Studies in the Nitrogen Metabolism of the Apple Fruit

CHANGES IN THE NITROGEN METABOLISM OF THE APPLE DURING THE NORMAL AND ETHYLENE-INDUCED CLIMACTERIC RISE IN RATE OF RESPIRATION

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During work carried out over a number of years, it has been found repeatedly that, in Bramley's Seedling apples which have been gathered before the onset of the respiration climacteric and placed in storage, there is a slow rise in net protein content during the period when the respiration climacteric occurs (see, e.g., Hulme, 1936*b*). It is not certain, however, that there is a direct connexion between these two phenomena; it may be that both are changes normally occurring in ripening fruit and that their concurrence may be fortuitous.

Kidd & West (1932) have demonstrated that the respiration climacteric can be induced even in immature fruits by treatment with ethylene. A net rise in protein content, therefore, in relatively immature apples during an ethylene-induced climacteric rise in respiration would be strong presumptive evidence that the two phenomena are intimately connected. The experiments described in this paper were designed, primarily, to ascertain whether this is the case. Data are also given for changes occurring during the 'normal' (i.e. not ethylene-induced) climacteric rise in respiration which occurs eventually even in immature fruits.

Data on the nitrogen metabolism of apples at various stages of development on and off the tree, which have accumulated in this laboratory over a number of years, have suggested that, of the soluble nitrogen fractions presumably involved in protein synthesis, asparagine nitrogen is most closely associated with rise or fall in protein. The present experiments provided an opportunity for studying such a relationship over a short specialized period in the ontogeny of the fruit. The period of the climacteric rise in respiration in immature fruits is especially suitable for such a study, since it embraces a relatively rapid rise in protein under conditions of constant content of total nitrogen.

EXPERIMENTAL

Materials and methods of sampling and storage

Bramley's Seedling apples at two stages of maturity were selected for the experiment. They were taken from 18 trees (18 years old) on East Malling stocks I and II on an unmanured plot at East Malling Research Station. The date

of petal fall was 17 May 1937, and the two series of samples were picked on 17 June (Series 1) and 3 August (Series 2). At each pick the requisite number of apples of uniform size was gathered, and each sample was made up from a definite number of fruits from each tree. This procedure ensures that the samples are uniform, each being representative of the group of trees as a whole. Series 1 consisted of sixteen samples, each of 22 fruits; the average weight per apple was 16.0 g. Series 2 consisted of twenty-one samples, each of 30 fruits; the average weight per apple was 102.5 g. Both series of fruits were, therefore, very immature, the normal commercial date of picking for this variety being about the end of September.

Immediately after preparation, three or four samples were rapidly halved, the seeds removed and the fruits of each sample placed in tins and frozen to -20° to await analysis (Hulme, 1936*a*). These fruits are the 'initial' samples. The remaining samples were each placed in their respective respiration chambers, which formed part of an apparatus for the determination of their rate of respiration in presence and absence of ethylene. This train of apparatus, which was situated in a constant-temperature room at 12° , was similar to that already described (Hulme, 1937), except that a T piece was inserted between the KOH bubbler and the respiration chamber so that ethylene could be introduced into the train at a definite rate through a needle valve and a calibrated bubble bottle. In the case of Series 1 the respiration chamber took the form of a 2 l. Buchner flask in place of the usual vacuum desiccator, and the CO_2 -absorption towers were replaced by Pettenkofer tubes.

Preparation of material for analysis and methods of analysis

Separation into peel and pulp tissue and disintegration of the separated tissues was carried out as already described (Hulme, 1936*a*). The seeds of the fruits were collected and their total N content determined so that an indication could be obtained of any possible migration of N between flesh and seed. This factor must be considered, since, in the immature fruits used in these experiments, the seeds were only partially developed and appeared to be still organically connected to the cortical tissue.

Total N was determined on a sample of frozen, ground tissue by the usual Kjeldahl method using 100% (v/v) H_2O_2 as catalyst in addition to $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

The various N fractions* were estimated as previously described (Hulme, 1936*a*), the rapid continuous vacuum

* It has been shown (Hulme, 1936*b*) that glutamine is absent from the apple fruit.

extractor (Hulme & Roach, 1936) being used for the 85% ethanol extraction of the tissue.

