THE INTER-RELATIONSHIPS OF THE ENZYMES AND THE ALKALOIDS OF BELLADONNA AND HYOSCYAMUS LEAVES.

by

James P. Todd.

May, 1931.

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of Glasgow University.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

INDEX.

Introductory.

The alkaloids have attracted much attention since the discovery of morphine by Sertürner over a century ago. While the chemistry of a great many of them is well understood, the problems connected with their role and mode of origin are still unsolved. They are generally considered to be connected with the formation of protein, either as stages in its synthesis or as bye products in its breakdown. On the other hand they are sometimes considered to be the products of reactions taking place in the cell as the result of conditions over which the plant has no control. This type of secondary reaction is frequently encountered in organic synthesis, where the formation of the main substance is accompanied by the formation of others in smaller amount but the occurrence of such secondary reactions in the plant cell does not seem feasible in view of the economy of energy and material so notable a feature of the metabolism of plants.

At the beginning of the last century it was believed that special vital powers were inherent in all living cells, but with the growth of organic chemistry and with the discovery of the enzymes this idea has been very much modified. Enzymes have been discovered which are capable

of synthesising and breaking down the carbohydrate, fat, protein and other constituents of the cell in vitro as well as in the living tissues. No such relationship has, so far, been found to exist between the enzymes and the alkaloids, but it does not seem improbable that such a relationship does exist.

It is the aim of this research to discover if such a relationship can be traced . With this object the fresh leaves of Atropa Belladonna and Hyoscyamus niger have been treated in order to encourage the enzymes contained in them to act under conditions with as few interfering factors as possible. An attempt has also been made to determine the specific enzymes involved in the changes in the amounts of the alkaloids.

The leaves of medicinal plants containing alkaloids are particularly suitable for such an investigation as the amount of the alkaloids is sufficiently large to enable accurate assays to be made. This renders it easier to detect small changes in the direction of either increase or decrease, following on any special treatment to which the leaves have been subjected.

In the living plant enzyme action is kept under strict control but during and after the removal of water, following the collection of the plant, the enzymes produce many far

reaching changes. These changes are sometimes of value in the preparation of plants of economic importance but are generally undesirable in medicinal plants, leading to loss of active principle and of other properties.

During storage, loss of active principle takes place. The evidence available from the literature regarding the loss of alkaloid in plants stored in the dried condition has also been considered and the possibility of enzyme action being involved, discussed.

A section dealing with the work of a group of plant physiologists has been included. The latter have been mainly concerned with showing that the alkaloids may be assimilated by growing plants and utilised as a source of nitrogen, and here the disappearance of alkaloids in the growing tissues is regarded from a different angle.

It is desired to express gratitude to Professors Ellis and Stockman and also to Dr Blodwen Lloyd for criticism and advice.

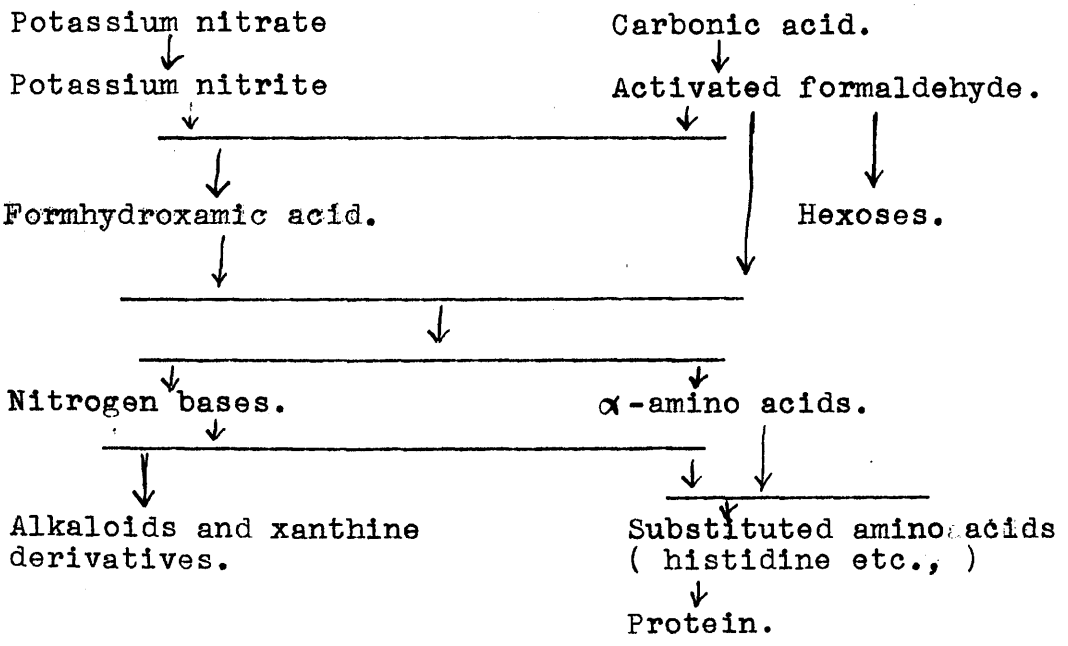
Chapter I.

THE NATURE AND THE ORIGIN OF THE ALKALOIDS.

In endeavouring to trace a relationship between the enzymes and the alkaloids of plants, it will be of interest to review some of the better known theories which have been advanced to explain the origin of the alkaloids. There seems little doubt that there is a connection between the alkaloids and the formation of protein, as many of the chemical groupings of the proteins are also present in the alkaloids, but it is undecided whether the latter are to be regarded as reserves of raw material in the synthesis of protein or whether they are in the nature of waste metabolites.

Baly, Heilbron and Hudson (I) consider that the plant exerts little vital control in the formation of the alkaloids. They consider that they are produced during the synthesis of nitrogenous compounds and that, given certain conditions, light and a sufficiency of the reacting substances, their formation is inevitable. They regard the following as representing the stages in photosynthesis, the alkaloids being produced in the process. The raw materials for the synthesis are potassium nitrate and carbonic acid both present in every plant.

Photosynthesis in the living plant according to Baly, Heilbron and Hudson.



The production of alkaloid according to the authors follows the production of an excess of the simple nitrogen bases; these being converted to alkaloids by methylation and condensation. The process being dependent on the action of light, the synthesis can only take place in the leaves.

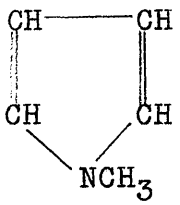
Since all the above conditions are satisfied in most ordinary green plants, (the presence of light, nitrate and CO_2), and as Baly and his collaborators consider that the above are the only possible courses which can be taken by the reactions, it is rather surprising to find the alkaloids limited , as they are, to a comparatively small

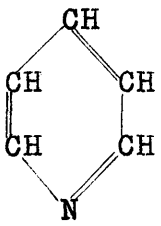
group of plants. If the hypothesis is correct an explanation must be sought for the comparative rarity of the alkaloids in ordinary green plants.

The authors allow that the plant is capable of a certain degree of control. This control must be largely enzymatic in nature. Gadamer (2) had previously stated as his opinion , that the primary products are the same for the alkaloids as for the proteins and that when the rate of assimilation is high, the alkaloids are formed as a nitrogen reserve. During periods of diminished assimilation of nitrogen, the enzymes which synthesised the proteins, attack the alkaloids and utilise the decomposition products for the formation of protein. This view is in agreement with the observed variation in the alkaloidal content of Belladonna leaves. During the flowering stage, when assimilation is intense , the alkaloidsincrease in amount and in Autumn as assimilation slows down, they gradually disappear. They are not rejected with the worn out leaves.

