XXV. BIOCHEMICAL CHANGES DUE TO ENVIRONMENT.

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OBSERVATIONS on the influence of climate on the adaptation of alpine plants have so far been limited to the more or less obvious macroscopic or microscopic changes and their accompanying physiological effects on assimilation, transpiration and respiration. The most extensive investigations were those of Bonnier [1888, 1894] carried out in the experimental gardens in Paris and in the Montblanc range (1060–2800 m.), and those of Kerner [1894] who grew plants from seed in the botanical garden of Vienna and on the summit of the Blaser (2195 m.), near Innsbruck. The main conclusions to be drawn from this work were that the whole habit of the plants grown at higher altitudes became more stunted and dwarfed than in the plains, the development of the subterranean parts was relatively greater and the internal structure of both stem and leaf was modified, tending to lead to an increased power of assimilation. Of the principal factors of the alpine climate which gave rise to these changes the largest share is ascribed to the more intense illumination by day accompanied by low night temperatures and a drier atmosphere. Many observers as well as Kerner and Bonnier note the greater intensity of colour of alpine flowers and Bonnier attempted to confirm this subjective observation by comparing the colour tints of flowers at different altitudes in the mountains of Austria-Hungary by means of a graduated colour scale or "chromometer."

The rapid progress which has been made in the last few years in our knowledge of the chemistry of flower pigments makes it possible to attack these questions from a different point of view. It was first suggested by Miss Wheldale [1909] that a relationship exists between the anthocyanins and the flavones (or possibly xanthoncs). Whilst the theoretical assumptions of Miss Wheldale [1911] demanded hydrolytic and oxidative processes for

the formation of anthocyanins from flavone glucosides, the experimental facts subsequently brought forward by Keeble, Armstrong and Jones [1913] led them to assume an interplay of reducing and oxidative enzymes. Combes [1913] was the first completely to contradict the oxidation theory and he showed that a reducing agent, presumably an enzyme, plays the fundamental rôle in the process. Everest [1914] confirmed this conclusion and was able to convert extracts of white and yellow flowers, probably containing flavone glucosides, without intermediate hydrolysis, into anthocyanins by making use of reducing agents, of which magnesium was the most suitable. Willstätter, in conjunction with Everest [1913] and with Mallison [1914] adduced definite proofs that all naturally occurring anthocyanins were glucosides of reduced flavones or flavonols, and furnished on hydrolysis the chromophoric component (anthocyanidin = reduced flavone or flavonol) and a carbohydrate (glucose or galactose)¹.

Shibata [1915] pointed out the universal distribution of flavone derivatives in the overground parts of plants, and in conjunction with Nagai and Kishida [1916] showed that alpine and tropical plants especially are rich in these substances. The Japanese investigators advanced the interesting suggestion that the presence in the peripheral tissue of flavone derivatives may possess an important physiological significance in absorbing the ultra-violet rays of sunlight and thus protecting the plant against the injurious action of these rays. They also demonstrated the fact that greatly diluted solutions of chemically pure flavone derivatives absorb the ultra-violet region of the iron arc light almost completely.

If the hypothesis of Shibata is correct one would expect that alpine plants grown at lower altitudes, in which ultra-violet light is known to be much less intense owing to its rapid absorption by the atmosphere, would not produce the flavone substances to the same extent as those grown in the Alps where the insolation is so much more intense.

In spite of the apparently adverse conditions (dampness of the atmosphere in winter and the absence of the protecting influence of a permanent layer of snow) alpine plants can be easily grown in this country and develop normally from the seedling to the flowering and fruiting stage, even in the neighbourhood of London where they are exposed to the influence of a smoke-laden atmosphere. One of the easiest to grow from seed is the well known Edelweiss (*Leontopodium alpinum*) and partly for this reason and partly because I had

¹ For detailed discussion and full literature of this subject see the monographs of Everest [1915] and Wheldale [1916].

a considerable number of specimens collected by myself in the Alps available for comparison, I selected this plant for the experiments to be described.

A large number of flowers from plants growing on my rock garden in a London suburb (80 m. above sea) were used in these experiments. They came from a plant collected six years ago on Mt. Colbricon, about 2000 m. above sea level (near San Martino di Castrozza), and from the seedlings of this plant, of which several generations had been raised. The plants are growing in different positions as regards aspect and soil, but in all cases care was taken that the soil contained a plentiful supply of calcium carbonate (mortar rubble), which is essential for the well-being of this and the majority of alpine plants.