Titrateable acid was determined in the pulp tissue by the method used previously (Hulme, 1936a).

Dry weight was determined by drying a sample of frozen ground tissue *in vacuo* at 60° for 48 hr. The results agreed closely with those obtained by exhaustive drying *in vacuo* at 25° over P_2O_5 .

Statistical treatment. Replicate samples were taken at intervals as follows: *Series 1.* Four initial samples; two samples at the 15 days' storage point. *Series 2.* Three initial samples; two at 27 days; two at 35 days and two at 55 days. Each series was treated as a separate entity in the statistical analysis of the results. Standard deviations, covering total errors, were calculated from the following equations (Fisher, 1928). Variance = $\Sigma \delta^2 / \text{degrees of freedom}$, δ being the deviation of replicates from their mean. Standard deviation, $\sigma = \sqrt{\text{variance}}$. Statistical analysis of the respiration data was considered unnecessary since determinations were made at very frequent intervals and replicate results agreed to within 2-3 mg. $CO_2/100 \text{ g./100 hr.}$, even during the period of rapid change associated with the climacteric rise in respiration.

Ethylene treatment

Series 1. The dosage of ethylene in all cases was c. 1.5% in the air passing over the treated fruits. This relatively

high concentration was used to obtain an immediate response to the introduction of the gas. All twelve samples had been respiring in pure air at 12° for 28 days when the first sample was treated with ethylene; several samples were treated at 36 days, and one final sample at 46 days from zero storage time. Some samples were never treated with ethylene. The time of treatment with ethylene and the time at which treated and untreated samples were frozen to await analysis will be seen from Fig. 1. Where several samples were treated at the same time, the subsequent rate of respiration shown in Fig. 1 is the mean rate of all these samples.

Series 2. Dosage was approximately the same as for Series 1, but all the samples to be treated were given ethylene at the same time, i.e. 20 days after placing in the respiration chambers. The points at which treated and untreated samples were frozen to await analysis are indicated in Fig. 2. The rates of respiration subsequent to ethylene dosage for both treated and untreated samples are the means of the rates for the samples remaining under observation. The number of samples represented by the rate shown in Fig. 2 decreased as samples were removed and frozen.

During the course of the experiments one or two fruits in some of the samples developed infection by moulds. As soon as this was noted the fruit concerned was removed and weighed and the necessary correction applied to the subsequent calculations of rate of respiration.

Table 1. *Apple weight and nitrogen content (per apple) of ethylene-treated and untreated samples, Series 1, stored at 12°; pulp and peel tissue*

Days in store	Ethylene treatment (+ or -)	Initial fresh wt. of whole fruit (g.)	Dry wt. (mg.)	Total N (mg.)	Protein N (mg.)	Ammonia N (mg.)	Asparagine N (mg.)	Amino acid N (mg.)	Residual N (mg.)	Titrateable acid* (mg.)
Pulp										
0	-	15.9	1393	12.35	8.70	0.05	2.32	0.32	0.96	256
3	-	17.0	1460	13.72	8.87	0.05	2.96	0.62	1.22	278
7	-	16.1	1338	12.80	7.68	0.05	3.08	1.05	0.93	261
15	-	15.6	1293	13.33	6.55	0.09	4.37	0.81	1.52	238
28	-	15.5	1237	12.50	6.15	0.12	3.92	0.68	1.61	209
36	+	16.2	1248	12.53	7.48	0.09	3.12	0.35	1.49	195
36	-	16.0	1143	12.78	6.80	0.13	3.90	0.54	1.43	199
38	+	15.9	1212	13.80	7.20	0.12	4.40	0.46	1.62	198
41	+	16.1	1164	12.54	7.51	0.11	3.31	0.38	1.23	190
46	+	16.3	1191	13.15	8.11	0.11	3.12	0.52	1.29	183
46	-	16.8	1256	14.09	7.47	0.17	4.26	0.46	1.71	184
64	+	15.3	1024	12.73	8.54	0.11	2.42	0.28	1.18	132
σ	.	1.13	93	0.64	0.40	0.017	0.25	0.058	0.11	21
Peel										
0	-	15.9	.	4.67	4.14	0.27	.	.	.	.
3	-	17.0	.	5.03	4.30	0.33	.	.	.	.
7	-	16.1	.	4.37	3.47	0.44	.	.	.	.
15	-	15.6	.	4.48	3.19	0.76	.	.	.	.
28	-	15.5	.	4.30	3.00	0.87	.	.	.	.
36	+	16.2	.	4.50	3.27	0.84	.	.	.	.
36	-	16.0	.	4.32	2.79	1.04	.	.	.	.
38	+	15.9	.	4.69	3.12	1.12	.	.	.	.
41	+	16.1	.	4.61	3.11	1.08	.	.	.	.
46	+	16.3	.	4.87	3.50	1.01	.	.	.	.
46	-	16.8	.	4.81	3.19	1.06	.	.	.	.
64	+	15.3	.	4.27	3.15	0.81	.	.	.	.
σ	.	.	.	0.39	0.32	0.049	.	.	.	.

