CXII. EXPERIMENTS ON THE ORIGIN AND FUNCTION OF BERBERINE IN *BERBERIS DARWINII*.

By BENJAMIN THORNTON CROMWELL.

From the Department of Biochemistry, University of Liverpool.

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THE classical researches of Pictet [1905; 1907] led him to conclude that alkaloids were waste products of the metabolic activity of the plant, analogous to urea, ethereal sulphates *etc.* of the animal body, and he supposed that they were produced in two stages (*a*) by the resolution of nitrogenous bodies such as proteins or chlorophyll into more simple basic substances, and (*b*) subsequent condensation of these simple bodies with the active products of metabolism, *e.g.* aldehydes, with the formation of the alkaloid nucleus. Research on these lines in recent years has been sporadic, and the results are difficult to correlate because of diversity in the nature and origin of the alkaloids studied. Thus Annett [1920] studied the effect of sodium nitrate on the production of latex by *Papaver somniferum* and found a large increase with but little variation in its morphine content. Mothes [1928] showed that the concentration of nicotine in the leaves of *Nicotiana* increased as the leaf developed, while Koenig [1931] found an increased production of alkaloid if nitrogenous fertilisers were applied. Ripert [1921] studied the effect of etiolation on the atropine and hyoscyamine content of young shoots of *Atropa* and found a considerable increase in both leaves and stalks. In addition, Sabalitschka [1923] and Sabalitschka and Jungermann [1925] observed no decrease in the alkaloid content of the seeds of *Lupinus* and *Strychnos* on germination. All these results tend to confirm Pictet's assertion that alkaloids are waste products, but throw very little extra light on the question of a possible connection between alkaloid production and protein metabolism.

In the present research an attempt has been made to unify some of these earlier findings by studying the metabolism of berberine in *Berberis Darwinii*. The experiments have been continued throughout two seasons, and have been planned to throw light on (1) the variation in berberine content with age, (2) the effect of applying nitrogenous fertilisers and of the withdrawal of essential elements, (3) the changes brought about by etiolation, (4) disturbances in the normal distribution of berberine brought about by ringing of the bark. The results obtained establish in certain cases an undoubted connection between protein breakdown and berberine synthesis.

EXPERIMENTAL.

Materials used. The plants were obtained as required from nurseries and the ages are calculated from the time of the first transplantation. The various plant members were cut into small pieces and dried at 105–110° for one day, finely powdered in a coffee mill and dried for the further period of one day.

Estimation of berberine. 4 to 5 g. of the powdered material were extracted with alcohol in a Soxhlet apparatus until the extract was no longer coloured yellow, and the berberine was estimated in the alcoholic extract by the method of Richter [1914] which is based upon the fact that berberine in one of its isomeric forms is soluble in ether. The normal and crystalline form (ammonium form) is soluble in water and insoluble in ether; by the action of alkali this is converted into the presumed "aldehyde form" which is soluble in ether and almost insoluble in water. The procedure was as follows.

The alcoholic extract was evaporated to dryness on a water-bath and the residue taken up with hot water, which precipitates resinous matter. The resin was filtered off and the aqueous solution evaporated to a small bulk (20 cc.). 10 cc. of 15 % NaOH solution were added and the alkaline solution was shaken with 110 cc. of ether for 10–15 minutes, 5 cc. of a 2 % solution of gum tragacanth being added prior to shaking. 55 cc., representing one half of the original volume of ether added, were removed for the subsequent estimation. The ethereal extract was then treated with a saturated solution of picrolonic acid in ether until precipitation was complete. The precipitate of alkaloid picrolonate was allowed to stand overnight and filtered through a Gooch crucible. The precipitate was washed with ether to remove picrolonic acid, dried at 105° and weighed. The weight multiplied by the factor 0.561 gives the equivalent amount of berberine. The accuracy of the method was tested by applying it to a commercial sample of berberine hydrochloride. A recovery of $100 \pm 5\%$ was obtained.

PART I.

The seasonal variation in the berberine content of roots and stems.

The first investigation was to determine whether the berberine content of the different tissues varied during the season otherwise than as a result of increased age. For this purpose a large number of 3- and 5-year-old plants were investigated periodically from October until the following August. It is to be noted that each series of estimations was carried out on one plant, and that the successive results have all been obtained on different plants, the age of which is believed to have been 3 or 5 years. These results are summarised in Table I.

