Effect of high doses of vitamin A palmitate on vitamin A aldehyde, esters and alcohol and carotenoid contents of hen's eggs

By P. A. PLACK

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

(*Received* 27 *August* 1962—*Revised* 10 *October* 1962)

The occurrence of vitamin A aldehyde in hen's eggs has recently been reported (Plack, 1960; Plack & Kon, 1961). Winterstein & Hegedüs (1960) were able to confirm this finding, but reported an amount per egg about one-third that given by Plack (1960). We have noted variations also in the vitamin A aldehyde content of fish eggs (Plack, Kon & Thompson, 1959; Plack & Kon, 1961; Plack, Woodhead & Woodhead, 1961) and wished to know whether such variations might be due to differences in diet.

A preliminary experiment with hens had shown that doses of 1·5 mg vitamin A/day gave an increase of 12–30% in the vitamin A aldehyde content of eggs. To obtain a greater effect, the doses used in the work now described were higher. Other workers (Cruickshank & Moore, 1937; Deuel, Hrubetz, Mattson, Morehouse & Richardson, 1943) also found that large supplements of vitamin A were needed to give any marked increase in vitamin A esters and alcohol contents of eggs, but Neff, Parrish, Hughes & Payne (1949) produced an effect with smaller doses after the hens had been given a suboptimal level of vitamin A.

With doses of 10 or 20 mg vitamin A/day, the amounts of vitamin A aldehyde, esters and alcohol in hen's eggs were significantly increased. The aldehyde form, together with esters and alcohol, was also found in the hens' livers.

EXPERIMENTAL

Animals. Six Light Sussex hens were used, all laying regularly though at the end of their first year. Their live weights varied considerably (see Table 1), but the variation was not reflected in the weights of the eggs. The birds were housed in single battery cages and given *ad lib.* a breeder's mash containing maize and dried grass and 2·5 mg added vitamin A/kg, so that a bird consuming 100 g diet/day would receive 250 μg vitamin A/day together with further amounts from the provitamin A carotenoids. Twice weekly the birds were inseminated with semen from Rhode Island Red cockerels. Eggs were collected daily and stored at 4° for up to 2 days before analysis.

Dosing. For 15 days the hens were not dosed with vitamin A. For the next 14 days each bird received the equivalent of 10 mg vitamin A alcohol/day by mouth and

* Grant-aided by the Development Fund.

for the following 14 days the equivalent of 20 mg/day. The vitamin A was given as vitamin A palmitate (Roche Products Ltd) since at normal temperatures this compound is an oil and can be freely mixed with the oil used as diluent. The molecular weights of vitamin A palmitate and vitamin A alcohol are as 1·84: 1, and the quantity of vitamin A palmitate used was 1·84 times the dose of vitamin A alcohol required. Solutions were prepared by weighing the vitamin A palmitate and making to the appropriate volume with hydrogenated arachis oil containing 0·001 % hydroquinone, so that the dose of 10 or 20 mg vitamin A was contained in 1 ml oil. The concentrations were checked by the Carr–Price test; the solutions were found to keep satisfactorily for a week in the dark at room temperature. The dose was administered into the back of the throat by means of a 1 ml syringe fitted with a short length of Polythene tubing.

Liver samples. Thirty days after dosing was ended, the birds were killed and bled, and their livers and gall-bladders were removed.

Chemicals. A.R. grade chemicals were used as supplied, except that diethyl ether and antimony trichloride were purified in the way described by Plack & Kon (1961) and Plack (1961) respectively. The latter reagent was dissolved in chloroform to give a 14 % (w/w) solution and used for determining the vitamin A aldehyde. For the Carr–Price test with vitamin A esters and alcohol a solution of antimony trichloride in chloroform, for vitamin A tests (British Drug Houses Ltd), was used.