The flowers which served for comparison with the lowland flowers were collected during a period extending over several years from various places in the central and eastern Alps (Switzerland, Tyrol, Dolomites), generally at a height of 2000 m., and had been preserved in the usual way¹.

The whole of the material was gathered approximately at the same stage of development of the plants (during middle and end of August) and was finally dried together for one month *in vacuo* over sulphuric acid.

The presence of flavone derivatives in the silvery hairs of the bracts of the Edelweiss inflorescence can easily be demonstrated by two reactions. An intense yellow coloration is at once produced on exposure of the flowers to ammonia vapour, a characteristic reaction of nearly all white flowers which was first made use of by Miss Wheldale. The second reaction is still more striking and may be demonstrated with as little as 0.2 g. of the flower bracts. The colourless, or slightly yellowish, alcoholic extract of the bracts gives on reduction with magnesium in acid solution (in the presence of mercury as a catalyst) an intense orange-red colour [Shibata, 1915].

Nature of the Chromogenic Substance. The isolation and identification of the chromogenic substance obviously demands a larger amount of material than was at my disposal. There can be little doubt that the substance belongs to the class of flavone derivatives, which occur in plants in the free state as well as in glucosidic combination. A very simple reaction makes it possible to decide whether the glucoside, the non-glucoside, or a mixture of the two is present in Edelweiss. In their work on flower pigments Willstätter and Everest [1913] devised the so-called "anthocyanidin" reaction which depends

¹ It had already been noticed by Shibata [1915] that flavones are not lost appreciably if the drying is done quickly, and that determinations can be made with any well preserved herbarium material, even if kept for a number of years.

on the fact that the glucoside (anthocyanin) remains entirely in dilute sulphuric acid solution when shaken with amyl alcohol, whilst the non-glucoside (anthocyanidin) passes quantitatively into the amyl alcohol layer. When this alcoholic layer is shaken with sodium carbonate solution the pigment descends quantitatively into the aqueous layer. It is apparent that this reaction may be applied to the reduced coloured extracts, in which the flavone or its glucoside originally present, is reduced either to anthocyanidin or anthocyanin. It was found that plants collected in the Alps contained traces of the chromogenic substance in glucosidic combination, whilst the London grown plants contained only free flavones¹. The reductions were carried out at a low temperature in order to avoid any acid hydrolysis of the glucoside, and the reduced solutions were diluted with water and freed from alcohol by shaking out with ether. On shaking with amyl alcohol the pigment passed quantitatively into it in the case of the homegrown plants, whilst a faint tint remained in the other cases even after repeated extraction with fresh amyl alcohol. The alcoholic solution showed the typical spectral band between λ 480– λ 520, allowing red and violet to pass. On shaking the amyl alcohol solution with sodium acetate the pigment remained in the alcohol, but it descended quantitatively into the aqueous layer on shaking with dilute sodium carbonate. The conclusion that the chromogen is present as a free flavone and not in glucosidic combination receives a certain amount of confirmation from the solubilities of the substance (insoluble in water, soluble in alcohol).

Quantitative Relationship of Flavone Derivatives in Edelweiss grown in London and in the Alps. The colour produced by the reduction of the colourless alcoholic extract of Edelweiss obviously offers a method for comparing quantitatively the amounts of flavone derivatives formed in the plant under different environmental conditions. This may be carried out either by comparing the colour intensity of the various extracts *inter se* or with a standard solution. The finding of a suitable standard makes the latter procedure difficult. An attempt was made to use the colour produced on reducing alcoholic apigenin (1. 3. 4'-trihydroxyflavone)² solutions of known strength by means of magnesium. The colour of the reduced solution, although

¹ The white flowers of alpine saxifrages and of *Dryas octopetala* also contained free flavones only. The majority of white non-alpine flowers which I examined, on the other hand, contained practically the whole of their chromogen in the glucosidic combination.

² Apigenin was obtained by the hydrolysis of the glucoside apiin, which I prepared from parsley. I am indebted to Dr Barger for another sample of pure apiin, which gave identical results.

resembling that of the reduced Edelweiss extracts to the naked eye, was found on comparison in Duboscq's colorimeter to differ in its tint to such an extent as to make exact comparison impossible.