Table I. *Mean values showing the seasonal variation in berberine content (g./100 g. dry tissue).*

Date	3-year-old plants			5-year-old plants		
	Roots (whole)	Main stem	Subsidiary branches	Roots (whole)	Main stem	Subsidiary branches
Oct.–Dec.	1.24	0.24	0.10	1.41	0.38	0.17
Jan.–Mar.	1.21	0.21	0.10	1.36	0.38	0.12
April–May	1.25	0.28	0.12	1.39	0.39	0.14
June–Aug.	1.34	0.36	0.13	1.57	0.46	0.17

From the figures in Table I it will be seen that the highest concentration of berberine occurs in the roots: the main stems contain about one-fifth of the quantity in the roots, and the subsidiary branches about one-tenth. Furthermore, there is little change in the berberine content of the tissues during the period October to March. From March onwards the figures rise to a maximum at the end of the summer.

It will be seen that the berberine accumulates in the tissues during the period of active photosynthesis, and the enhanced level is maintained until the

following spring, for the 5-year plants contain more berberine in all their tissues than the 3-year plants.

Further, figures for the distribution of alkaloid in 7-year-old plants were obtained from plants used for cultural experiments. In these experiments the initial concentrations, before the application of the various nutrient solutions to the plant, were determined by removing from each plant samples of subsidiary roots, main stems and subsidiary branches. In addition, one plant, age 30 years, was also investigated as follows in January 1931: height of plant 7 ft. 6 in.: secondary branch analysed 5 ft. 6 in. above ground: main stem analysed at levels 5 ft., 4 ft., 3 ft., 1 ft. 6 in. above ground: sections cut at 1 ft. 6 in. above ground and of main root 3 in. below soil surface. These results are set out in Tables II and III, while Table IV records the average figures for the berberine content of 3, 5, 7 and 30-year-old plants.

Table II. *Concentration of berberine in tissues of 7-year-old plants*
(g./100 g. of dry tissue).

Number	Subsidiary root	Main stem	Subsidiary branches
3	2.14	0.53	0.06
5	1.22	0.36	0.08
7	2.06	0.43	0.14
8	1.57	0.64	0.13
9	1.88	0.91	0.20
10	1.94	0.43	0.11
11	1.58	0.48	0.12
12	1.86	0.40	0.09
13	1.83	0.47	0.11
14	2.45	0.37	0.07
15	1.96	0.47	0.09
16	1.65	0.41	0.11
17	1.81	0.40	0.13
Average	1.84	0.48	0.11

Young active growing shoots. Mean value 0.04 %.

Young parenchymatous roots. Mean value 1.41 %.

Table III. *Distribution of berberine in tissues of a 30-year-old plant*
(g./100 g. of dry tissue).

	Bark	Wood
Tertiary branch	—	0.33
Secondary branch	—	0.76
Main stem apex	2.67	—
	2.75	0.76
	3.58	0.85
Soil level	4.93	1.53
Root (surface)	11.17	2.86
(1 foot below surface)	10.94	2.60
(secondary branches 6 inches below surface)	—	2.80

Table IV. *Average concentration of berberine in various tissues of*
3, 5, 7 and 30-year-old plants.

Age (years)	Roots (whole)	Roots (subsidiary)	Main stem	Branches (subsidiary)
3	1.27	—	0.29	0.12
5	1.44	—	0.40	0.15
7	—	1.84	0.48	0.11
30	bark 11.0	—	2.67-4.93	0.33-0.76
	wood 2.80	2.80		

All these figures are consistent in showing the progressive increase in the berberine content with increase in age.

In no plant was sufficient berberine present in the leaves to enable a chemical estimation by this method to be carried out. Attempts were therefore made to detect it by means of the colour reaction with chlorine water and hydrochloric acid, which varies from pink to red according to the amount of alkaloid present. In none of the young leaves except in those of the 30-year-old plant could berberine be detected by this means. On the other hand it was found in the old leaves of all plants irrespective of age.

Histological distribution of berberine in the various tissues.

Very young roots (parenchymatous) triarch root bundles. Alkaloid is most abundant in a ring of parenchymatous tissue surrounding the vascular bundle. Where phellogen is developed this forms the distal boundary of the berberine-containing tissue. The walls of the xylem elements where developed are impregnated with the alkaloid, but the phloem is free.

Fibrous roots. Further penetration of the xylem (which has developed to a considerable extent) has taken place. Ring of tissue surrounding the vascular bundles still the richest in berberine, but many cells of the cortex show the presence of alkaloid. The phloem is free.

Main and subsidiary roots. Alkaloid is distributed throughout the section of the root with the exception that the phloem remains free.