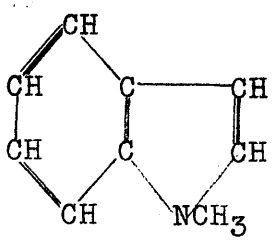
Pictet (3) held the view that the origin of the alkaloids lay in the decomposition, not in the synthesis of the proteins. The decomposition yielding pyrrole derivatives which by condensation with other substances or by intramolecular transformations resulted in the of the alkaloids. Pyrrole, for example, would be converted

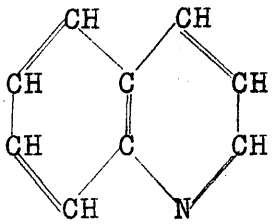
to a pyridine derivative by methylation of the nitrogen and then migration of the carbon atom of the lateral chain. Thus N-methyl pyrrole would give pyridine:-



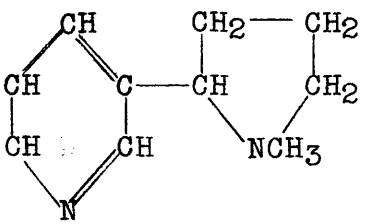


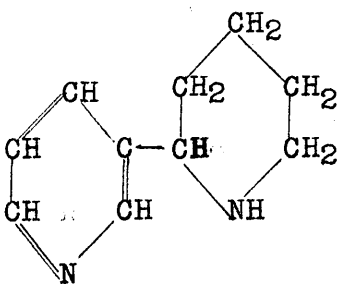
Pictet was able to perform this reaction by heat. Similarly Methyl indole would give quinoline.





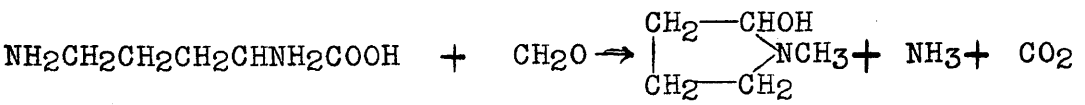
These views received considerable support by the isolation by Pictet and Rotschy (4) from tobacco, of three bases, in addition to nicotine, one of which, nicotimine, is an isomer of nicotine. But while nicotine contains a pyrrolidine nucleus methylated at the nitrogen atom, nicotimine appears to be a pyridyl-piperidine.



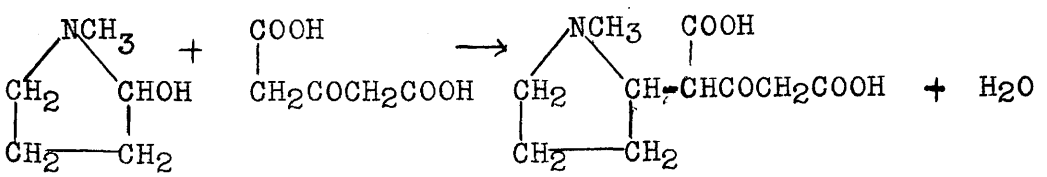


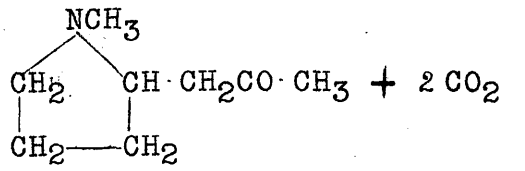
There seems to be the same relationship between these two compounds as exists between methyl pyrrole and pyridine. Pictet and Court (5) isolated pyrrolidine and N-methyl pyrroline from tobacco and a number of other plants. This was taken as additional proof of the theory.

Robinson (6) from his work on tropinone, considers that the alkaloids arise as the result of a series of simple reactions such as aldol condensations, oxidations and dehydrations. The raw materials,(formaldehyde, ammonia, amino acids and acetone dicarboxylic acid), for building up the alkaloids, either occur as such in the plant or in the combined state. These highly reactive bodies undergo a series of transformations ultimately leading to the alkaloid. Thus the pyrrolidine grouping would be produced by the condensation of formaldehyde with a diamino acid such as ornithine.



This compound would yield the alkaloid hygrine by condensation with acetone dicarboxylic acid and the subsequent elimination of CO_2





Hygrine

In the various theories of the origin of the alkaloids, with the exception of that of Gadamer, little mention is made of the enzymes. Control is assumed to be necessary but how the control is applied is not at all made clear. There can be little doubt that the reactions, many of which are of the type catalysed by the enzymes, are accelerated by these bodies. Many reactions taking place in the living cell are controlled and modified in a manner which is at present beyond our understanding. Raper (7) mentions the case of the enzyme amylase and the glycogen of the liver which in the living cell react only under strict control whereas in vitro interaction continues until all the glycogen is hydrolysed. The autolysis of plant tissues also illustrates this control or rather its disappearance. On the death of the plant the tissues are literally digested by the contained enzymes.

It would also appear from the theories outlined, that chemical synthesis in all the different plant families should be on exactly similar lines. These syntheses although similar in many respects lead also to many varying products. The different types of alkaloids help to illustrate this point. We find certain groups of alkaloids restricted

to a plant family, e.g. the morphine group in the Papaveraceae or the atropine group in the Solanaceae. The occurrence of an alkaloid such as berberine in several families is unusual. It seems, therefore, that while the mechanism of synthesis in plants ~~in plants~~ shows a general resemblance, each family has evolved along lines of its own.

The effect of cultural conditions on the alkaloidal content of plants.

A study of the effect of cultural conditions does little to help in the problem of the relation of the enzymes to the alkaloids. The Belladonna plant on account of its economic importance has been much investigated with a view to ascertaining the cultural conditions yielding more alkaloid. In Belladonna the improvement in conditions leads to a more vigorous plant and the largest and healthiest plants yield weight for weight the greatest amount of alkaloid. Poorly developed plants contain in proportion less alkaloid than well grown specimens. The addition of (8) fertilisers increases the yield, with the curious exception of nitrates (9) which when added to the soil in large quantities were found to decrease the amount of alkaloid yielded by Belladonna plants.

With increased intensity of illumination there is

a progressive increase in the alkaloidal content .

Deluard (I0) gives the following results:-

	Alkaloidal content
Plants grown in the shade.....	0.39%
Plants grown in moderate light.....	0.42%
Plants grown in sunshine.....	0.65%

In such researches so many factors are involved that it is difficult to trace those directly responsible for any change in the results. In Belladonna, the improvement in cultural conditions leads to improvement in the vigour of the plant but does not increase our knowledge of the factors responsible for the increase in alkaloidal content beyond that they are produced in largest amount by healthy plants.

The alkaloids accelerate respiration.

Palladin (II) has shown that the alkaloids increase the rate of respiration of plants immersed in solutions containing them. Is it possible that the alkaloids function as respiratory catalysts? In support of this possibility it may be mentioned that they are found in greatest amount in the regions of most intense metabolic activity such as the growing point, the fruit and the leaves; thus the amount of alkaloid is greatest during the flowering and fruiting stages and gradually decreases thereafter. The amount of alkaloid is greatest when respiration is most active.

The absorption and decomposition of alkaloids by plants.

Many attempts have been made to grow plants in culture solutions containing solutions of the various alkaloids in place of the usual nitrates or ammonium salts. The object in these experiments was to determine the part played by alkaloids in the living plant. In general the the various experimenters concluded that alkaloids could be assimilated and utilised as a source of nitrogen.

The concentration of the alkaloidal solution is important and some of the discrepant results obtained can be explained by the use of too strong solutions which seem to have a poisoning effect on the tissues. A similar effect is noted in the latter part of this work where certain concentrations of the alkaloid seemed to inhibit the action of the enzymes.

Knop and Wolf (I2) found that the extract of a plant is poisonous to the plant and cannot therefore be absorbed. They experimented with Maize and Buckwheat growing in culture solutions differing from ordinary water cultures in that the only sources of nitrogen were the nitrogenous bases urea, morphine, cinchonine, quinine and caffeine. These were found to be toxic to the plants in varying degree morphinebeing the least harmful. Nitrates of quinine and cinchonine were not harmful. From their experiments

they concluded that since the alkaloids are absorbed from the nutrient solution and since they are not found in the leaves they must be assimilated by the plants.

