SUMMARY

1. Reaction of an esterified protein hydrolysate with benziminoethyl ether gave a mixture of products containing the oxazolines derived from serine and threonine, and substituted imino ethers derived from amino-acid esters lacking the hydroxyl group in the β -position.

2. Complete separation of the oxazolines from the substituted imino ethers was not attempted because of the close proximity of the various boiling points. Alkaline hydrolysis of the oxazoline fraction, followed by a series of pH changes on the resulting solution gave a mixture of DL-benzoylserine and L-benzoylthreonine free from other amino-acid derivatives. The chemical and stereochemical changes involved are discussed.

3. Serine and threonine were separated from one another on a small scale by partition chromatography on a starch column and on a larger scale, as their benzoyl derivatives, by countercurrent distribution.

4. The yield of L-threonine obtained on a small scale was about 20% of the amount originally present in the protein.

5. L-Serine was isolated from silk sericin by an alternative method which was found not to be of general application.

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The Distribution of Ascorbic Acid in Developing Salmon (*Salmo salar* L.)

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Although attention has been focused upon physical and chemical changes that take place in the yolk and growing tissues of the developing salmon (and trout) egg, there are practically no references to vitamin content of the egg during these changes. The present investigation was limited to assays of ascorbic acid in two early embryonic stages, and one later, larval, stage of development in the salmon, and to its histological distribution. No estimations were made of the amounts of dehydroascorbic acid that might be present.

METHOD

The eggs were fertilized on 1 December 1947, and kept in running water at the following temperatures: 1–15 December 1948, 9–11°, average 9.9°. This covers the period for sample 1 (9 December) and sample 2 (15 December). 1 December–9 March 1948, 7–11°, average 9.0°. This covers the period from fertilization to the date of sample 3.

Fifty eggs or larvae were taken for each sample. The method followed was that worked out by Mapson (1943). Formaldehyde was used, as first recommended by Lugg (1942), to differentiate ascorbic acid from other substances

which might interfere with subsequent titration by 2:6-dichlorophenolindophenol. Mapson (1943) studied the effects of pH, concentration of formaldehyde and time, in order to distinguish titration values for sulphur-containing substances, ascorbic acid and certain non-specific substances (reductones) which occur in dehydrated food. The full method was applied to the present estimations, although it is not usual to find reductones in fresh tissue.

It is difficult to estimate ascorbic acid in different parts of the fertilized egg because of the semi-liquid nature of the yolk. It can be done, in a limited manner, if the yolk is first

RESULTS

With the two earlier samples the titrations showed no error due to the presence of sulphur compounds or reductones, since the values found by the method of Mapson (1943) were the same as those obtained by direct titration of a sample of the metaphosphoric acid extract with the indophenol reagent. The amount of ascorbic acid thus found was small. For

Fig. 1.

Fig. 1. Sagittal section through blastoderm cells, periblast and yolk of salmon egg 8 days after fertilization. Objective $\frac{1}{4}$. Final magnification, $\times 288$. The black dots and masses are presumed to indicate the distribution of ascorbic acid.

Fig. 2.

Fig. 2. Sagittal section of salmon egg 14 days after fertilization, showing position of blackened oil globules. Objective A.A. Final magnification, $\times 9.4$. BC, blastoderm cell; BL, blastoderm; O, oil globules; PB, periblast; R, rifts in yolk; YK, yolk.

hardened by some fixative which also preserves the ascorbic acid. Consequently fifty eggs were taken, 19 days after fertilization, placed in a 4% (w/v) solution of HPO_3 in a closed vessel under N_2 , and kept on ice for several hours. The blastoderm, together with the underlying periblast and a small amount of yolk, was then carefully removed from each egg by means of a razor-blade and treated as one sample. A second sample consisted of the rest of the yolk, yolk-sac and the protoplasmic vitellin membrane which lies between the outer membrane or chorion and the yolk. In order to handle these samples as quickly as possible they were not weighed.