Extraction of vitamin A. Eggs were weighed and broken open, and their yolks were separated, weighed and freeze-dried in a centrifugal freeze-drier of the type described by Record & Taylor (1958). The samples were left overnight in the freeze-drier under greatly reduced pressure. Next morning the weights of dried yolk were determined, and the dry material was ground in a mortar, transferred to a Soxhlet thimble and extracted with 150 ml diethyl ether for 4 h. This extraction removed most of the fat, pigments and vitamin A esters and alcohol, but only a negligible amount of vitamin A aldehyde (see Plack, 1960). Most of the diethyl ether was removed from the extract on a steam-bath and the final portion under reduced pressure; the residue was dissolved in n-hexane and the volume made to 40 ml with the same solvent.

The solid residue from the Soxhlet thimble was transferred to a blender (Atomix, Measuring and Scientific Equipment Ltd) and blended with 200 ml light petroleum (b.p. 40–60°) in an atmosphere of nitrogen, and 60 ml ethanol and 30 ml water were added. Blending was continued for 2 min, and the light-petroleum layer was separated. The aqueous residue was extracted twice more with 200 ml lots of light petroleum, and the three extracts were combined. Any fine solid matter was allowed to settle and the supernatant extract was evaporated to dryness in a clean flask on the steam-bath, the last portions of solvent being removed under reduced pressure. The residue was weighed and dissolved in n-hexane, and the volume was made to 20 ml with the same solvent.

In a separate flask, 10 ml of the diethyl ether extract were combined with 5 ml of the light-petroleum extract, that is, one-quarter of each.

Livers, in portions of 20 g, were extracted with light petroleum in the same way, the residues from the extracts were weighed and dissolved in n-hexane, and the

volume was made to 100 ml. With the liver from hen C, which contained a high percentage of lipid (see Table 1), the extract was made to 400 ml.

Determination of vitamin A. The analyses were restricted to the aldehyde, ester and alcohol forms of vitamin A_1, since preliminary experiments had shown that negligible amounts of vitamin A_2 were present. A portion of the liver extract, equivalent to 1 g lipid, was subjected to a mild saponification at room temperature overnight and extraction of the non-saponifiable residue (see Plack & Kon, 1961). The whole of this saponified liver extract, or a 5 ml portion or a suitable smaller volume of the light-petroleum extract from eggs, was chromatographed by the method of Plack *et al.* (1959), as modified by Plack & Kon (1961), to separate the vitamin A aldehyde. The solvent was removed from the aldehyde fractions, the residue was weighed, and a suitable volume of chloroform was added. Allowance was made for the volume of the lipid residue, which was calculated from its weight and an approximate density of 0·9 g/ml. The Carr–Price test was then carried out: to 0·5 ml of the chloroform solution 2 ml of antimony trichloride in chloroform were added, and the maximum extinction of the blue-green colour produced was measured in a Beckman model DU spectrophotometer. A value of $E^{1\%}_{1\,cm}$ (666 mμ) = 4150 (Plack & Kon, 1961) was used for calculating the concentration of vitamin A_1 aldehyde.

For the determination of vitamin A_1 esters and alcohol, a 4 ml portion of the combined extract from eggs or a suitable smaller volume or 1 ml of the liver extract was used. Esters and alcohol were separated by the chromatographic method of Thompson, Ganguly & Kon (1949) as modified by Kon, McGillivray & Thompson (1955). The ester fractions were saponified and extracted, as described by Plack (1956), except that 60% (w/w) aqueous KOH solution was used, and were subjected to further chromatography by the method of Thompson *et al.* (1949). Solvent from the ester fractions (now in the form of vitamin A alcohol) and from the original vitamin A alcohol fractions was removed as described on p. 236, and appropriate volumes of chloroform were added to the residues. To 0·5 ml of the chloroform solution, one drop of acetic anhydride and 2 ml of the commercial solution of antimony trichloride in chloroform were added. The extinction of the blue colour at 620 mμ was read on the instrument described by Thompson (1949), and a value of $E^{1\%}_{1\,cm}$ (620 mμ) = 5000 for vitamin A alcohol was used for calculating the results. The vitamin A alcohol fractions contained carotenoid pigments, and the maximum readings obtained in the Carr–Price test were corrected, as described by Thompson (1949), before calculation of the results. Values for vitamin A esters have been expressed as equivalent weights of vitamin A alcohol.