Reveil (I3) carried out extensive experimental work on alkaloidal assimilation by plants. Some of this is described below. He used solutions of the salts of the alkaloids equivalent to I-I000 of the free base, these solutions being added to the ordinary nutrient medium used in water culture experiments.

I. Two plants of the Balsam species which had been germinated in ordinary soil were moistened with (a).. plain water and (b).. with a solution of atropine. The latter opened its flowers on the I7th. July and its fruits ripened on the 28th. July, whereas the control plant flowered on the 29th July and fruited on the I2th. August.

II.. A shoot of Cherry Laurel placed in a solution of atropine showed no imcrease of growth, and died in about two months. No trace of atropine was found in the tissues.

III.. Aquatic mints were placed in water containing atropine. They developed very well and atropine disappeared from the nutrient solution. The alkaloid could be detected inthe leaves and stem, but when the plant was dried no trace of atropine could be found.

IV.. Hyacinth bulbs placed in solution of atropine flowered in the usual way. Atropine was found in the

flowers and leaves. When the bulbs were transferred to water, the alkaloid disappeared in four days.

V.. A Crocus corm placed in sand which had previously been calcined and moistened with a I-IOOO solution of atropine developed and showed atropine in the leaves and in all parts of the corm not buried.

VI.. Barley placed in previously calcined sand and watered with solution of atropine germinated more rapidly than a control and gave the reactions of alkaloids. When the plants were no longer watered with the alkaloidal solution they lost their alkaloid little by little and in ten days ceased to give any reaction for alkaloid. Similar experiments were made with morphine, narcotine, codeine, nicotine, quinine, cinchonine, strychnine and brucine. All of these were absorbed and apparently broken down by the plant; the less stable alkaloids such as atropine and codeine disappeared sooner than the others. The plant does not seem to be able to exert any selective influence, since it absorbs alkaloids whether they are harmful or not. Reveil found that quinine and cinchonine were toxic while ~~while~~ morphine codeine and nicotine were harmless.

Reveil concluded that added alkaloid can be utilised by growing plants, and further, that plants supplied with alkaloid mature more rapidly than controls.

De Varigny (I4) investigated the effect of atropine on Lentils and Water Cress seeds grown on purified sand. He found that they germinated more rapidly but were less vigorous than controls grown in distilled water. The average weight of the plants grown with atropine was 0.40 gm. that of the controls I.00 gm. De Varigny found further that when solutions of atropine were added to plants grown in earth they had an accelerating effect which increased in inverse ratio to the concentration.

In earth

I...Weight of water cress plant at the end of ten days.

Control.....weight.....I.25 gm

In solution of atropine (I-200).....weight.....I.25 gm

In solution of atropine (I-800).....weight.....I.400 gm.

In sand.

2...Weight of water cress plant at the end of ten days.

Control.....weight.....0.80 gm

In solution of atropine (I-I00).....weight.....0.36 gm

In solution of atropine (I-200).....weight.....0.45 gm

In solution of atropine (I-400).....weight.....0.70 gm

In solution of atropine (I-800).....weight.....0.70 gm

Comparing I and 2 it is seen that the alkaloid favours growth when the plants are grown in soil better than in sand. The explanation suggested is that the nitrates of the soil assist in the absorption and transformation of the alkaloid. Carr (loc. cit.) has shown that the addition of nitrates to the soil

lowers the yield of alkaloid. The soil also possesses great adsorbing powers not found in sand and this by lowering the active concentration would produce the same effect as a dilute solution.

Comère (I5) using Ulothrix and Spirogyra as test plants found that growth was possible in a nutritive medium containing no nitrogen other than the salts of certain alkaloids which were gradually added. He found that they were absorbed without apparent selective absorptive action. Some were readily assimilated as for example atropine, cocaine, and quinine; others, however, such as strychnine were markedly toxic.

The most marked feature of the above experiments is the disappearance of the alkaloids from such diverse types of plants. It is not conceivable that mechanism exists in these plants for dealing specially with these bodies but rather that the presence of foreign matter in the tissues stimulates the normal mechanism with the ultimate utilisation of the products. It is suggested that the most probable fate of the absorbed alkaloids is oxidation, and considerable support is lent to this theory by the finding of Palladin (loc.cit.) that the immersion of a plant in solution of quinine causes a great increase in its respiratory activity.

THE LOSS OF ALKALOID IN PLANTS DURING STORAGE.

During storage of dried plants loss of active principle is known to take place. As the plant in the dried condition is not affected by many factors influencing the living plant the evidence of the change being enzymatic is more clear. The loss of glucoside which frequently occurs is well known to be caused by slow hydrolysis brought about by enzymes;loss of alkaloid is harder to detect and the cause has not been explained.

Enzyme action is not inhibited in dried plants in the so called air dry condition in which they retain about ten per cent of moisture. In the presence of this remaining moisture the delicate colour of flower petals rapidly fades, whereas if the flowers are rendered perfectly dry before storage and precautions taken to prevent access of moist air, the colour can be kept almost indefinitely. Similarly Digitalis leaves which have been stored over quick-lime and are therefore perfectly dry, retain their therapeutic activity,but a loss of activity results if the leaves are stored in the air dry condition. All such changes cannot be considered as due to enzyme action. There is,however, little doubt that many of the changes taking place are the direct result of their action.

Bourquelot (I7) First drew attention to the effects produced by the oxidases in contact with the phenols. Amongst the phenols he included morphine which he stated was converted to an insoluble form. Debourdeaux (I8) had previously mentioned that the morphine in Opium seemed to undergo chemical change, becoming insoluble in water and at the same time being lessened in amount to a varying degree. He suggested that the change was due to an oxidase acting in the presence of air. Recently, Abraham, Digby and Rae (I9) found a similar decrease in in the morphine content of Opium. At times, however, there was actually a gain; they satisfied thamselves that this apparent anomaly was not due to experimental error and suggested enzymes as the cause of both phenomena. This loss in the morphine content of Opium was confirmed by Annet and Singh (20) and the presence of the oxidase in both fresh and dried Opium was demonstrated.The fluctuation in the morphine content is more difficult to explain although enzyme action is generally understood to be reversible. In this connection it is interesting to note that Heinrici (2I) claimed to have increased enormously the amount of morphine in the expressed juices of papaveraceous plants by the action of yeasts, diastase, pepsin, oxidases, potassium permanganate and hydrogen peroxide. In fourteen days the alkaloidal content of fresh extracts of Papaver somniferum was increased from

I.8% and 2.5% to 8.7% and I3.2% respectively, by these substances.

Ergot loses potency very rapidly. The British Pharmacopoeia requires that this drug should not be more than one year old and that it should rendered thoroughly dry and stored in air tight containers. The pharmacopoeias of other countries have similar requirements but it is doubtful if this is sufficient safeguard against deterioration in such an important drug. Wokes and Elphick (22) have shown that both the solid and the liquid extracts of Ergot undergo steady deterioration, half the initial activity being lost in a year or less. Enzyme action is not entirely responsible for the loss of activity , for the specific alkaloid ergotoxine was found, when isolated, to undergo deterioration both in the dry condition and in solution. The rate of deterioration increased with rise of temperature and it also occurred in vacuo although it was more rapid in air. Ergotoxine has an abnormally high molecular weight and is colloidal, and the deterioration has not been explained.