For the histological demonstration of ascorbic acid in the stages examined, eggs and larvae were immersed in a 10% (w/v) solution of AgNO_3 in 10% (w/v) acetic acid in total darkness (Barnett & Bourne, 1942). The AgNO_3 solution penetrates the egg envelope slowly, and the yolk is so resistant that it has to be left in the solution for several hours. The tissue was subsequently fixed in $\text{Na}_2\text{S}_2\text{O}_8$ before bringing to the light and embedding in paraffin wax for section cutting.

the sample of 9 December it was 0.0106 mg./egg of average weight 153.3 mg., i.e. 140 i.u./100 g. For 15 December, it was 0.0103 mg./egg of average weight 157.4 mg., i.e. 131 i.u./100 g.

A different result, however, was obtained from a later sample taken on 9 March, when the larvae had been hatched 50 days and the yolk almost completely absorbed. After keeping the metaphosphoric acid extract in the dark at 20° for 1 hr. it was found that the titration value had fallen from 0.035 to 0.02 ml. of 2:6-dichlorophenolindophenol (strength 0.1 ml. equivalent to 0.04 mg. of ascorbic acid). According to Mapson (1943) such a fall is due to the presence of a substance similar to dihydroxymaleic acid. Further titrations at pH 0.6 gave the same result as that obtained in the blank. That is, no ascorbic acid was present, and the reading 0.02 ml. obtained in the preliminary stage was a measure of sulphur com-

pounds which had reacted to formaldehyde at the particular hydrogen-ion concentration.

The method used, therefore, indicated that in the two earlier samples of fertilized salmon eggs ascorbic acid was the only substance present capable of reducing 2:6-dichlorophenolindophenol. In the advanced larvae of 9 March, however, reduction was brought about by material other than ascorbic acid.

The assumption that some ascorbic acid would be retained in the tissue when eggs were fixed in a 4% solution of metaphosphoric acid, before cutting away the blastoderm, was found to be correct. The metaphosphoric acid extract and the two samples were estimated separately with the following results:

Extract	0.41 mg. ascorbic acid from total wet weight of 7.432 g.
Blastoderm	0.123 mg. ascorbic acid.
Yolk and yolk sac	0.123 mg. ascorbic acid.

There was an equal residue of ascorbic acid in each sample but the yolk was the bulkier sample of the two. The results show that ascorbic acid was in the yolk and the yolk-sac as well as in the blastoderm and periblast, although the actual amounts are not indicated.

Examination of histological material showed that in both the early samples there had been reaction to the silver nitrate solution in the multicellular blastoderm. In prepared sections the majority of the cells contain blackened deposits of silver, either few in number or clustered together; they occur in the cytoplasm. It is not possible to connect them with yolk granules as appears to be the case in the chick (Barnett & Bourne, 1940-1); such granules are less obvious in blastoderm cells of the salmon. There was blackening, also, in the underlying protoplasmic layer of periblast. There were further deposits in the yolk immediate to the periblast where the texture is less homogeneous than in the central yolk (Fig. 1).

DISCUSSION

It is possible that substances in the tissue other than ascorbic acid reacted in a similar way to the silver nitrate solution. There is some agreement, however, between the general distribution of ascorbic acid in the egg as demonstrated by the titration method used, and the distribution of blackened granules of metallic silver in the prepared sections. It is assumed, therefore, that some of the granules, at least, result from ascorbic acid in the tissues.

Since there are no granular depositions in the yolk, other than those adjacent to the blastoderm, it may be assumed that the presence of the granules connected with activities of the blastoderm cells. This part of the yolk would be included in the estimation designated 'blastoderm'. Barnett & Bourne (1940-1)

report a like deposition in the chick, and a similar laying down of material in yolk adjacent to actively dividing cells was found in the glycogen content of developing salmon (Daniel, 1946).

Although it has been demonstrated, by titration, that ascorbic acid is also present in the rest of the yolk-sac, it is difficult to form an opinion from histological preparations about its distribution. There is a general tendency for metallic silver to be precipitated wherever there are rifts or interfaces in the yolk, even in tissue that has been fixed for short periods (Fig. 2). Incidentally, there are similar deposits in sections that were passed through water and ethanol before being immersed in silver nitrate solution. They occur also in the late larval stage of 9 March, although quantitative analysis indicated that no ascorbic acid was present.