Main stem. The distribution is similar to that of the root but the upper sections show a fall in concentration more especially in the bundle sheaths and (where present) pith. The phloem is free.

Young shoots. These show the presence of chlorophyll corpuscles in the ring internal to the phellogen—no berberine could be detected in the bundle sheaths or vessels.

Etiolated shoots. These appear to have a greater concentration of berberine at the base than at the apex and are richer than the woody stems from which they spring. In section, the ring of tissue (inner-cortex) immediately surrounding the vascular bundles and interior to the phellogen is engorged, the wood fibres show definite impregnation, while the vessels and phloem are quite free.

DISCUSSION OF RESULTS.

Examination of the distribution of alkaloid throughout the various organs and tissues reveals the fact that berberine occurs in largest amounts in (a) organs of the plant which normally grow in absence of light, *i.e.* roots and rhizomes, (b) cortical tissues, the older and external cells of which are constantly being cast (in this connection it is worthy of note that *Berberis* possesses a deep-seated phellogen and consequently a large cortical area), (c) tissues which are known chiefly to perform mechanical functions, *e.g.* sclerenchymatous bundle sheaths and xylem. These observations lead to the deduction that in plants grown in normal conditions of environment the alkaloid is deposited in tissues which take no definite part in metabolic activities and which are made up principally of dead cellular elements. In etiolated shoots however, the highest concentration of berberine occurs in the ring of living parenchymatous cells surrounding the vascular bundles. It appears that this ring of cells surrounding the vascular bundles is the tissue in which berberine first makes its appearance and with increasing age of the organ penetration proceeds in a radial direction externally into the cortex and internally into the structural framework of the xylem

elements. It was consistently found that old tissues possessed a high concentration of alkaloid, young actively growing shoots containing very low percentages.

Analysis of roots, main and subsidiary stems of plants 3, 5, 7 and 30 years of age throughout the year showed that the berberine content does not vary to an appreciable extent, the values in all plants examined remaining comparatively constant from October until the following March, when a definite rise took place and continued throughout the summer months until August when analyses were discontinued. As the plant increases in age it is apparent that accumulation of alkaloid takes place in the specified tissues from year to year. Moreover, during the winter and spring months no utilisation appears to take place, a fact which indicates that berberine cannot be considered as a reserve food material, which is deposited in the roots and rhizomes for use when growth processes are resumed. The gradual increase recorded during the summer months coincides with the period of most active growth, when the assimilatory area of the plant is increasing rapidly. In the interpretation of the foregoing results cognisance is taken of the effect of variations in proportions of alkaloid-containing tissue to the total amount of tissue included in the sample. For example in portions of the stem where much secondary thickening has taken place and where the proportion of wood to the richest berberine-containing tissue is large there would be a tendency for low values of alkaloid to be recorded, and the reverse would hold good for tissues such as parenchymatous etiolated shoots, where the portion of berberine (by weight) to the dry weight of the whole organ would be comparatively large. In these experiments, although the older plants possessed much greater bulk, the values for alkaloid percentages showed a steady rise with age, and errors which may have arisen from variations in the proportion of berberine-containing tissues to the total tissue of the organ do not vitiate the conclusions of the experiments. The facts of distribution give considerable support to the view that in *Berberis Darwinii*, the alkaloid is a product of metabolic activity which once formed does not again enter into the nutritive cycle of the plant.

PART II.

Cultural experiments.

Seven, 5 and 3-year-old plants, after samples of the stems and subsidiary roots had been taken, were potted in coarse river sand which had been thoroughly washed free from fine sediment, and watered at fortnightly intervals with one of the following culture solutions. Each solution (8 litres), contained 2 g. of magnesium sulphate, a trace of ferric chloride and (except (4)) 2 g. of potassium dihydrogen phosphate; with the addition of (1) 6 g. of calcium nitrate and 2 g. of potassium nitrate; (2) 6 g. of ammonium nitrate and 2 g. of calcium nitrate; (3) 2 g. of potassium nitrate and 4 g. of ammonium nitrate; (4) 6 g. of ammonium nitrate and 2 g. of calcium phosphate; (5) 2 g. of calcium phosphate; (6) 2 g. of calcium phosphate and 8 g. of asparagine; (7) 2 g. of calcium phosphate and 8 g. of urea; (8) as (5), with 1 litre of 1 % solution of formamol; (9) as (1) with 20 g. of glucose. The results of the culture experiments, which are described in detail below, are given in Tables V and VI.

The effect of different sources of nitrogen on the yield of berberine.