Several workers have drawn attention to the loss of alkaloid which takes place in Colchicum during drying. Deterioration does not as a rule take place in seeds, since they are organs in a resting condition and their vital activities are barely manifest. Catillon (23) has

shown that in dry seeds the enzymes are inactive but Blau (24) concluded that there was a loss of colchicine in Colchicum seeds after long storage. He found that the alkaloid is chiefly located in the seed coats and seems to disappear. Twenty year old seeds, not carefully stored contained 0.19% of alkaloid whereas carefully stored batches, thirty years old, gave 0.202% on analysis. His method of assay gave an average value of 0.504% for one year old seeds generally. Loss of alkaloid has been shown to take place in the green parts of this plant. Enzymatic activity is to be expected in those parts of the plant which contain large amounts of moisture and which were in active growth at the time of collection. Such enzyme action seems to take place during the comparatively short time taken to dry this plant. De Jean (25) found that tinctures made from the fresh flowers were relatively much more potent than tinctures made from the same batch when dried; similar results were obtained from the fresh and dried corm. The existence of enzymes capable of affecting the amount of alkaloid is thus indicated. The work of Grier (26) gives support to this. He determined the alkaloidal content of some old galenical preparations which were more than forty years old. No loss of colchicine had taken place.. It appears therefore that in the preparations from which enzymes have been excluded the alkaloid is stable.

Leseur,(27) using different methods confirmed the findings of De Jean (loc.cit.) He found that preparations from the fresh drug are more active than those made from the dried drug . In the latter the enzymes seemed to be inactive and both cold and boiling alcohol were equally effective as solvents for the active principles. With the fresh drug,however, boiling alcohol was the better solvent since the enzymes were active and their activity was not inhibited by cold alcohol.

The leaves of Pilocarpus may lose as much as 50% of the original alkaloid when stored in a damp place (Tunman and Jenzer, 28) but when stored in a dry place very little loss takes place. The presence of moisture would assist the action of the enzymes.

Another example of loss during storage, more of interest than of application to the present problem, is noted by Griffiths, (29), who, examining leaves of Erythroxylon truxillense obtained from ancient graves in Peru, of pre-Christian era, found that all the alkaloid had disappeared.

Loss of alkaloid in soft extracts.

Soft extracts have been shown by Ribaut (30) and also by Fricotel (3I) to undergo gradual loss of the contained alkaloid. The former found a progressive loss in soft solanaceous extracts when kept over a period of

four years. He attributed the loss to the presence of moisture. Support is given to a possible action by enzymes by the findings of Rosenthaler and Meyer (32) that loss of glucoside in soft extracts of Gentian, Cascara, and Rhubarb, made from the dried drug , is lessened by using boiling alcohol in place of cold alcohol, thereby preventing enzyme action. Fricotel also found loss of alkaloid to take place in soft extracts of Conium, Opium and Aconite. In Grier's experiments quoted above no loss of Colchicine was found to have taken place in the old extract whereas in the other extracts loss of the alkaloid occurred. It should be emphasised that in the preparation of Extract of Colchicum the expressed juice is boiled. This would of course inactivate the enzymes. The other extracts are not heated other than in the evaporation.

Summary of the first part.

I. The theories regarding the origin of the alkaloids are, to a great extent, based on purely chemical or photochemical reactions which take little account of the action of the enzymes. These theories do not explain the different directions taken by the syntheses in the different plants.

2. Enzyme action while bringing about many changes in the cell has no obvious connection with the formation of the alkaloids.

3. When plants are grown in solutions of alkaloids, the latter are absorbed and made to disappear from the tissues. The general vigour of the plant is improved.

4. Some of the alkaloids absorbed by plants are more susceptible to oxidation than others. The more easily oxidised alkaloids are assimilated by the plant with less difficulty than the others.

5. In dried plants diminution of the amount of the alkaloids also takes place. This is most marked in the case of morphine, an easily oxidised alkaloid.

6. There seems to exist a connection between the alkaloids and the nitrates as the presence of nitrates in the soil produces plants with lower alkaloidal content. The nitrates of alkaloids seem to be more easily absorbed.

THE INTER-RELATIONSHIPS OF THE ENZYMES AND THE ALKALOIDS OF BELLADONNA AND HYOSCYAMUS LEAVES.

Part 2.

Experimental.

The following plan has been adopted in tracing the relation between the enzymes and the alkaloids of Belladonna and Hyoscyamus leaves.

I.. The main types of enzymes present in the leaves have been determined.

2.. The different methods of inactivating the enzymes have been tried and an improved method adopted.

3.. Using this method the enzymes have been inactivated, the leaves dried, and the changes produced in the amount of alkaloid and in other characters determined.

4.. In this series of experiments the action of the enzymes has been assisted and the time of action prolonged. The properties of this leaf after drying have also been determined

5.. To eliminate the possibility of the loss of alkaloid which was found to occur being due to simple chemical change, the stability of atropine under the conditions of experiment has been confirmed.

6.. Experiments have also been carried out with the specific alkaloid of the leaves and with:-

(a). The enzymes contained in the juice of the leaves.

(b). The enzymes of the leaf residue after removal of the soluble enzymes.

(c). The oxidising enzymes tyrosinase and peroxidase which are present in the leaf.

DETERMINATION OF THE ENZYMES PRESENT IN BELLADONNA AND HYOSCYAMUS LEAVES.

No special comment has been made on the enzymes of solanaceous leaves, they do not seem to differ from the enzymes found in foliage leaves generally. Lepinois (33) recorded the presence of an oxidising enzyme and Onslow (34) classes the Solanaceae among the families containing both oxidases and peroxidases. There is no mention in the literature of any specialised enzymes capable of interaction with the alkaloids. The other enzymes are common to most green leaves.

The following quantitative tests were carried out for enzymes.

I. Diastatic enzymes.

Samples of the fresh leaves were divided into three portions, a, b, c.

(a). Extracted with water without preliminary treatment.

(b). Dried rapidly in a current of warm air and then extracted as (a).

(c). Maintained in the moist condition at 32' C.for three days then extracted in the same way as (a) and (b).

The extracts were heated with a little Fehling's solution, precipitation of cuprous oxide occurred in each tube.

(a). Very faint precipitate.

(b). Slight precipitate, denser than in (a)

(c). Distinct precipitate.

Diastatic enzymes are present which convert the starch of the leaf into sugars.

2. Oxidising enzymes..

a.....Tyrosinase.

Fresh Belladonna and Hyoscyamus leaves were pounded with sand under 96% alcohol and drained on a Buchner funnel using a water pump and a rubber dam (thin sheet rubber tied over the funnel) to complete removal of the alcohol and to avoid air as much as possible. This was repeated until a leaf residue was obtained free from alcohol soluble matter. The grey coloured residue was used in the following tests.

Five gm. of the residue suspended in I5 c.c. of phosphate buffer, pH 8, and 0.I gm of tyrosine added. The tubes were placed in an incubator at 32' C along with a boiled control. The test solutions gradually turned through reddish brown to brownish black and a precipitate was ultimately produced. The control darkened slightly but the difference was marked.

A solution of para-cresol similarly treated was oxidised to a reddish yellow compound by the leaf residue. Tyrosinase is present.

A very convenient method for the demonstration of the oxidising enzymes consisted in adding a little of the dry powdered leaf to a solution of the appropriate phenol absorbed by a piece of filter paper. Distinct colourations were obtained with solutions of guaiacol, catechol, and purified guaiacum solution when compared with a little of the leaf which had been treated under pressure with alcohol vapour. The colouration given by the test leaf was much intensified by the addition of dilute solution of hydrogen peroxide. Peroxidase is thus present.

Catalase. The evolution of a gas which proved to be oxygen on the addition of a few drops of solution of hydrogen peroxide to the freshly expressed juice shows the presence of the above enzyme.

Proteolytic enzymes are difficult to prove present in Belladonna and Hyoscyamus as the natural constituents of the leaf interfere with the tests. The usual method of allowing the leaves to undergo autodigestion for several days darkened the colour of the juice so much that tests for tryptophane were unsuccessful. (the leaves were placed in an oven and maintained at 32' C. for

several days. It is assumed that proteases are present.

4. Chlorphyllase.

During the experiments with the fresh leaves, which were maintained at 32° C. for several days, the green colour gradually faded if air was present. The leaf in which the enzymes had been inactivated did not show this loss.
Chlorophyllase is present.

Further experiments with the enzymes are carried out in later part of the work.