Determination of carotenoids. A sample of 1 ml of the combined extract from eggs was added to 4 ml n-hexane; the solution was centrifuged briefly to clear it, and read at 450 mμ on the Beckman spectrophotometer. The content of carotenoids was calculated by means of the factor $E^{1\%}_{1\,cm}$ (450 mμ) = 2500.

Calculation of effect of dosing. Values for vitamin A and carotenoids in each egg analysed during the three periods when the hens were not dosed with vitamin A and when they received 10 or 20 mg/day have been expressed as percentages of the mean values for eggs from the same hen laid during the period when it was not dosed with

vitamin A. Mean values of these percentages were taken for the eggs analysed that were collected on the same day from the six hens. Usually these mean values were for four eggs, but occasionally a value for a single egg has been used, in which instance no range is given in Fig. 1.

RESULTS

All the hens appeared healthy throughout the experiment, although they tended to scour. Hen B began to moult while it was receiving 10 mg vitamin A/day, but the moulting was probably unrelated to the treatment. The number of eggs laid during each period is shown in Table 1; most of the hens laid slightly fewer eggs during the period of dosing with 20 mg vitamin A/day. Analysis of the livers 1 month after dosing had been stopped showed that hen C had a particularly fatty liver, containing over 40 % of lipid, and the content of lipid in the liver of hen B was also high (Table 1).

Table 1. *Numbers of eggs laid, live weight of bird, liver weight and percentage of lipid in liver of the six hens*

	No. of eggs laid during					
Hen	15 days of period without dosing	14 days of dosing with vitamin A (10 mg/day)	14 days of dosing with vitamin A (20 mg/day)	Live weight at end of experiment (kg)	Wet weight of liver (g)	Percentage of lipid in liver
A	13	13	12	3·80	63·9	7·4
B	8	7*	0†	3·50	85·6	17·4
C	10	6	8	3·50	107·9	41·6
D	11	11	9	2·95	37·9	9·3
E	11	8	3	1·97	52·6	9·1
F	11	11	8	2·40	40·9	6·0

* Hen B began moulting before the end of this period.
† Hen B moulting. Dosing stopped after 6 days of this period.

Table 2. *Mean values for weight of egg and of yolk and for amounts of vitamin A aldehyde, esters and alcohol and of carotenoids in the eggs from six hens while not dosed with vitamin A*

	Hen A	Hen B	Hen C	Hen D	Hen E	Hen F	Hens A–F
No. of eggs analysed	7	7	7	6	6	8	41
Weight of egg (g)	61·6	69·2	69·4	64·4	61·7	61·6	64·7
Weight of yolk (g)	20·6	23·7	22·9	18·8	22·2	19·4	21·2
Vitamin A aldehyde (μg/egg)	17·4	21·7	25·7	22·5	25·1	19·0	21·7
Vitamin A esters (μg/egg)	22·3	34·0	27·7	25·1	25·7	19·4	25·5
Vitamin A alcohol (μg/egg)	99·1	150	129	102	125	126	122
Carotenoids (μg/egg)	433	666	492	436	465	451	491

In Table 2 are given mean values for the weights of egg and yolks and the contents of vitamin A aldehyde, esters and alcohol and carotenoids for the six hens during the initial period when they were not being dosed with vitamin A. Statistical analysis of these results showed that there were significant differences ($0{\cdot}01 > P > 0{\cdot}001$ for vitamin A aldehyde, $0{\cdot}001 > P$ for the other values) between hens for all the values.