Potassium and calcium nitrates together as source of N (Solution 1). The results of the application of this solution to plants of 4, 5 and 7 years of age for the period of 9 months from November 1931 to August 1932 are set forth in Table V.

Table V. *Culture experiments with various sources of nitrogen.*

Culture solution	Plant	Age	Berberine contents g./100 g.					
			Control samples			After experiment		
			Root	Main stem	Sub-sidiary branches	Root	Main stem	Sub-sidiary branches
(1) Containing inorganic nitrate	(a)	4	1.58	0.27	—	1.57	0.21	—
	(b)	5	1.56	0.37	—	1.48	0.74	—
	(c)	7	2.5	0.69	0.13	2.01	0.69	0.13
(9) Containing inorganic nitrate with glucose	(a)	3	1.31	0.40	0.13	1.56	0.77	0.22
	(b)	3	1.19	0.23	0.11	1.21	0.32	0.19
(2) Excess of ammonium nitrate Do. with glucose	(a)	7	1.57	0.64	0.13	1.66	0.45	0.13
	(b)	7	1.94	0.43	0.11	1.83	0.34	—
	(c)	7	1.86	0.40	0.09	2.05	0.78	—
	(d)	5	1.52	—	—	1.60	0.39	—
(6) Asparagine as source of N	(a)	7	1.22	0.36	0.08	1.62	0.58	0.16
	(b)	5	1.63	0.27	—	1.85	0.35	—
(7) Urea as source of N	(a)	7	1.69	—	0.11	1.68	0.47	0.10
	(b)	5	1.45	0.16	0.03	1.66	0.24	0.10
(8) Formamol as source of N	(a)	3	1.22	0.41	—	1.84	0.35	—
	(b)	3	1.25	0.37	—	1.56	0.54	—

Solution 1 containing a preponderance of the nitrates of calcium and potassium was given to three 7-year-old plants. From November until March no new growth was made, but from March to August the plants produced strong healthy shoots, masses of fibrous roots and flowered profusely.

With the exception of the main stem of (b), organs of the plants to which excess of nitrates was applied show a slight decrease in the amount of alkaloid present. This result is the more significant when compared with the results in Table II above, which showed that plants growing in ordinary garden soil accumulated berberine during the summer months. This point will be discussed later.

Glucose in addition (Solution 9) was used with two 3-year-old plants from February to August 1931. Growth was strong and the plants flowered twice during the season. This addition has caused a definite increase in the berberine content of both roots and stems of plants 3 years of age. The increase is slightly greater than could be accounted for by accumulation during the summer months in normal untreated plants, a fact which points to the conclusion that the addition of glucose to nitrate solutions causes the production of larger absolute amounts of berberine than is usual in untreated plants.

Ammonium nitrate as source of N (Solution 2). Three plants of 7 years (a, b, c), and one of 5 years (d) in age were watered with this solution for a period of 8 months extending from the beginning of January until the end of August. Previously plants (c) and (d) had received glucose from August to October. Strong rank growth commenced in April and continued until the plants were examined in August. Two of these plants (c) and (d) which had received glucose did not produce flowers, one (a) produced flower buds which aborted, while the remaining plant (b) flowered late but profusely. Fibrous root development was excessive. The ammonium nitrate is not responsible for increased berberine production in roots as the slight rise indicated in the table is normal for plants of this age.

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In two plants (*a*) and (*b*) the concentration in the main stems showed a definite decrease, while the stems and roots of (*c*) and (*d*) showed an increase, but it is significant that the latter plants were grown in a shaded situation and had received glucose.

The interpretation of these cultural experiments is difficult. The results with the application of the nitrates alone might be explained on the basis that the profuse growth resulting caused the utilisation of all the carbohydrate and nitrate available for protein synthesis, the carbohydrate being the limiting factor. When glucose is also applied however, there is an excess of both nitrate and glucose available, with possibly a production of amino-acids in excess of those required for protein synthesis and a resulting removal of this excess by increased berberine production. Such reasoning as this would entail that berberine synthesis would occur in those conditions in which soluble nitrogen and carbohydrate were available in excess of the immediate requirements of the plant. In this connection, it is to be emphasised that during the active growing period under the normal conditions there is a regular increase in berberine content from year to year.