* Expressed as malic acid.

RESULTS

The primary data for the fruit used and the content of the various nitrogen fractions of the fruit and the total nitrogen content of the seeds during storage with and without ethylene treatment are shown in Tables 1-3. Owing to the small amount of material available, it was only possible to determine total, protein and asparagine+ammonia nitrogen in the peel of the fruit of Series 1.

The levels of significance as between two results in each series of samples (the 'significant difference'), calculated on the basis of a 20% probability ($P=0.05$) are $\sigma \times 3.17$ for Series 1 and $\sigma \times 2.94$ for Series 2. These values may be approximated to $\sigma \times 3$, so that in both series a difference between two results greater

than three times the appropriate standard deviation may be regarded as significant.

An examination of the appropriate significant differences shows that in both series there is no real variation, due to uneven picking, in the weight of the average apple throughout the series and that, as would be expected, there is no significant change in the total nitrogen content during storage. Changes in the fractions of nitrogen are therefore more readily apparent when expressed as a percentage of total nitrogen. The standard deviations for the various nitrogen fractions expressed on this basis are shown in Table 4.

The changes in protein nitrogen, as a percentage of total nitrogen, together with the corresponding changes in rate of respiration are shown in

Table 2. *Apple weight and nitrogen content (per apple) of ethylene-treated and untreated samples, Series 2, stored at 12°; whole fruit (obtained from separate results for pulp and peel) and peel tissue*

Days in store	Ethylene treatment (+ or -)	Initial fresh wt. of whole fruit (g.)	Dry wt. (g.)	Total N (mg.)	Protein N (mg.)	Ammonia N (mg.)	Asparagine N (mg.)	Amino-acid N (mg.)	Residual N (mg.)	Titrat-able acid* (mg.)
Whole fruit										
0	-	103.0	14.12	53.62	32.25	0.10	12.17	4.35	4.76	1603
3	-	105.4	14.93	52.00	31.40	0.13	10.92	3.92	5.62	1616
8	-	103.6	14.54	52.68	33.54	0.10	10.07	3.81	5.26	1559
20	-	102.3	13.95	51.96	32.93	0.13	10.02	3.40	5.49	1513
21	+	101.6	13.66	51.50	32.27	0.14	9.50	4.06	5.52	1532
23	+	104.7	13.81	50.18	33.00	0.11	8.75	3.68	4.64	1576
27	+	102.1	13.35	52.39	34.41	0.10	9.54	3.33	5.00	1504
27	-	102.7	13.65	52.22	31.67	0.14	11.72	3.75	4.84	1545
29	-	102.0	13.32	50.85	32.11	0.16	9.94	3.18	5.41	1541
35	-	103.0	13.68	54.27	33.48	0.20	11.98	2.69	5.86	1501
38	-	102.4	13.34	51.50	34.69	0.19	9.44	2.83	4.36	1485
38	+	103.3	13.27	50.54	35.30	0.14	8.05	2.62	4.44	1400
41	-	103.0	13.20	53.94	35.37	0.19	10.92	2.41	5.05	1464
55	-	102.4	13.08	52.69	37.20	0.24	8.54	1.81	4.93	1383
56	+	101.3	12.62	52.66	38.03	0.33	7.81	1.79	4.62	1291
70	-	104.0	12.66	50.63	37.46	0.43	6.40	2.20	4.14	1239
σ	.	1.50	0.34	2.06	1.37	0.017	0.75	0.33	0.39	30
Peel										
0	-	103.0	2.18	11.96	10.06	0.02	0.83	0.54	0.51	.
3	-	105.4	2.17	11.99	10.22	0.03	0.62	0.50	0.62	.
8	-	103.6	2.32	12.95	10.93	0.02	0.69	0.63	0.68	.
20	-	102.3	2.15	11.96	10.04	0.03	0.66	0.47	0.76	.
21	+	101.6	2.12	11.48	9.63	0.03	0.68	0.44	0.71	.
23	+	104.7	2.09	11.08	9.46	0.02	0.55	0.45	0.60	.
27	+	102.1	2.12	11.89	10.22	0.02	0.63	0.45	0.57	.
27	-	102.7	2.09	11.07	9.04	0.03	0.76	0.43	0.71	.
29	-	102.0	2.09	11.10	9.31	0.03	0.70	0.47	0.59	.
35	-	103.0	2.07	11.33	9.48	0.03	0.77	0.36	0.69	.
38	-	102.4	2.10	11.31	9.83	0.02	0.56	0.43	0.46	.
38	+	103.3	2.06	11.54	10.30	0.03	0.41	0.23	0.57	.
41	-	103.0	2.08	11.67	10.08	0.02	0.66	0.28	0.62	.
55	-	102.4	2.06	11.99	10.79	0.03	0.37	0.23	0.56	.
56	+	101.3	2.01	12.14	10.93	0.03	0.35	0.21	0.62	.
70	-	104.0	2.00	12.03	10.90	0.04	0.30	0.31	0.48	.
σ	.	.	0.030	0.41	0.40	0.005	0.049	0.033	0.052	.