The complete extraction of the flavone at first offered some difficulties. Exhaustive extraction with alcohol in a Soxhlet's apparatus introduced variable amounts of greenish-yellow decomposition products of chlorophyll which could not be completely removed by shaking out with light petroleum. Although small in amount they were sufficient to influence the tint of the reduction colour. The following procedure was finally adopted. Quantities of 0.5 or 1.0 g. of the plant organs were repeatedly extracted with twenty parts of 90 per cent. alcohol at 70–75° for some minutes, until the decanted extract no longer gave any colour after reduction. At least six extractions were found to be necessary. Each extract was filtered through a small filter-paper (2 cm.) into a graduated test tube and 1 cc. conc. HCl was added to every 4 cc. of the extract. After the addition of a few drops of mercury the acid solution was reduced by magnesium powder, added gradually in small amounts until the maximum colour intensity was reached. After reduction the coloured extracts were mixed and made up to 50 or 100 cc. respectively with 90 per cent. alcohol.

It became at once obvious that the colour intensity of the reduced extract from the plants collected in the Alps was much stronger than that of the plants grown in the lowland, so much so that it became necessary to dilute it largely before comparison in the colorimeter. Estimations were made by means of Duboscq's and Hellige's colorimeters. Owing to the presence of small amounts of chlorophyll decomposition products, the colour of extract (2) could not be matched in Duboscq's apparatus, but the slight difference in tint did not interfere with the estimation in Hellige's wedge colorimeter. The results of many readings, in which the standard was set at different heights, were as follows:

Alpine Flowers	Flowers from London Plants			
	(1)	(2)	(3)	(4)
100	25.2	26.0	28.1	21.6

It will be seen therefore that the reduction colour, which serves as an index of the amount of flavone originally present, reaches on the average in London grown plants only 25 per cent. of that produced in the plants grown at higher altitudes.

Distribution of Flavone derivatives in Edelweiss plants. Whilst the flower-heads as a whole contained a large amount of flavone, it was found that the

stems of plants collected in the Alps contained much less and the stems of London grown plants were quite devoid of chromogenic substance. On the other hand the fruit (achene with its pappus) is extremely rich in it. Extracts of fruits and the corresponding flower bracts were compared quantitatively with the result that the reduction colour of the flower bracts was found to be a little more than half (54 per cent.) of that of the fruits. It seems therefore that the flavone derivatives are stored up in the seed and in accordance with this it was found that the inflorescence gathered in November had lost with its fruit the whole of its chromogenic substance. Vegetative leaves as well as roots of plants examined in December, were also devoid of chromogen.

The Flavone Contents of Edelweiss as a Biological Factor. The distinguishing feature of this plant, especially of its inflorescence, consists in its covering by long woolly hairs which give rise to the idea of whiteness expressed in its name. As an explanation for the biological significance of this pubescence it has been assumed that the air entangled between the matted hairs checks excessive transpiration and helps the plant to exist under very dry or desert conditions. The fact that under the influence of the moist English climate the plant continues through many generations to produce this characteristic feature is to some extent against this explanation, and together with the results of the above described observations tends to lend support to the suggestion of Shibata. The growing season of the alpine plants coincides with the maximum intensity of chemical sunlight in the year and the necessity of some protective mechanism of the living protoplasm against the injurious action of ultra-violet light is apparent. The flavones dissolved in the cell sap may well exert this protective action, as they do when examined in ordinary solution. The same plants grown in London under conditions in which the insolation is much less intense, apparently adjust themselves to the altered conditions by producing the protective substances in smaller amounts. In this connection it is interesting to note the behaviour of some other white alpine flowers which I had occasion to examine in February, a month in which the amount of ultra-violet light is extremely low. Extracts of the petals of *Saxifraga burseriana* and of *S. Boydii alba* (*burseriana* × *aretioides*) which flowered in my garden in the first half of February, gave practically no colour on reduction. On the other hand the white flowers of saxifrages (*S. cotyledon*, *S. longifolia*, *S. lingulata*, *S. Wallacii*), examined later in the season, contained flavones. The petals of *Dryas octopetala* and *Campanula pusilla alba*, also gave intense reduction colours when examined in May and July respectively.

SUMMARY.

The inflorescence of Edelweiss (*Leontopodium alpinum*) contains a chromogenic substance, probably a flavone, which is not in glucosidic combination. Plants grown in London at an altitude of 80 m. contain only one fourth of the amount found in plants collected in the Alps at an average altitude of 2000 m. These facts illustrate the biochemical adaptation of alpine plants to changed environment and speak in favour of the assumption that the biological significance of flavones in the plant consists in their protective action against the injurious influence of ultra-violet light.

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