Asparagine as the sole source of nitrogen (Solution 6). Two plants, (*a*) 7 years and (*b*) 5 years of age were given the asparagine solution during the periods January to August 1932 and April to August respectively. Both plants made vigorous growth and flowered freely. Of the two plants taken for experiment, the 7-year-old plant was noteworthy on account of a low alkaloid percentage in both root and stem. The effect of asparagine on this plant was to cause a very definite increase in berberine content in all parts, far greater than could be accounted for by annual accumulation. The 5-year plant did not show as great an increase, which might partly be accounted for by its much smaller leaf area. (One 7-year-old plant died before experimental samples were taken, due to absence of fibrous root development).

The berberine increase observed is due directly to the asparagine and it might occur in one of two ways. Asparagine yields aspartic acid on deamination and it is not unreasonable to suppose that on deamination this might yield malic acid. The production of this malic acid from an external source would possibly entail an economy of amino-acids or carbohydrate from which it would normally be formed, and cause an increased berberine production. On the other hand, asparagine or aspartic acid may be utilised directly in the building up of the alkaloid.

Urea as sole source of nitrogen (Solution 7). Samples of the root and stem of 7-year and 5-year-old plants were taken in April 1932 and the plants were watered with urea solution until the end of August. During this period both plants showed free healthy growth and flowered. Although growth was prolific the alkaloid content was not increased. The slight increase which occurred in plant (*b*) was not sufficient to show that urea acts differently from ammonium nitrate in its influence on alkaloid production, for this slight rise would have taken place in normal circumstances.

Formamol as sole source of nitrogen (Solution 8). Two 3-year-old plants were treated from August 1931 to July 1932. Growth was very slow and flowers were not produced. The application of formamol led to a definite increase in the berberine content of the roots of both plants under experiment. The stem of (*b*) also showed an increase, while that of (*a*) showed a fall in concentration of alkaloid which may be accounted for by assuming that during the period of experiment downward translocation was taking place into the rootstock.

The application of formamol in an acid soil is equivalent to the presentation

of formaldehyde and ammonium citrate. The plants to which this substance was applied showed very little growth and produced no flowers, yet analysis of their roots and stems gave high results for the concentration of berberine. Here then a significant increase occurred with very restricted growth. These experiments however have in common with the ones already described, in which an increase in berberine resulted, the fact that in every case there was provided a carbohydrate or possible carbohydrate intermediate (glucose, succinic acid, citric acid) along with nitrogen (nitrate, asparagine, ammonia). It might be argued that in the formamol experiments, because growth was very restricted, there would be little protein synthesis and that in these plants protein breakdown would preponderate because the leaves were ageing. This being so, an increase in berberine might be expected to arise from the breakdown products and the effect of the formamol treatment was merely to increase the berberine by limiting growth in this manner. On the other hand, the results from experiments in which little or no growth occurred because of deprivation of calcium or potassium must be borne in mind, because in these experiments no such increase occurred.

This group of experiments may be summarised therefore by saying that increased berberine production has been observed only when there has been supplied to the plant a substance or substances which provide it with an external source of nitrogen and simple organic acids, and that the increase resulting is independent of growth.

Effects of withdrawal of essential elements.

Culture solution containing no nitrogen (Solution 5). Five 7-year (*a* to *e*) and two 3-year-old (*f* and *g*) plants were deprived of nitrogen for the following periods: (*a*) and (*b*) November 1931 to April 1932: (*c*) and (*d*) November 1931 to June 1932: (*e*) November 1931 to August 1932: (*f*) and (*g*) May to August 1932.

Table VI. *Culture experiments with nitrogen-free, calcium-free and potassium-free solutions.*

		Berberine contents g./100 g.							
		Control samples					After experiment		
Culture	Solution	Plant	Age years	Root	Main stem	Sub- sidiary branches	Root	Main stem	Sub- sidiary branches
(5)	No nitrogen	(a)	7	1.83	0.47	0.11	2.22	0.86	0.17
		(b)	7	2.45	0.36	0.07	2.15	0.40	0.11
		(c)	7	1.96	0.46	0.09	2.34	0.29	0.10
		(d)	7	1.65	0.41	0.11	1.66	0.42	0.11
		(e)	7	1.81	0.39	0.13	2.02	0.39	0.12
		Mean values		1.94	0.42	0.10	2.07	0.47	0.12
		(f)	3	1.13	0.24	—	1.26	0.24	—
		(g)	5	0.99	0.16	—	0.97	0.18	—
	Mean values		1.06	0.20	—	1.11	0.21	—	
(3)	No calcium	(a)	3	1.43	0.19	—	1.59	0.21	—
		(b)	5	1.81	0.22	—	1.96	0.32	0.11
(4)	No potassium	(a)	3	0.84	0.20	—	0.86	0.21	—
		(b)	3	0.87	0.17	—	0.88	0.17	—

In all cases no growth took place, and during the winter the leaves of plants (*a*), (*b*) and (*c*) turned yellow and were cast. No young leaves appeared in spring and the plants became moribund and diseased. Plants (*d*), (*e*), (*f*) and (*g*) retained their leaves but made no growth.