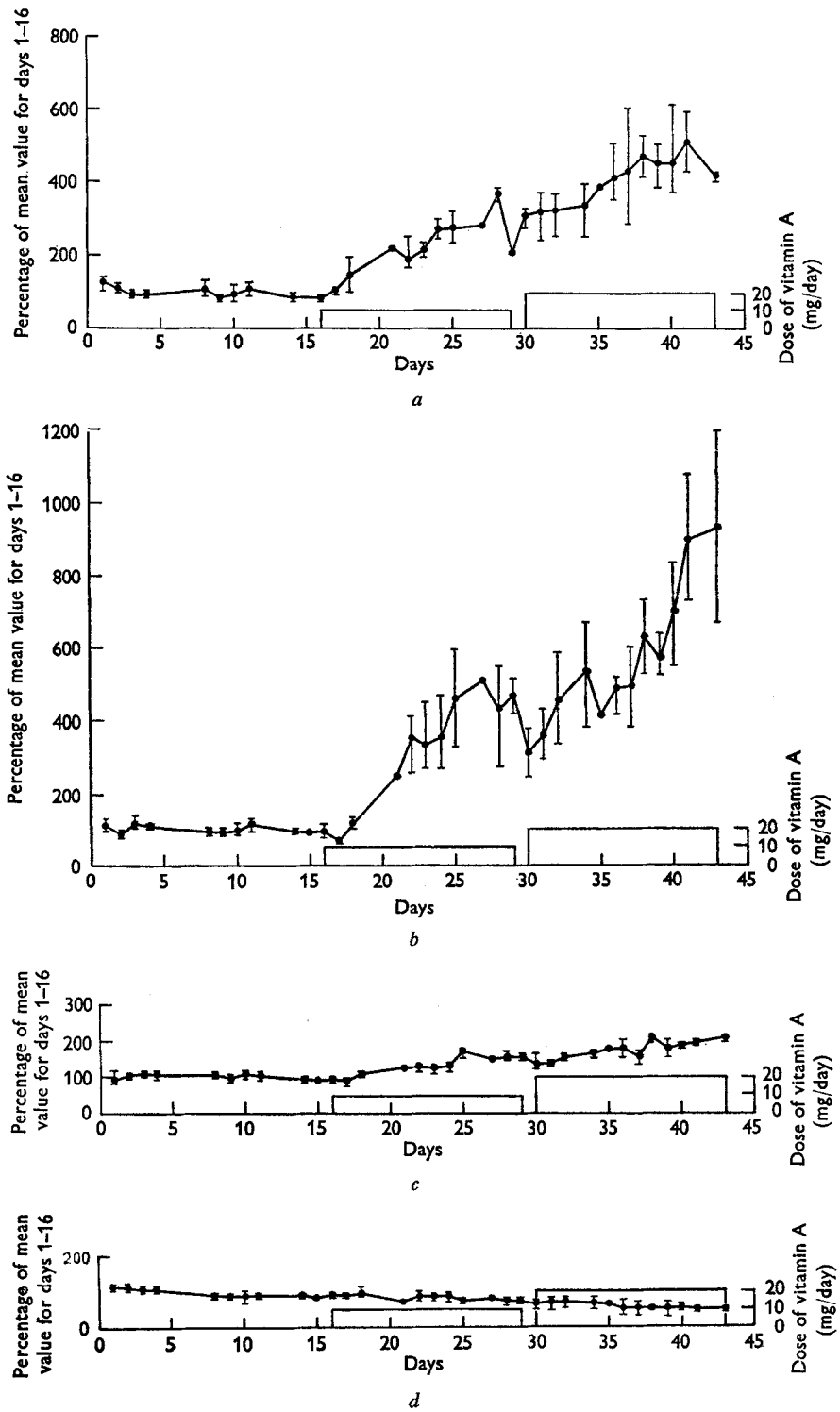

Fig. 1. Mean values and ranges for the vitamin A and carotenoid contents of eggs from hens dosed with vitamin A. The values are expressed as percentages of the mean value for eggs laid while the hens received no dose of vitamin A (see p. 237). *a*, vitamin A aldehyde; *b*, vitamin A esters; *c*, vitamin A alcohol; *d*, carotenoids.

The weight of yolk was used as the concomitant variable in analyses of covariance for vitamin A aldehyde, esters and alcohol and for carotenoids. Within hens the regression was significant only for vitamin A alcohol ($0{\cdot}05 > P > 0{\cdot}01$), and mean values for this substance, adjusted for yolk weight, were still significantly different ($0{\cdot}001 > P$) between hens. The within-hen regression of yolk weight on total weight was calculated, and the regression coefficient was $0{\cdot}219 \pm 0{\cdot}046$ (34 df), $0{\cdot}001 > P$. The values for vitamin A and carotenoids in Table 2 have therefore been given as μg/egg and not as μg/g yolk.

Mean values for forty-one eggs from the six hens are included in Table 2.