The above enzymes, with the possible exception of the oxidases, act only on specific substrates. Heinrici (loc. cit.) claimed to have produced morphine by the addition of diastases and pepsin to extracts of papaveraceous plants. It is not generally considered, however, that these enzymes react with alkaloids. To confirm this dilute solutions of the alkaloid atropine were added to solutions of the following enzymes:- (a). Diastase, (b). pepsin, (c). a mixture of pancreatic enzymes, (Liquor Pancreatis B.P.). In each case the alkaloid was all recovered after maintaining at a temperature of 32° C. for ten days.

THE METHODS OF INACTIVATING THE ENZYMES.

In addition to simple drying, methods of preserving vegetable tissues have been employed for centuries. Most of these methods have either arrested enzyme action or destroyed the enzymes, although the presence of the latter, as such, was not recognised. In the preservation of plants for medicinal purposes, the process by which the enzymes are inactivated is known as stabilisation.

The following methods of inactivating enzymes have been used experimentally:-

(I). Preservation by means of sugar.

Enzyme action is arrested by pulping the fresh tissues with sugar. The Confection of Roses of the Pharmacopoeia is an example of the use of this method. The preservative action of sugar has been well demonstrated by Carles (35) who pounded the fresh Kola-Nut with an equal weight of sugar and was thus enabled to keep the tissues white for seven years. On removal of the sugar by washing, the seeds immediately assumed the bright red colour characteristic of the Sun-dried seed. Carles claimed that the process is as effective as sterilisation with boiling alcohol. Belladonna leaves pulped with sugar

have remained a dark green colour for four years and have remained perfectly sound. The alkaloidal content was not determined as the sugar interfered seriously with attempts to dry the leaves to constant weight and also as the ethereal ammoniacal extracts of the leaves emulsified very badly when shaken with dilute acid. This difficulty was later overcome. The reaction for atropine could still be obtained after three years.

2. The following modification of the method of pulping with sugar has been suggested by Golaz (36) as suitable for every day use. The fresh tissues are pulped with sugar and immersed immediately in a mixture of glycerine and boiling alcohol. The glycerine absorbs the water and prevents the lowering of the destructive effect of the alcohol by dilution, on the enzymes.

3. Stabilisation with steam under pressure.

This method was employed by Arnould and Goris (37) in studying the chemistry of the fresh Kola Nut. The fresh tissues are treated with steam under pressure in an autoclave so that the enzymes are effectively destroyed. The process has the disadvantage that plant tissues are generally unable to withstand such severe treatment and become covered with a mucilaginous layer which renders drying very difficult. Belladonna leaves treated in

in this way at half an atmosphere pressure above normal were reduced to a pulpy mass which made further treatment almost impossible.

4. Stabilisation with alcohol or its vapour.

Bourquelot (38) used boiling alcohol to destroy the enzymes in fresh plants and was thus able to detect the presence of hitherto unsuspected glucosides as these had been hydrolysed during the ordinary drying processes. The method while useful as a pioneer process had the disadvantage that the dilution of the alcohol with the water of the tissues, prevented complete inactivation of the enzymes. To overcome the difficulty it was necessary to use large volumes of alcohol. Bourquelot's method consisted of the addition of the fresh tissues a little at a time to boiling 96% alcohol containing a little calcium carbonate to neutralise the plant acids.

The method was tried with Belladonna leaves. A noticeable feature was the completeness of the solvent action on the cell contents. The latter seemed to dissolve completely out leaving the cellular tissues as a grey coloured mass, the alcohol becoming deep green. The contrast between the fresh leaf treated thus and the same leaf treated in a similar way after drying is striking. In the latter case the leaf residue after boiling with the alcohol was brown in colour; much of the cell contents

seemed to have been rendered insoluble during the drying process.

The method of Perrot and Goris.

Perrot and Goris (39) modified Bourquelot's method by using alcohol vapour under pressure in an autoclave. They found that after two minutes with a pressure of an atmosphere above normal, the enzymes were destroyed and the product could be kept indefinitely.

Dhers and Pugol (40) modified the above method by using two autoclaves joined together and heated by steam. A valve controlled communication and after raising a pressure of three atmospheres in the first autoclave, this valve could be opened and a pressure of one and a half atmospheres obtained in the second autoclave containing the drug.

In the various stabilising processes reviewed, heat is the essential factor for the destruction of the enzymes. In those processes which only suspend enzyme action, sugar and glycerine are shown to be useful. Alcohol vapour in conjunction with heat is the most effective method as it destroys the enzymes effectively, leaves the tissues in a convenient condition for further manipulation and induces no great changes through elevation of the temperature. As the result of the

experiments carried out with the various methods advocated for the inactivation of the enzymes in plant tissues the following apparatus was devised. With it control of the temperature and pressure over a wide range was possible.

Apparatus used in the experimental inactivation of the enzymes.

I. An autoclave fitted for heating by gas but adapted to electricity to minimise the risk of fire. The heating element consisted of a winding of Nichrome wire, the heating surface being suitably insulated and lagged by means of asbestos.

2. The stabilising chamber, a heavy cast iron vessel also heated by a winding of Nichrome wire and well insulated. The temperature was indicated by Cambridge type dial thermometer and the pressure was read of on a pressure gauge.

3. A variable resistance in series with the windings of both vessels allowed control of the temperature over a wide range.

The autoclave communicated with the stabilising vessel by a flexible copper tube which was heavily heat insulated with asbestos rope to prevent the condensation of the alcohol in its passage to the stabilising vessel.

Condensation of the alcohol released from the stabilising vessel was accomplished by an exhaust flask attached to a pet-cock in the foot of the stabiliser, the side arm of which opened into a condenser cooled by a water jacket. By this arrangement the alcohol condensing on the leaves or water displaced by the alcohol from the leaves was collected and after evaporation returned to the appropriate batch of leaves after they had been powdered.

The procedure for stabilisation consisted first in heating the autoclave until alcohol vapour escaped freely from the escape valve communicating with the outer air. Meanwhile the stabiliser had been heated to a temperature approximating to that of the autoclave. The leaves were then placed loosely in a wire basket which fitted the stabiliser but remained clear of the bottom and sides. Next the cover was replaced and the alcohol vapour allowed to enter at the desired pressure. At the end of the allotted time, the communicating valve was closed and the alcohol vapour blown off and condensed. The leaves were then removed and dried in the drying chamber.

The chief difficulty of this method is that condensation of the alcohol takes place in the stabiliser and although this was reduced in amount as more experience

was gained in working the apparatus, the difficulty was never quite obviated. The condensate was mainly aqueous but contained also a variable amount of alcohol with a little of the alkaloid of the leaves in solution.

A second disadvantage at first encountered was the tendency of the alcohol to wash out some of the alkaloid by displacing the water of the cells; by the method of trapping the condensate in the stabiliser loss of alkaloid was prevented as it was recovered from the exhaust flask. In a trial experiment, assays of the alkaloid in the condensate gave an average value of 0.03 gm of alkaloid from 100 gm. of the leaf, calculated on the dried leaf. In subsequent stabilisations, the condensed alcohol was evaporated and returned to the appropriate batch of leaves after they had been dried and powdered. It was possible to reduce the amount of alcohol condensed by raising the temperature of the stabilising vessel but as the leaves were subjected to this high temperature for some time before the entrance of the alcohol, this was considered inadvisable. The elevation of the temperature had a tendency to cook the leaves in the same way as the treatment with steam.

Tests of the ability of the apparatus to inactivate the enzymes of the leaves.

The apparatus was tested by subjecting fresh leaves

to the vapour of alcohol under pressure of one half of one atmosphere above normal for five minutes. The peroxidases have been shown by Aurousseau (4I) to resist inactivation in this way more than any of the other enzymes, they are also easily detected and were therefore chosen as the indicators of the efficiency of the apparatus.

The test was carried out by placing a drop of the buffered indicator on a piece of filter paper and then allowing a little of the powdered leaf to rest on the spot of indicator for some time. For comparisom a little of the leaf which had been dried without alcohol vapour pressure was used in the same way. A colouration was obtained with the untreated leaf which on the addition of solution of hydrogen peroxide was much intensified. No colouration was obtained with the stabilised leaf showing that the treatment had inactivated the enzymes.