Although the results of these experiments in the absence of nitrogen are somewhat erratic, ample proof is given that the alkaloid value does not fall even when conditions are optimum for utilisation of all available nitrogen. The figures in some cases show an increase during the time the plant is drawing upon its nitrogenous reserves, indicating that in conditions which appear to be unfavourable to the synthesis of alkaloid, its production still persists. These observations permit the conclusion to be drawn that the plant is unable to utilise the nitrogen of its alkaloid even when conditions are most favourable. The experimental results of Mothes [1928] showed that in *Nicotiana*, nicotine was still synthesised in conditions unfavourable to the growth of the plant. During nitrogen starvation, the nitrogenous reserves in roots, rhizomes and stems would be drawn upon for re-synthesis of protein at the growing points, until eventually complete exhaustion would take place, after which all soluble nitrogen would be derived from the breakdown of older leaf proteins. It seems remarkable that even in these circumstances the synthesis of berberine should still continue, and again it is apparent that there exists a parallelism between the synthesis of the alkaloid and the degree of protein breakdown prevailing.

Culture solution containing no calcium (Solution 3). From April to August 1932 one 3-year-old (*a*) and one 5-year-old plant (*b*) were deprived of calcium with the result that during the season no leaf or shoot growth took place, although flowers were produced in abundance. Deprivation of calcium, while checking the production of new growth did not seem to affect the seasonal rise in berberine content. The slight rise in concentration recorded in both root and stem has been shown to take place in plants growing in normal conditions.

Now calcium is considered to be an essential factor in the mechanism of protein synthesis and also in the building up of the cell wall, so that plants deprived of it usually show stunted growth and predisposition to disease. In the present experiment although the plants did not produce new growth they remained quite healthy, and analyses showed that during the period of experiment the concentration of berberine increased by an amount compatible with the normal rise during the summer months. During this period protein degradation would proceed normally, but the intermediates would not be capable of recombination to form protein at the growing points as lack of calcium inhibits this re-synthesis. The soluble nitrogen it is presumed would show a high value and a rise in alkaloid was observed.

Culture solution containing no potassium (Solution 4). During the same period as in the calcium experiments, two 3-year-old plants were watered with potassium-free solution. Again no new growth was observed, and one only (*a*) of the two plants flowered. The alkaloidal content of plants grown in the potassium-free medium remained unaffected as compared with controls, but growth processes being entirely checked, the rise in berberine content during the summer months was not so pronounced as under normal conditions.

Since potassium is considered to be an important factor in carbohydrate synthesis and translocation there would be a tendency towards carbohydrate deficiency in potassium-starved plants. From the experiments the evidence that carbohydrate concentration is of great importance in the determination of berberine concentration is convincing, and the experimental conditions of these plants would be similar to those in the set of experiments on the effect of nitrates without addition of carbohydrate. It appears that alkaloid concentration does not rise because there is a deficiency of carbohydrate in relation to the amount of available nitrogen.

PART III.

Effects of etiolation.

One plant (*a*) 7 years of age and 5 plants (*b* to *f*) 3 years of age were potted in sand, placed in complete darkness for varying periods and watered at fortnightly intervals with culture solutions 1 and 2, (*a*) and (*c*) receiving solution 2 (ammonium nitrate) and (*b*), (*c*), (*e*) and (*f*) solution 1 ($\text{Ca}(\text{NO}_3)_2$ and KNO_3). The results are recorded in Table VII.

Table VII. *Effects of etiolation.*

Plant	Period of experiment	Berberine content g./100 g.						
		Before experiment			After experiment			Etiolated shoots
		Root	Main stem	Sub-sidiary branches	Root	Main stem	Sub-sidiary branches	
(<i>a</i>)	Mar.-Aug.	2.06	0.43	0.14	2.07	0.53	0.26	1.5
(<i>b</i>)	May-Aug.	0.61	0.20	—	1.42	0.31	—	1.36
(<i>c</i>)	April-Aug.	1.13	0.23	—	2.12	0.50	—	1.2
(<i>d</i>)	June-Aug.	0.83	0.13		1.25	—	—	0.85
(<i>e</i>)	June-Aug.	0.97			—	—	0.05 *	0.72
(<i>f</i>)	May-Aug.	1.12	0.17	—	1.98	0.29	—	1.2

* Young shoots.

