum. The stability of $E_{\max}$ varied slightly with different preparations, and it is probably also dependent on the rate of mixing the reagent and the test solution, and on temperature.

At 20°, a saturated chloroform solution of antimony trichloride contains about 20% (w/w), so that the 25 and 30% solutions were supersaturated. At lower temperatures, the commercial solutions of about 14% sometimes crystallize out. It seemed sensible from the result of this experiment to use the same concentration of antimony trichloride used for other purposes, namely 14% (w/w).

Quantitative aspects of the reaction. With a 14% solution of purified antimony trichloride and no acetic anhydride, the maximum value of the extinction at 741 mµ increased linearly with concentration of vitamin A₂ aldehyde up to 10 µg./ml. (final concentration of aldehyde in reaction mixture, 2 µg./ml.). A mean value for $E_{1\text{cm}}^{1\%}$ (741 mµ) of 4200 (range 4000-4400) was obtained with five preparations of antimony trichloride solution and solutions of vitamin A₂ aldehyde prepared from four separate weighings. With these same solutions, $\lambda_{\max}$ varied over the range 739-743 mµ. Solutions of the crystals of m.p. 75-77° gave values within these ranges.

Concentrated solutions of the aldehyde, m.p. 66-68°, gave an initial fleeting red colour in this reaction, which immediately changed to green-blue.

Fig. 1 shows a series of curves drawn by the recording spectrophotometer at various times after mixing chloroform solutions of vitamin A₂ aldehyde and purified antimony trichloride. They are not true absorption curves, since during the time of scanning one curve (35 sec.) the extinction diminished slightly. With the high speed of scanning used, there is also a slight displacement of $\lambda_{\max}$ to longer wavelengths. As the extinction at $\lambda_{\max}$ diminished, the shoulder at about 700 mµ became more pronounced.

Reaction with antimony pentachloride

Preliminary experiments with antimony pentachloride in a diphasic system showed that the $\lambda_{\max}$ of the green-blue colour varied from 705 to 724 mµ with increasing concentrations of vitamin A₂ aldehyde. The rate of fading was rapid and the specific

extinction coefficient not so great as with antimony trichloride. Antimony pentachloride in chloroform gave no colour reaction, though with the 15 and 1.5% (v/v) solutions the yellow colour of the aldehyde disappeared.

Reaction with glycerol dichlorohydrin

Vitamin A₂ aldehyde gave a blue-green colour with glycerol dichlorohydrin, rising to a maximum intensity about 2 min. after mixing, and starting to fade a few seconds later. With final concentrations of aldehyde in the reaction mixture of 0.27–4.33 µg./ml., $\lambda_{\max}$ of the coloured product was constant at 618 mµ. Over this range of concentrations, the colour intensity was proportional to the concentration of aldehyde, and the mean $E_{1\text{cm}}^{1\%}$ (618 mµ) was 1340. Vitamin A₁ aldehyde with glycerol dichlorohydrin gave a coloured product with $\lambda_{\max}$ 653 mµ, with a shoulder at 610 mµ.

DISCUSSION

Vitamin A and its related compounds are usually determined either by measuring their absorption of light in the ultraviolet or by a colorimetric reaction, of which the Carr–Price test (Carr & Price, 1926) with antimony trichloride is the most widely used. Each method has certain advantages, but in this Laboratory we prefer the colorimetric technique, since it is more sensitive and less subject to interference when small quantities of vitamin A in extracts from natural materials are to be measured. For the determination of vitamin A₂ aldehyde, there is another reason for choosing a colorimetric method. The aim was to devise a method for the determination of vitamin A₁ and A₂ aldehydes in the presence of each other (see Plack & Kon, 1961); in the ultraviolet, the wavelengths of their respective absorption maxima vary according to the solvent used, but are separated by only about 20 mµ, whereas in the colour reaction with antimony trichloride the difference between their maxima is much greater, 75 mµ in the absence of acetic anhydride, so that the detection of the two aldehydes in mixtures is easier and the determination of each more accurate with this test.

The Carr–Price test with antimony trichloride is applied in slightly different ways in different laboratories; the amount of acetic anhydride used varies, and so does the proportion of reagent to sample; the instrument available for measuring the colour intensity may use a small or broad band width and may determine whether the maximum intensity can be read or whether a reading at some fixed time after mixing reagent and sample must be used. Extinction coefficients must therefore be determined for the conditions used, and for the purpose of defining them for this Laboratory a

sample of crystalline vitamin A₂ aldehyde was prepared. Henbest *et al.* (1955) were unable to obtain crystalline material by their method, but I was fortunate enough to obtain spontaneous crystallization which gave a product of m.p. 66–68°, considerably different from the two values previously reported, 77–78° (Farrar *et al.* 1952; Cama *et al.* 1952) and 61° (Cama *et al.* 1952), and yet from its absorption characteristics apparently pure all-*trans*-vitamin A₂ aldehyde. Vitamin A₁ aldehyde is known to exist in dimorphic forms, with m.p. 57 and 65° (Robeson, Blum, Dieterle, Cawley & Baxter, 1955) and it may be that all-*trans*-vitamin A₂ aldehyde exists in three different crystalline forms.

The reactions of solutions of the crystalline aldehyde with antimony trichloride in the presence of acetic anhydride gave, even with purified materials, green-blue colours whose $\lambda_{\max}$ varied with concentration of aldehyde. This variation (Table 2) possibly accounts for the discrepancies in $\lambda_{\max}$ reported in the literature. Two other colour reactions were tested in an effort to overcome this variation. With antimony pentachloride, in a diphasic system, there was still variation of $\lambda_{\max}$ with concentration of aldehyde; antimony pentachloride in chloroform produced no colour with vitamin A₂ aldehyde, although Murata & Nagashima (1960) have recently used this reagent successfully for determination of vitamin A₁ alcohol. With glycerol dichlorohydrin $\lambda_{\max}$ did not vary with concentration of aldehyde, but vitamin A₁ aldehyde gave a higher $\lambda_{\max}$ than vitamin A₂ aldehyde and, for the latter, the specific extinction coefficient was low compared with that in the Carr–Price test; the reagent therefore seemed less suitable than antimony trichloride, provided that the irregularities with the latter could be overcome.

Further experiments with purified antimony trichloride showed that omission of acetic anhydride from the reaction mixture led to values of $\lambda_{\max}$ that were constant over a range of concentrations of vitamin A₂ aldehyde and remained stable for at least 5 min. (Table 2). Acetic anhydride is usually added in the antimony trichloride reaction to remove any traces of water and avoid clouding, but Carr & Price (1926) in their original communication do not mention its use, and in the reaction reported here it is essential to omit it. The omission did not lead to any cloudiness in the solutions.

Conditions have thus been found for a quantitative colorimetric test for vitamin A₂ aldehyde with antimony trichloride. The wavelength of maximum absorption is not affected by changes in aldehyde concentration, the colour intensity is stable for a short time, and varies linearly with the concentration of vitamin A₂ aldehyde. With the range of values for $E_{1\text{cm}}^{1\%}$ (4000–4400, mean 4200)

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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