Table 3. *Concentrations (μg/g wet weight) of vitamin A aldehyde, esters and alcohol in the livers of four of the hens 1 month after dosing had been stopped*

	Hen C	Hen D	Hen E	Hen F
Vitamin A aldehyde	1·4	2·5	1·3	1·8
Vitamin A esters	526	628	346	336
Vitamin A alcohol	5·7	5·8	8·5	8·2

Since there were significant differences between hens in the amounts of vitamin A and carotenoids in eggs, the effects of dosing have been calculated first for eggs from individual hens and mean values of the percentage changes for eggs from all the hens have then been obtained (see p. 237). These combined results are shown in Fig. 1. The dosing was not continued sufficiently long at either level for steady values to be reached, but when the hens received 10 mg vitamin A/day the amounts of vitamin A aldehyde, esters and alcohol in eggs reached 275, 300–500 and 150 %, respectively, of the mean values for eggs from the undosed hens. These values correspond to increases of about 40, 50–100 and 60 μg in the amounts of vitamin A aldehyde, esters and alcohol per egg. The amount of carotenoids during the same period fell to about 75 % of that in eggs from undosed hens. Further changes in vitamin A and carotenoid contents of eggs took place during the period when the hens received 20 mg vitamin A/day, the values for the amounts of vitamin A aldehyde, esters and alcohol and of carotenoids in eggs reaching about 450, 600–900, 200 and 50 %, respectively, of those in eggs from undosed hens.

The livers of hens C–F were analysed for vitamin A aldehyde, esters and alcohol at the end of the experiment, and the results are given in Table 3. Vitamin A esters comprised 97–99 % of the total vitamin A, vitamin A alcohol 1–2·5 % and vitamin A aldehyde less than 1 %. The amounts of total vitamin A in the livers were 57·5, 24·1, 18·7 and 14·2 mg for hens C, D, E and F, respectively.

DISCUSSION

The vitamin A contents of the livers of hens C–F 1 month after dosing had been stopped (see above) were not noticeably high. Hens and cockerels from our flock, not separately dosed with vitamin A, have liver stores of the order of 20 mg vitamin A, and only hen C, with 57·5 mg, had appreciably more in its liver. Each of these four hens received total doses of 420 mg vitamin A, so that only a small percentage of the dose was

stored in the liver. Some vitamin A may have been lost from the liver in the month after dosing had been stopped, although the hens were receiving an adequate quantity of vitamin A in their diet.

A dose of 10 mg vitamin A/day was sufficient to produce a marked increase in the vitamin A aldehyde contents of eggs such that, for individual hens during the last 8 days of this period, the lowest value for the vitamin A aldehyde content of an egg was above the highest value obtained during the 15 days when the hens were not dosed with vitamin A. When the dose of vitamin A given to a hen was increased to 20 mg/day, the vitamin A aldehyde content of its eggs increased still further. The vitamin A content of the hen's diet therefore affected the vitamin A aldehyde content of its eggs.

Increases in the amounts of vitamin A esters and alcohol, measured separately, in eggs of hens dosed with vitamin A have been noted previously (Neff *et al.* 1949), and attention need only be drawn here to the large percentage increases in the vitamin A ester contents of eggs and the much smaller percentage increases in vitamin A alcohol contents. These changes are no doubt a reflection of the blood pattern. With large doses of vitamin A, whether as ester or alcohol, vitamin A ester is formed in the intestinal wall and circulates in the blood for some time before absorption by the liver. The normal form of blood vitamin A is the alcohol, which is produced in the liver by hydrolysis of vitamin A esters, and its concentration is kept at a steady level by some regulatory mechanism in the liver. With high doses of vitamin A, therefore, the ester form may predominate over the alcohol form in the blood, and with some eggs, laid when the hens were receiving 20 mg vitamin A/day, the ratio of the ester to alcohol forms of vitamin A exceeded unity, and the ratio of vitamin A alcohol to total vitamin A fell below 0·4. Normal values for these two ratios, from the mean values for forty-one eggs given in Table 2, are 0·21 and 0·7.