It is possible that masses of blackened material which lie in many oil vacuoles of the yolk may be connected with the presence of ascorbic acid, since the latter is not manifest in the surrounding yolk itself. Conditions under which a water-soluble vitamin, such as ascorbic acid, could be allied with oil globules call for further examination. In the meantime, two possibilities are presented:

(1) *Attraction of ascorbic acid to the oil globules.* This suggestion is prompted by an experiment of Barnett & Fisher (1944) with 5% gelatin, drops of olive oil and ascorbic acid to give a concentration of 200 mg./100 ml. The mixture was emulsified and pieces left in acidified silver nitrate for 10 min. Examination under the microscope showed that some of the oil globules had a heavy precipitate of metallic silver attached to the surface. The investigators claimed that this localization is a physico-chemical phenomenon occurring after the precipitation. Their figure, however, shows that the blackening is almost wholly confined to the surface of the oil globules, and is different in kind from that found under somewhat similar conditions in yolk oil globules (Fig. 2).

(2) *Affinity between cholesterol and ascorbic acid.* Recent work on the adrenal gland has suggested an affinity between cholesterol and ascorbic acid (Long, 1947). It has been put forward by Zwemer, Lowenstein & Pines (1940) that the adrenal gland contains water-soluble steroids which are associated with ascorbic acid. Long states that approximately 90% of adrenal cholesterol is present in ester form. According to M. Scott (unpublished), only free cholesterol is present in oil obtained from yolk of developing salmon larvae. Conditions therefore would appear to be even more favourable for a linkage with ascorbic acid.

Finally, there may have been reaction between the silver nitrate solution and the original oil content of the vacuoles; in fact the amount of material involved suggests this to be the case. Smears of highly

unsaturated cod-liver oil, which is free from ascorbic acid, shows an intense black deposit after immersion in acidified silver nitrate in the dark (although not *in situ* in the liver), and to a limited extent this is also true of oil removed from salmon yolk.

In the late larval stage of 9 March, the various organs are almost completely differentiated. Although no ascorbic acid was found by estimation at this stage, there appears to be some deposition of metallic salts in the tissue. In section the issue is confused by the presence of black pigment, but there are certain discrete deposits near the vertebral column and in cells of the yolk-sac.

SUMMARY

1. It was found by the titrimetric method of Mapson (1943) that ascorbic acid was present in early

stages of developing salmon (8 and 14 days after fertilization), but absent in a late larval stage in which most of the yolk had been absorbed. It was contained both in the blastoderm region and in the yolk and yolk-sac.

2. Sections obtained from material fixed in acidified silver nitrate show that ascorbic acid may be present in blastoderm cells, in the underlying periblast and also in adjacent yolk. In this respect the distribution is much the same as in early stages of chick development as recorded by Barnett & Bourne (1940-1).

3. There are intense deposits of metallic silver where oil globules have been situated in the main part of the yolk. These may be due, in part, to ascorbic acid present in the yolk, although there is also evidence in favour of a reaction between the silver nitrate solution and the oil globules.

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Studies in Biochemical Adaptation. Effect of Diet on the Intestinal Phosphatase of the Rat

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Verzár & McDougall (1936) suggested that the absorption of glucose and fructose is accompanied by phosphorylation by the intestinal mucosa. Part of the evidence upon which this theory was based was that iodoacetate decreases the rate of absorption of glucose, and this evidence has been criticized by several later writers (e.g. Klinghoffer, 1938). Again, Verzár supposed that an adrenal cortical hormone was responsible for the phosphorylation, and this has been criticized (Cori & Cori, 1941). Nevertheless, most workers accept the view that phosphorylation of glucose and fructose is an important stage in their absorption.

It has also been suggested that fats are phosphorylated before absorption (e.g. Sinclair, 1929). Verzár & McDougall (1936) claimed that this was controlled

by the adrenal cortex, but, as with their theory of carbohydrate absorption, this view has since been severely criticized. Frazer (1948; see also Frazer, 1946) has suggested that fat may be absorbed as the unchanged triglyceride, although this is not universally accepted (Lundbaek & Maaløe, 1947). Most workers are agreed, however, that at least some phosphorylation of fat occurs during absorption (Schmidt-Nielsen, 1946).

It might be considered that diets with varying proportions of carbohydrate and fat might possibly stimulate the small intestine to produce varying amounts of phosphatase, since it is known that the amounts of some digestive enzymes are affected by variations in the degree to which they are called upon to carry out their work. A study was therefore