* Expressed as malic acid.

Table 3. *The number, weight and nitrogen content of the seeds of treated and untreated fruits of Series 1 and 2*

Days in store	Ethylene treatment (+ or -)	No. of seeds per apple	Average wt. per apple (mg.)	Average wt. per seed (mg.)	Total N	
					Per apple (mg.)	Per seed (mg.)
Series 1						
0	-	5.0	117	23	0.57	0.113
3	-	4.5	110	25	0.53	0.120
7	-	4.6	117	25	0.57	0.122
15	-	4.5	96	21	0.44	0.097
28	-	4.4	89	20	0.33	0.075
36	+	4.9	74	15	0.25	0.050
36	-	4.9	72	15	0.23	0.052
38	+	5.1	73	14	0.26	0.049
41	+	4.1	60	14	0.19	0.044
46	+	4.5	63	14	0.21	0.044
46	-	4.2	65	15	0.23	0.051
64	+	4.2	45	11	0.24	0.050
σ	.	0.33	10	2.2	0.046	0.0136
Series 2						
0	-	4.9	194	40	1.77	0.364
3	-	4.4	181	41	1.69	0.385
8	-	4.8	183	38	1.85	0.385
20	-	4.0	128	33	1.37	0.352
21	+	4.7	166	35	1.70	0.361
23	+	4.8	162	34	1.84	0.383
27	+	4.6	154	33	1.79	0.391
27	-	4.9	144	30	1.59	0.326
29	-	4.5	136	30	1.47	0.328
35	-	4.6	128	28	1.33	0.293
38	-	4.1	123	30	1.57	0.386
38	+	5.2	139	27	1.57	0.300
41	-	4.9	133	27	1.50	0.305
55	-	4.3	121	28	1.57	0.366
56	+	4.3	114	26	1.54	0.355
70	-	.	.	.	.	.
σ	.	0.51	13	1.7	0.147	0.0319

Figs. 1 and 2. With the treated samples the rate of respiration immediately before introduction of ethylene does not fall exactly on the line representing the mean respiration of all untreated samples (continuous line).

Changes in protein in relation to changes in rate of respiration. In view of the fact that there is no significant change in the absolute amount of total nitrogen in the fruit during storage, the results presented in Figs. 1 and 2 indicate clearly that, over the period of the respiration climacteric, whether ethylene-induced or whether occurring as a normal phenomenon in the ontogeny of the fruit, the net amount of protein in both peel and pulp rises significantly. In Series 1 the significant difference for protein nitrogen as a percentage of the total nitrogen is 4.2 for whole fruit and 4.5 for the peel. In all cases the rise in protein nitrogen in the whole apple following ethylene treatment is more than twice the significant difference. In the peel, all the rises are significant except that after the first ethylene treatment; even in this case there is no indication that the rise has ceased when the only treated sample was frozen for analysis. In Series 2,

where the significant differences are 2.2 and 2.0% respectively, the rise in protein nitrogen subsequent to ethylene dosage is highly significant for both whole fruit and peel. In this series also the onset of the normal climacteric is followed by a highly significant rise in protein nitrogen in both whole fruit and peel.