The ratio of the vitamin A aldehyde content to the vitamin A alcohol content in eggs did not remain constant with the changes in the two components produced by dosing the hens with vitamin A. It is unlikely, therefore, that the vitamin A aldehyde in eggs is produced from the alcohol by a simple enzymic equilibrium, and the finding of the aldehyde form in hen's blood (Plack, unpublished observation) suggests that the aldehyde form in the egg is derived from the hen.

The fall in carotenoid contents of eggs from hens receiving high doses of vitamin A (Fig. 1) has been noted previously by Deuel *et al.* (1943). From the values in Fig. 1, the carotenoid content appeared to be falling before the hens were dosed with vitamin A (days 1–15) so that factors other than high levels of vitamin A may be contributing to the effect.

Winterstein & Hegedüs (1960) have previously reported the occurrence of vitamin A aldehyde in the livers of the pig, cow, horse and rabbit, in quantities of up to 2 μg/g wet weight. Hen's liver has now been found to contain vitamin A aldehyde at concentrations of the same order. The mild saponification procedure, necessary to change the large excess of vitamin A esters, which interfere with the chromatographic separation of the aldehyde, into vitamin A alcohol, results in a loss of some 20% of the aldehyde (Plack & Kon, 1961), so that the values given in Table 3 are only a first approximation.

SUMMARY

1. Six Light Sussex hens were given a breeder's mash containing 250 μg added vitamin A/100 g together with carotenoids derived from dried grass and maize. For the first 15 days they were not dosed with vitamin A, for the next 14 days they received the equivalent of 10 mg vitamin A alcohol/day, given as the palmitate, and for the final 14 days the equivalent of 20 mg vitamin A alcohol/day. Each dose, in 1 ml arachis oil, was given by mouth.

2. Eggs laid by the hens were analysed for amounts present of vitamin A aldehyde, esters and alcohol and carotenoids. The vitamin A aldehyde content of eggs, as well as their contents of vitamin A esters and alcohol, increased significantly when the hens were dosed with vitamin A, whereas the carotenoid content fell.

3. With differences in the three forms of vitamin A in eggs, the ratio of vitamin A aldehyde content to vitamin A alcohol content did not remain constant, so that the two compounds were not in simple enzymic equilibrium.

4. Vitamin A aldehyde was present in hen's liver at a concentration of 1–2·5 μg/g wet weight, representing less than 1 % of the total vitamin A.

I thank Mr C. M. Ward for technical assistance, and Dr M. E. Coates, Mr G. F. Harrison, MPS, and the staff of the henhouse, Nutrition Department, National Institute for Research in Dairying, for their co-operation.

REFERENCES

Cruickshank, E. M. & Moore, T. (1937). *Biochem. J.* **31**, 179.
Deuel, H. J. Jr., Hrubetz, M. C., Mattson, F. H., Morehouse, M. G. & Richardson, A. (1943). *J. Nutr.* **26**, 673.
Kon, S. K., McGillivray, W. A. & Thompson, S. Y. (1955). *Brit. J. Nutr.* **9**, 244.
Neff, A. W., Parrish, D. B., Hughes, J. S. & Payne, L. F. (1949). *Arch. Biochem.* **21**, 315.
Plack, P. A. (1956). *Biochem. J.* **64**, 56.
Plack, P. A. (1960). *Nature, Lond.*, **186**, 234.
Plack, P. A. (1961). *Biochem. J.* **81**, 556.
Plack, P. A. & Kon, S. K. (1961). *Biochem. J.* **81**, 561.
Plack, P. A., Kon, S. K. & Thompson, S. Y. (1959). *Biochem. J.* **71**, 467.
Plack, P. A., Woodhead, A. D. & Woodhead, P. M. J. (1961). *J. mar. biol. Ass. U.K.* **41**, 617.
Record, B. R. & Taylor, R. (1958). *Biochem. J.* **68**, 420.
Thompson, S. Y. (1949). *Brit. J. Nutr.* **3**, 43.
Thompson, S. Y., Ganguly, J. & Kon, S. K. (1949). *Brit. J. Nutr.* **3**, 50.
Winterstein, A. & Hegedüs, B. (1960). *Hoppe-Seyl. Z.* **321**, 97.

Printed in Great Britain