(*a*), (*b*), (*c*) and (*f*) cast their leaves and produced strong brilliant yellow shoots from the leaf axils and rootstocks, some of which attained a length of 18 inches, the average length being about 12 inches. Investigation of the anatomy of these etiolated shoots showed full development of vascular bundles with the surrounding ring of cells very rich in berberine. Plant (*d*) was ringed at a level of 6 inches from the surface of the sand. Etiolated shoots were not produced from the stem above the ring, but the plant put forth from the stem below the ring two strong shoots which contained a high percentage of berberine.

Plant (*e*) had produced young and strong shoots before being placed in darkness and these shoots showed rapid growth in the absence of light. It was observed that while the berberine concentration of that portion of the shoots which had grown under normal conditions was very low, the etiolated upper portions showed a high concentration.

Plant (*f*), after 3 months' growth in the dark was exposed to full light. Chlorophyll gradually appeared in the etiolated shoots which lost their yellow colour. Etiolated shoots of (*f*) after exposure to light for 2 weeks showed a berberine percentage of 0.47. Histological examination showed a disappearance of berberine from the upper portions of the shoots while the lower portions remained rich.

From Table VII it is apparent that growth in darkness is responsible for a marked increase in the concentration of berberine in all parts of the plant which were examined. The etiolated shoot was found to show a very high concentration in relation to the age of the tissue, and comparison with the values of young normal shoots reveals the fact that the influence of light is a very important factor in the production or location of the alkaloid. This result is in agreement with that of Ripert [1921] for the atropine and hyoscyamine contents of *Atropa*. The question thus arises as to whether the berberine which appears in such concentration in the etiolated shoot of *Berberis* is the result of translocation from the stem and root or whether it is synthesised *in situ* during the rapid growth

of the shoot. Consideration of the behaviour of plants (*d*), (*e*) and (*f*) affords support to the supposition that the alkaloid is produced in the actively growing etiolated tissue. Those parts of the shoots of plant (*e*) which had grown in normal conditions of light intensity showed very low concentrations of alkaloid, but in the region of the continued growth of the shoots after removal of the plant to darkness, the berberine concentration was found to be high, a fact which seems definitely to favour the view that alkaloid is produced in the growing tissues of the etiolated shoot. Plant (*d*) was ringed 3 inches above the soil surface and placed in darkness. No growth took place above the ring, but shoots which contained a high percentage of berberine were produced from the stem below the ring. As the removal of bark would prevent any downward translocation of berberine, the high value attributed to the shoots can only be explained as due to either synthesis in the region of growth or translocation upwards from the stem and root. Examination of the behaviour of etiolated shoots of plant (*f*) when exposed to light tended to confirm the view that the high concentration of alkaloid in the etiolated shoot is due to synthesis in the growing region and not to translocation, as the extended apical growth region (where growth had taken place in presence of light), showed practically an absence of berberine.

Rhizomes which had appeared above the ground (as shoots) presented an interesting appearance on histological examination, which revealed the existence of an abrupt fall in berberine content in the region of the stem immediately above the ground. In the portions of rhizome below the surface of the soil, cells of the cortex and xylem appeared rich in alkaloid but at the level of half an inch above the surface, the corresponding tissues of the young stem were practically devoid of berberine. This observation strengthens the assumption that the alkaloid chiefly accumulates in tissues which are not exposed to light.

These experiments on the effects of etiolation appear to justify the deduction that the connection between protein breakdown and alkaloid synthesis is very close, as increase of the former appears to lead to increase in the amount of alkaloid produced. With regard to the region of synthesis of berberine it is suggested that this takes place in the etiolated shoot itself from products of protein breakdown and sugar residues translocated upwards from the stems and roots (*cf.* plant (*d*)).

PART IV.

Ringing experiments.

In the following experiments a narrow ring of bark was removed from the main stem at a level of 1 ft. from the ground. The ring was vaselined and bound with tape. At the end of the periods stated analyses of the root and portions of the stem above and below the ring were made. The results are set out in Table VIII.