Tests were similarly carried out with leaves under a pressure of one quarter of one atmosphere above normal, but unless the leaves were very carefully arranged so that no area was present through which the alcohol was unable to penetrate, the results indicated that the enzymes were not properly inactivated.

The indicators used, consisted of solutions of para-phenylene diamine and guaiacol in phosphate buffers

at a pH of 8. A solution of Guaiacum resin in alcohol was also used. The latter was purified before use by boiling with charcoal to remove any traces of peroxide in the resin.

The appearance of the stabilised leaves.

The leaves after stabilisation by this method were much deeper in their shade of green than the control. On drying the depth of colour was retained and the leaves were harsh, brittle and rather unsightly. They dried very rapidly in a current of warm air and in each experiment were dry several hours before the control batch of leaves. The alcohol vapour seemed to have affected the permeability of the cellular tissue so that water vapour escaped more easily.

On microscopic examination the stabilised leaves were found to have suffered structural disintegration. This applied particularly to leaves which had undergone treatment at high temperatures and pressures. There was even destruction of some of the characteristic features on which reliance is placed for the microscopical identification of the leaves in the powdered state. The typical solanaceous stomata and the striations of the cuticle were unrecognisable. As was to be expected, the more resistant calcium oxalate cells had suffered little change

and the cells containing it were readily seen after removal of the colouring matter which obscured detail more than in the untreated leaf. With increased pressures of one quarter to one half of one atmosphere the microscopic features were much less affected and differed chiefly from the ordinary leaf in the depth of the green colour.

Chevalier (42) considers that in the fresh state the constituents are in the form of soluble, very complex, easily decomposed compounds readily oxidised during the drying process or even split by the so-called neutral solvents. Even the mineral constituents he considered to be in the same state and these latter are the first to be altered passing to the crystalline form by the action of heat or air. He therefore holds that the crystalline principles as isolated from plants are the result of more or less violent decompositions , the product varying with the method of extraction.

In drugs stabilised by alcohol vapour the inactivation of the enzymes seems to preserve the tissues in a condition approximating to that of the fresh plant. The colloidal state of the original cell contents is largely retained and this proved to be a very troublesome point in the estimation of the alkaloids as intractable emulsions were formed.

THE EFFECT OF THE DESTRUCTION OF THE ENZYMES ON THE ALKALOIDS.

The determination of the amount of alkaloid in the leaves.

The assay of Belladonna and Hyoscyamus leaves in the ordinary dried condition does not present any special difficulty. Some of the methods seem at times to yield emulsions in the course of the assay which by preventing clean separations lower the accuracy of the processes. In general the development of emulsions can be avoided by using relatively large volumes of the solvents and avoiding violent shaking during the shaking of the immiscible solvents with one another.

In the present work the attempts to carry out assays of stabilised leaves were at first made quite impossible by the formation of such emulsions; not the emulsions generally encountered in work of this kind where a layer of the solvents emulsifies at the interface , but an emulsion which involved the whole of both solvents and did not separate on standing for several weeks. On microscopic examination they were found to be of the oil in water type, the oil being represented in this case by the volatile solvent, (chloroform or ether). Many different processes were tried including those of the British Pharmacopoeia 1914, the United States Pharmacopoeia, Auenmuller's method (43), Panchaud's method (44) and others but none was found to overcome the difficulty.

Experiments were also carried out on the lines suggested by Clayton (45). Calcium oleate was added to the mixed solvents before they were agitated together in the hope that as this substance forms emulsions of the water in oil type the opposing influence of the emulsifying agents would cancel one another and prevent emulsification. A certain degree of success was achieved but this method was abandoned for a process which by dissolving away the troublesome substances prevented the trouble arising.

It must be emphasised that emulsification only occurred to any extent in the assay of stabilised leaves, although special modifications had to be adopted for the fresh juice and leaves. The emulsification took place when any attempt was made to extract the ether or ether chloroformic mixtures obtained in extracting the leaves. The substance causing the emulsification was found to be chlorophyll and similar ether soluble bodies which exist in the fresh leaf as hydrated sols, the dispersion medium being the watery cell sap. The trouble was overcome by the following process in which the interfering substances are dissolved out by treatment with ether, without dissolving the alkaloids which are fixed by the previous addition of a little sulphuric acid.

The steps in the process are as follows:-

(I). The removal of ether soluble matter in acid solution.

The finely powdered leaves dried to constant weight in a hot water oven, of this I0 gm. taken for each assay. This was triturated in a small glass mortar with three c.c. of I0 per cent sulphuric acid. The acid was completely absorbed forming a crumbly powder which was transferred to a 250 c.c. flask with a closely fitting rubber stopper. (Rubber is slightly affected by ether but was found to be more satisfactory than glass stoppers which do not prevnt the escape of ether vapour so well.) The mortar used in the absorption of the acid was swept out with a small camel hair brush.

To the contents of the flask I00 c.c. of ether (S.G. 0.720) were added, the flask securely stoppered and shaken for one hour in a shaking machine. The etheral solution of inert matter was drained off through a plug of cotton in a dry funnel. The contents of the flask were allowed to dry for 24 hours, the plug of cotton returned to the flask and any trace of leaf residue brushed in.

The ether thus drained off yielded no reaction for alkaloid when tested in the usual way with Meyer's reagent.

Extraction of the alkaloid from the purified leaf.

To extract the alkaloid from the purified leaf, I00 c.c.

of ether and 25 c.c. of chloroform were added to the contents of the flask, then three c.c. of a I0 per cent solution of ammonia and the flask rapidly shaken in the shaking machine for thirty minutes. Eighty c.c. of this solution were taken on every occasion, representing six and a half gm. of the original substance. Filtration was carried out in a closed funnel, the filtrate being caught and measured in a flask with a narrow neck ringed at the desired volume. The ethereal filtrate was transferred to a separating funnel rinsing in with a few c.c. of ether. The amount of ether lost during filtration was negligible.

Extraction of the alkaloids from the ethereal solution as sulphates.

The ethereal solution was extracted once with 20 c.c. of N/I sulphuric acid, and then thrice with I0 c.c. Each lot of acid was filtered as it was drawn off. Theoretically only three extractions are necessary to extract practically all the alkaloid, but on practical grounds the fourth extraction is an advantage as with it the stem of the separator and the filter can be rinsed free of the previous extractions.

Extraction of the alkaloids with chloroform.

The mixed acid solutions were made alkaline with ammonia and extracted with chloroform as above. The mixed chloroformic solutions were washed with a little

water and filtered into a wide-mouthed beaker flask. The filter was washed with five c.c. of chloroform and the chloroform was finally evaporated in a current of warm air.

Removal of volatile bases.

The volatile bases consisting of pyrrolidine derivatives and a trace of pyridine (46) were removed by heating on a water bath for thirty minutes. Markwell and Walker (47) have shown that this heating has no deleterious effect on the alkaloid.

Titration of the residue.

The residue, which was obtained in most of the assays as a crystalline mass, was dissolved in 10 c.c. of neutral absolute alcohol and 25 c.c. of boiled, cooled, distilled water were added, two drops of solution of methyl red as indicator and then five c.c. of N/10 sulphuric acid. Back titration was carried out with a solution of sodium hydroxide which had been protected from CO_2. The alkaline solution was freshly prepared from a concentrated solution for each set of determinations and its neutralising power ascertained.

The effect of the destruction of the enzymes on the alkaloidal content of Belladonna and Hyoscyamus leaves.

The leaves were divided into three or more portions one being dried and retained as the control and the others treated with the vapour of alcohol under pressure.

Table I. Belladonna Leaves.

Batch I.