It appears, however, that in both peel and pulp tissues of both series the rise in respiration precedes by some hours the rise in protein content (Fig. 1, ethylene treatment at 36 hr. and Fig. 2, both ethylene-treated and untreated samples). The immediate rise in protein following ethylene treatment at 36 hr. in the peel of the fruit of Series 1 is not significant. It is interesting to note that in experiments designed to determine the relative rate of respiration of peel and pulp tissue it has been found (Hulme, unpublished data) that the peel of fruits of 100 g. average weight respire at least three times as rapidly as the pulp tissue. As the present data show, the protein content per unit dry weight of peel tissue is at least twice that of pulp tissue and forms a higher percentage of the total nitrogen (see also Hulme & Smith, 1938).

Changes in the nitrogen content of the seeds. It is clear that the average number of seeds per apple does not vary significantly throughout all the samples (Table 3). The total nitrogen of the seeds

Table 4. Standard deviations for various nitrogen fractions expressed as a percentage of total nitrogen

Tissue and fractions		Standard deviation (σ)
Series 1		
Whole apple:		
Protein N		1.39
Asparagine + ammonia N		1.04
Pulp:		
Protein N		1.78
Ammonia N		0.13
Asparagine N		1.23
Amino-acid N		0.37
Residual N		0.37
Peel:		
Protein N		1.53
Asparagine + ammonia N		0.30
Series 2		
Whole apple:		
Protein N		0.73
Ammonia N		0.021
Asparagine N		0.97
Amino-acid N		0.81
Residual N		0.40
Peel:		
Protein N		0.67
Ammonia N		0.053
Asparagine N		0.33
Amino-acid N		0.33
Residual N		0.44

Fig. 1. The change in the rate of respiration (top graph) and the change in content of protein N in the whole apple and in the peel during the storage of apples of Series 1 in air and in ethylene. An E above an arrow indicates the point at which ethylene was given to samples whose subsequent history is indicated by an interrupted line.

per apple falls rapidly in Series 1 during the first 36 days of storage, but there is no evidence of any change during the climacteric rise in respiration whether ethylene-induced or not. The fate of the nitrogen lost in the early stages of storage is not

Fig. 2. Changes similar to those depicted in Fig. 1, but for the fruit of Series 2.

clear. In Series 2 there is no significant change in the nitrogen content of the seeds at any time. Thus no transfer of nitrogen from seeds to flesh tissue occurs at any time during the storage of the fruit.

Fig. 3. Relation between protein N and the non-protein N fractions over the whole period of storage of Series 1 fruit, both normal and ethylene-treated.

Fig. 4. Relation between protein N and the non-protein N fractions over the whole period of storage of Series 1 and 2 fruits, both untreated and ethylene-treated. The results shown are for the whole fruit.

Changes in the fractions of the non-protein nitrogen during increase or decrease in protein. Since total nitrogen is constant, it may be expected that all the forms of non-protein nitrogen will be affected to some extent by change in content of protein. A cursory examination of the data presented in Tables 1 and 2 suggests that the changes in asparagine nitrogen are most closely related to rise and fall in protein. The relationship between protein nitrogen and the non-protein nitrogen fractions is shown in Figs. 3 and 4. The close correlation between rise in protein nitrogen and fall in asparagine nitrogen is striking. Furthermore, the change in asparagine nitrogen per unit protein (or vice versa) is the same for peel and pulp and covers a wide range of protein levels. The values for asparagine nitrogen in Fig. 4, Series 1, are rather high compared with those of Series 2 owing to the inclusion of ammonia nitrogen in the former case. It is clear that, at both stages of

maturity examined, a similar relationship exists throughout the fruit between fall in asparagine and rise in protein.