Table VIII. *Ringing experiments.*

Berberine content g./100 g.				
Plant	Period of experiment	Root conc.	Stem below ring	Stem above ring
(<i>a</i>)	May 1931–Oct. 1931	0.37	0.22	0.08
(<i>b</i>)	May 1931–Oct. 1931	0.31	0.23	0.13
(<i>c</i>)	Aug. 1931–Jan. 1932	1.21	0.36	0.21
(<i>d</i>)	Oct. 1931–Mar. 1932	0.88	0.21	0.18
(<i>e</i>)	Jan. 1932–June 1932	1.20	0.27	0.15
			Mean 0.26	0.15
(<i>f</i>)	Mar. 1932–Aug. 1932	1.83	0.94	0.48

Leaf and shoot growth on the stem below the ring was normal; above the ring no growth took place, the leaves assumed a pale green colour in the spring and summer but in September rapidly turned brilliant red.

In all plants except one (*d*) young shoots and leaves were removed from the stem below the ring as quickly as they appeared. The shoots and leaves which appeared below the ring in the case of (*d*) were allowed to remain.

Analysis of leaves above the ring revealed the presence of large amounts of tannin and carbohydrates, the latter including both reducing and non-reducing sugars, while the stem below the ring was relatively poor in these substances. In two instances (*a*) and (*b*) root development was completely inhibited and the root bark cast, and in the remaining plants fibrous root development was lacking.

The mean values for stems of the 3-year-old plants (*a*, *b*, *c*, *d*, *e*) show a high figure for alkaloid values above the ring when compared with figures for the corresponding tissues of unringed plants of this age (compare Table I, subsidiary branches, 3-year-old plants).

This fact is borne out by histological examination. Possible translocation of alkaloid in a downward direction therefore appears to be checked by the removal of the ring of bark.

The recent work of Maskell and Mason [1928; 1929] on the paths of translocation of food materials in plants has established the fact that the phloem is the tissue through which elaborated carbohydrate and nitrogenous substances pass in both upward and downward directions. Removal of a ring of bark from the stem would therefore impede the translocation of these substances to and from the root and rhizomes. The plants on which this experiment was made quickly lost their capacity for growth, and the leaves on the branches above the ring showed ageing. It was observed that in all tissues above the ring, accumulation of carbohydrate, tannins and anthocyanins took place, and, although analysis of these tissues for nitrogenous substances was not carried out, it is reasonable to assume that they would also accumulate in this circumstance. After the experimental period the roots in most cases gave evidence of starvation, casting of bark took place, and fibrous root development was completely inhibited. Analysis of parts above and below the ring gave varying results. The roots of plants in which decortication had taken place naturally were poor in alkaloid, while the mean values for the portions of stems above the ring were higher than the mean of this tissue in normal plants. Root and stem concentrations otherwise were normal for plants of this age. Unfortunately these experiments do not definitely elucidate the problem of the possible translocation of berberine, as the high mean value of the stems above the ring may be explained as being due either to obstruction of the alkaloid in downward movement or to an increased production, the result of the accumulation of carbohydrate and protein degradation products in the upper parts. Absence of fibrous root development would cause a deficiency of inorganic nitrogen in the upper region of the plant, as absorption of water could not take place to any extent. It would therefore be expected that the demand for soluble nitrogen in the tissues above the ring would be partially satisfied by disruption of the elaborated protein of leaves and stems. Carbohydrates being abundant, conditions favourable for the synthesis of berberine would be realised. Satisfactory conclusions regarding the transportation of berberine cannot be drawn from the results of these experiments and further work is necessary before the problem of the exact mode and place of berberine synthesis can be resolved.

SUMMARY.

1. In *Berberis Darwinii* the alkaloid berberine appears to be a waste product of metabolism as suggested by Pictet, for it accumulates in tissues which take no part in metabolic activities, and which are made up principally of dead elements. Synthesis of the alkaloid takes place in summer during the period of active growth, and no decomposition occurs during the winter or spring, so that the absolute amount present in the plant increases year by year.

2. The application of asparagine as a nitrogenous fertiliser leads to an increase in berberine content, whereas with ammonium salts and nitrates glucose must be given in addition. Growth on a nitrogen-free medium to the point of exhaustion does not bring about the decomposition of berberine, the normal increase with age was, in fact, observed, suggesting that synthesis of the alkaloid is connected with protein decomposition.

3. Etiolation leads to the production of shoots rich in berberine. If the bark be ringed these shoots appear only below the ring, suggesting that the berberine is synthesised from products translocated upwards from the root system.

4. Ringing experiments with plants grown in the light show an accumulation of berberine above the ring, suggesting that synthesis has taken place from products that are normally translocated from the leaves downwards to the root system.

5. Although the evidence is not conclusive, it appears that berberine is definitely a product of protein metabolism. It is suggested that synthesis is brought about by condensation of the products of protein breakdown and sugar residues.

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