A. Stabilised at one and a half atmospheres pressure above normal.

Per cent alkaloid.....	0.308
.....	0.310
.....	0.289
Average for lot A.....	0.302

B. Stabilised at one atmosphere pressure above normal.

Per cent alkaloid.....	0.285
.....	0.300
.....	0.305
Average for lot B.....	0.296

C. Control. Dried in air without treatment.

Per cent alkaloid.....	0.306
.....	0.285
.....	0.277
Average for lot C.....	0.289

The effect of the destruction of the enzymes on the alkaloidal content of Belladonna and Hyoscyamus leaves.

Table 2. Belladonna Leaves.

Batch 2. divided in three portions.

A. stabilised at one atmosphere pressure above normal.

Per cent alkaloid.....	0.379
.....	0.383
.....	0.402
Average for lot A.....	0.388

B. stabilised at one half of one atmosphere pressure above normal.

Per cent alkaloid.....	0.404
.....	0.397
.....	0.432
Average for lot B.....	0.4II

C. Control dried in air without treatment.

Per cent alkaloid.....	0.404
.....	0.402
.....	0.390
Average for the control	0.399

The effect of the destruction of the enzymes on the alkaloidal content of Belladonna and Hyoscyamus leaves.

Table 3. Belladonna Leaves.

Batch 3. Divided in three portions.

A. Stabilised at one atmosphere pressure above normal.

Per cent alkaloid.....	0.635
.....	0.681
.....	0.661
Average for lot A.....	0.659

B. Stabilised at one half of one atmosphere pressure above normal.

Per cent alkaloid.....	0.673
.....	0.634
.....	0.625
Average for lot B.....	0.643

C. Control dried in air without treatment.

Per cent alkaloid.....	0.660
.....	0.656
.....	0.638
Average for the control.....	0.651

The effect of the destruction of the enzymes on the alkaloidal content of Belladonna and Hyoscyamus leaves.

Table 4. Hyoscyamus Leaves.

Batch 4. Divided in two portions.

A. Stabilised at one atmosphere pressure above normal.

Per cent alkaloid..... 0.139
..... 0.155
..... 0.135

Average for lot A..... 0.143

B. Control dried in air without treatment.

Per cent alkaloid..... 0.135
..... 0.143
..... 0.147

Average for the control..... 0.143

In the assay of the Hyoscyamus leaves 20 gm. was taken instead of 10 gm. on account of the lower alkaloidal content of these leaves. The other quantities were then taken in proportion.

Discussion of the results obtained by inactivating the enzymes of fresh Belladonna and Hyoscyamus leaves.

Dhers and Pugol (loc.cit.) found that by inactivating the enzymes of Tobacco leaves and Broom tops a loss of alkaloid could be detected in a control which had been dried in air. One of the main objects in the stabilisation of the leaves in this case was to repeat this work with plants containing alkaloids of a different type from those of Tobacco and Broom which are volatile, non oxygenated , and unstable in air. The alkaloids of Belladonna and Hyoscyamus are non volatile, oxygenated and are comparatively stable in air.

An inspection of the figures on pages 46 to 49 show that with Belladonna and Hyoscyamus this gain in the alkaloidal content of the stabilised plant does not take place. It is noteworthy that sparteine and coniine are so much more readily oxidised by air than atropine; The catalytic oxidation by enzymes seems to be involved for it must be remembered that the plant after inactivation of its enzymes by alcohol vapour is exposed to the simple oxidising influence of the air during the time taken to remove the moisture from the tissues. In Broom and Tobacco with their easily oxidised alkaloids the time during the drying is sufficiently long to allow of the

oxidation of the alkaloid. In Belladonna and Hyoscyamus with their less easily oxidised alkaloids the time taken by careful drying is not sufficient to bring about a loss. Proof of this is given in the next part in which by prolonging the time during which the enzymes are allowed to act in the presence of air a distinct loss takes place.

The stabilised drug differs in many respects from the drug which has been dried in air without treatment. The latter differs greatly in the solubility of many of its constituents. Thus the stabilised leaf dropped into boiling alcohol parts with most of its colouring matter like the fresh leaf, whereas the leaf which has been dried first, leaves a brown coloured residue. This is further support to the conclusion in the paragraph above that oxidation in the leaf during the removal of water is largely brought about by the enzymes.

The stabilised leaf also yields its soluble matter in a form resembling that in which it exists in the fresh leaf. This is evident from the tendency to produce emulsions with immiscible solvents and the aqueous acid solution. The colloidal cell juices of any fresh plant produce similar emulsifcation.

In the light of the above, the ordinary drying process seems to affect the colloid condition of the cell

contents. Substances which are normally present in the colloidal condition seem to pass to non-colloidal forms as they lose moisture in the drying leaf. The change does not seem to be reversible. In the stabilised leaf the drying does not prevent the imbibition of moisture when this is brought in contact with the cell contents, and from this aspect the inactivation of the enzymes of leaves is undesirable.

THE AUTOLYSIS OF BELLADONNA HYOSCYAMUS LEAVES.

In the last series of experiments with the enzymes and the alkaloids of Belladonna and Hyoscyamus leaves, the enzymes were inactivated and, as a standard for comparison was necessary, the leaves carefully dried were chosen as the control. In the present series the the action of the enzymes has been encouraged as far as possible and the action on the alkaloids again noted. The rapidly dried leaf has again been chosen as control since in the last series of experiments it was shown that it suffers no visible loss of alkaloid during careful drying.

Certain plants are only of value economically when subjected to conditions which favour the action of the enzymes in them. The required conditions are, (a), the elevation of the heat level to that temperature which induces the greatest activity of the enzymes; (b), the prevention of loss of moisture without which the action of the enzymes ceases. Commercially, Tobacco leaves are treated in this way and a very elaborate technique has been evolved for their preparation. As Tobacco, Belladonna and Hyoscyamus are all members of the same

family it is interseting to survey briefly, the methods used in furthering enzyme action in Tobacco leaves with a view to the application of such methods to the present problem.

The methods employed in the different countries differ in detail but the general principles outlined above are always observed. In the most extensively used method the leaves are hung in barns the ventilation of which is under strict control. Heated air from fires placed outside is passed in through flues. The leaves pass through three stages, I.. in which the enzymes are very active and the colour of the leaf changes gradually to lemon yellow; 2.."Fixing" the colour , accomplished by raising the temperature of the curing shed and at the same time allowing free ventilation which had previously been restricted to prevent loss of moisture. 3.. The leaf is dried by a still further increase in temperature.

In stage I, the accelerating of the action of the enzymes, the temperature commences at 33' C. and is gradually raised towards the end of this stage to 53' C. This later elevation of the temperature rapidly brings enzyme action to a stop thus preventing further change in colour . The continued elevation of the temperature in stage 2 finally accomplishes this (53'-7I' C.)

Still further elevation of the temperature with free ventilation completes the drying of the leaf.

The process takes from four to six days to accomplish and it is stated that the barns are redolent with the vapour of ammonia and nicotine while "curing" is proceeding.

It is sometimes stated that bacteria and yeasts assist in the process and are responsible for many of the changes. (48). While these organisms are invariably present and are capable of modifying the process they are not responsible as is shown later in the experiments with Belladonna and Hyoscyamus leaves. The latter were induced to undergo changes similar to those produced in tobacco, under sterile conditions.

In the first of the present series of experiments carried out with Belladonna and Hyoscyamus leaves, no precautions were taken to ward off the attacks of moulds and bacteria and the leaves were rendered unfit for further treatment in a few days by their attacks. It is difficult to understand ~~the~~ how it is possible to carry out large scale "curing" without serious interference from microorganisms.

By dusting the surfaces of the leaves with finely powdered thymol it was found possible to keep the leaves in the warm atmosphere of an incubator without interference from either bacteria or moulds. Under these conditions the leaves rapidly developed a rich brown colour, never yellow, and the odour became rather like that of the heavier qualities of tobacco.

The leaves were loosely arranged in large Bulloch jars which, while allowing free entrance of air, did not allow them to dry. The temperature was maintained at 32° C. during the process by keeping the jars in an incubator as used in the culture of bacteria.