This general close relationship is confirmed on examination of the correlation coefficients for protein nitrogen on asparagine plus ammonia nitrogen, amino-acid nitrogen and residual nitrogen. Asparagine gives a coefficient (r) of almost unity in every case. The correlation coefficients for amino-acid nitrogen and residual nitrogen are also fairly high, the former not consistently so. Little is yet known of the composition of the residual nitrogen, although it is certain that basic amino-acids are present only in small amounts in the apple (Hulme, 1937).

Titratable acid. The determination of titratable acid has been carried out as a matter of routine in the study of the nitrogen metabolism of apples which has been proceeding in this laboratory for some years. The results obtained in the present study are included in the tables, since it is clear that immediately before the onset of the respiration climacteric (normal and ethylene-induced) there is a rise in the amount of titratable acid in these immature fruits. This rise is, however, only significant in the case of the samples of Series 2.

DISCUSSION

The above data are insufficient for the development of a theory of the paths of protein synthesis in the apple fruit; the most that can be said is that deamidization of asparagine appears to be closely connected with the synthetic processes. Of interest in this connexion is the fact that, while free ammonia nitrogen never forms much more than 1% of the total nitrogen, it does tend to rise significantly during active protein loss and when the rate of protein synthesis is slowing down (Series 1 at day 28; Series 2 towards the end of the storage period).

SUMMARY

1. Evidence is produced that during the climacteric rise in rate of respiration in immature apples, whether occurring normally or whether induced by treatment of the fruit with ethylene, there is a highly significant rise in the net protein content of the fruit.

2. A strong negative correlation is shown to exist between change in protein content and change in asparagine content, not only during the rise in protein which accompanies the respiration climacteric, but also during the steady fall in protein content which precedes the respiration climacteric in very young fruits. The other forms of soluble nitrogenous constituents show some negative correlation with change in protein content, but the correlation is less consistent.

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The Algae

4. THE LIPOCHROMES OF THE MALE AND FEMALE GAMETES OF SOME SPECIES OF THE FUCACEAE

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Biochemical work done hitherto on the algae has been concerned chiefly with the vegetative thallus. Mainly because of the difficulties of securing pure material in sufficiently large quantities, very little consideration has until recently been given to the pigmentation of the reproductive bodies. Among the lower plants, the male gametes of many algae are known to be yellowish in colour, in contrast to the rather more green female gametes, and this difference is especially marked in heterogamous and oogamous plants.

The members of the family Fucaceae (class Phaeophyceae), so abundant on many parts of our British shores, reproduce oogamously by means of minute motile, biciliate sperms and larger non-motile eggs. The dioecious members of the Fucales are especially convenient for study, in that large quantities of both male and female gametes can be obtained in a pure state and free from any possibility of contamination by gametes of the other sex. In the same family, monoecious species also occur, and thus provide material for comparison with the dioecious types.

In the Fucaceae, the antheridia and oogonia are produced within flask-shaped, sunken cavities, the conceptacles. These occur at the periphery of somewhat swollen and mucilaginous receptacles, which in some species are modifications of some of the dichotomies of the thallus as in *Fucus serratus*, *F. vesiculosus* and *F. spiralis*, or in other genera, of the apices of special lateral deciduous fruiting bodies, as in *Ascophyllum nodosum*.

The gradual extrusion of groups of mature eggs and sperms through the openings (ostioles) of the conceptacles, normally occurs between the tides, and is probably brought about in part by the expansion of mucilage, together with contraction on desiccation. In nature, the gametes are removed from the surface of the conceptacles by the incoming tide and fertilization thus takes place outside the parent plant.

The numerous minute biciliated sperms contain a small yellowish chromoplast, and the mature aggregates of sperms therefore appear orange-yellow, which colour they impart to the entire receptacle. This affords a means of distinguishing the male plants in the dioecious species of the Fuci at the time of gamete discharge. In the preparation of material for the present experiments, however, colour alone was not considered to be a sufficiently safe criterion, and a section of a receptacle of every plant collected was examined microscopically, in order to confirm the accuracy of the separation of the male and female plants. Moreover, plants were selected from all parts of the zone of their distribution on the Aberystwyth shore.

The marked orange colour of the exudate of the male plants of the dioecious members of the Fucaceae in their fruiting season, in striking contrast to the olive-green colour of the female-plant exudate, suggested that the colouring matter of the chromatophores of the male and female gametes might possibly be connected with the reproductive process. It seemed feasible that the light-absorbing