During this treatment the alkaloids suffered a diminution in amount as shown by the following tables.

The effect of the prolonged action of the enzymes on the alkaloidal content of fresh Belladonna and Hyoscyamus leaves.

Table 5. Belladonna Leaves.

Batch 3 divided in two portions.

A. Maintained et 32' C. for 240 hours.

Per cent alkaloid..... 0.525
..... 0.537
..... 0.538

Average..... 0.533

B. Control dried in air without treatment.

Per cent alkaloid..... 0.660
..... 0.656
..... 0.638

Average..... 0.65I

Loss of alkaloid in I00 gm. of the treated leaf calculated on the leaf dried at eighty degrees Cent.

..... 0.II8 gm

Percentage loss of alkaloid as above..... I8.I

The effect of the prolonged action of the enzymes on the alkaloidal content of Belladonna and Hyoscyamus leaves.

Table 6. Belladonna Leaves.

Batch 5. divided in four portions.

A. Maintained at 32' C. for 72 hours.

Per cent alkaloid	0.588
.....	0.584
.....	0.597
Average	0.590

B. Maintained 32' C. for I20 hours

Per cent alkaloid	0.570
.....	0.545
.....	0.545
Average	0.553

C. Maintained at 32' C. for 2I6 hours.

Per cent alkaloid	0.520
.....	0.506
.....	0.535
Average	0.520

D. Control dried in air without treatment.

Per cent alkaloid	0.65I
.....	0.642
.....	0.638
.....	0.660
Average	0.648

Loss of alkaloid in I00 gm . of the dried leaf 0.I28 gm

Percentage loss of alkaloid as above..... 20.I

The effect of the prolonged action of the enzymes on the alkaloidal content of fresh Belladonna and Hyoscyamus leaves.

Table 7. Hyoscyamus Leaves.

Batch. 4. divided in three portions.

A. Maintained at 32° C. for 96 hours.

Per cent alkaloid.....	0.II7
.....	0.II7
.....	0.II5
Average.....	0.II7

B. Maintained at 32° C. for 2I6 hours.

Per cent alkaloid.....	0.099
.....	0.099
.....	0.II2
Average.....	0.I04

C. Control dried in air without treatment.

Per cent alkaloid.....	0.I26
.....	0.I04
.....	0.I45
Average.....	0.I25

Loss of alkaloid in I00 gm. of the treated leaf calculated on the leaf dried at eighty degrees Cent.

..... 0.02I gm.

Percentage loss of alkaloid as above.....I6.8

The effect of the continued action of the enzymes seems to be to cause a diminution in the amount of the alkaloid in the leaves; up to 20% is lost in this way in 10 days. Experiments carried on for longer periods showed no great increase in this figure. The loss seems to result from enzyme action. To avoid the possibility of simple hydrolysis of the alkaloid having taken place experiments are carried out in the next section which show that under the conditions of the experiment it is quite stable.

The leaf after treatment was rich brown in colour and had a pleasant rather treacly odour. Commercially Belladonna leaves are sometimes met which are brown in colour instead of green. This may be taken to indicate that insufficient care has been exercised during the operation of drying.

When the brown coloured leaf obtained in the above experiments is treated with boiling alcohol a certain amount of green colour is extracted but the residue remains deep brown and is quite unlike the exhausted tissues left when fresh or stabilised leaves are similarly treated.

On assay, emulsification hardly occurred at all and any of the processes commonly used gave no trouble. The

process described earlier was adhered to throughout for the sake of uniformity.

The added thymol did not interfere with the assays as it was volatilised when the powdered leaf was heated to constant weight in the hot water oven.

THE STABILITY OF ATROPINE IN SOLUTIONS OF VARYING HYDROGEN ION CONCENTRATION.

Squire (49) states that atropine in aqueous solution, slowly decomposes when heated on the boiling water bath. As the heating is continued, the solution gradually ceases to give the characteristic precipitate with mercuric chloride and the alkaline reaction gradually disappears. Henry (50) also states that atropine in aqueous solution gradually deposits resinous decomposition products as the result of oxidation. The time necessary to bring about the oxidation is not mentioned. The writer was unable to obtain the formation of resinous decomposition products when an aqueous solution of atropine alkaloid was exposed to the air for two months. The oxidation must therefore be very slow.

It is interesting to note that a museum specimen of the alkaloid which has been exposed to light in a sealed glass tube contained in a glass fronted case, for about five years, shows a brown colour on the side turned to the light. The same colouration can be observed in other alkaloids stored in the same case and also on the

side exposed to the light.

To ascertain the effect of heating at 32' C. on solution of atropine under conditions similar to those existing in the leaf, the pH of the freshly expressed juice was determined. Freshly collected Belladonna leaves were chilled by placing them in a glass container and immersing in a mixture of ice and salt. The leaves were then pulped by passing them through a mincing machine and then pressing in a hand screw press. The mincing machine and the press were coated with hard paraffin to avoid metallic contamination. The juice darkened very rapidly on exposure to air. The juice of leaves,which had been treated with alcohol vapour under pressure to inactivate the enzymes, did not darken when expressed and the value obtained for the pH agreed substantially with that from the untreated leaf. The latter had to be diluted with freshly distilled water,to enable readings to be obtained,on account of its colour. Since the fresh juice could be diluted without affecting the pH to any extent tests were carried out on it in preference to that from the stabilised leaf.

The freshly expressed juice was diluted one part to five of the distilled water. The leaf which had been dried

and then remoistened gave the same value as the fresh leaf but the leaf in which the enzymes had been allowed to act for some time showed a gradual increase in pH. For the determinations a solution of methyl red in phosphate buffer solutionswas used. As it was only necessary to obtain approximate results a colorimetric method was deemed sufficiently accurate for the purpose.

Determination of the pH of fresh and autolysed leaves

pH of the fresh leaf juice .....6.2 average.
pH of dried leaf remoistened.....6.2 average.
pH of the leaf after autolysis for seven days
.....6.5 average.
Autolysis for ten days.....6.7 average.

Atropine in phosphate buffer solutions with varying pH.

A series of phosphate buffer solutions was prepared having a range extending from the acid to the alkaline side of the observed values for the cell sap. These were as follows... pH.. 4.49, 4.94, 5.9I, 6.47, 6.98 and 7.73.

To I00 c.c. of each of the buffer solutions was added 25 c.c. of a two per cent solution of atropine alkaloid. (The alkaloid was assisted into solution by

the careful addition of N/I0 solution of sulphuric acid.) A previous range using a saturated solution of the pure base in the buffer was rather unsatisfactory as the amount of alkaloid proved inconveniently small for accurate analysis. The addition of the sulphate was found by experiment, to have very little effect on the value of the buffer.

The solutions after preparation were immediately divided in two portions, one half being placed in the incubator at 32' C. and the other half assayed by the addition of solution of ammonia . The process then followed on the usual lines for the estimation of the alkaloid. At the end of ten days the solutions were withdrawn from the incubator and yielded the following figures on assay.

Stability of atropine alkaloid in phosphate buffer solutions of varying pH.

Two gm. of atropine alkaloid in water dissolved by the addition of N/I0 sulphuric acid. 25 c.c. diluted to I00 c.c. with buffer solution and 25 c.c. taken for each assay

pH of buffer.	Amount of alkaloid recovered.	
	Control.	Solution for test
4.49	0.I27	0.I23
4.94	0.I22	0.I24

(contd., overleaf)

pH of buffer.	Amount of alkaloid recovered.	
	Control.	Solution for test.
5.9I	0.I24	0.I24
6.47	0.I24	0.I24
6.98	0.I22	0.I26
7.73	0.I24	0.I24

Each of the figures given in the table is the result of three assays averaged. It will be observed that no loss of alkaloid takes place under the conditions stated. It may therefore be concluded that loss of alkaloid on autolysis is not the direct result of hydrolysis resulting from the acidity of the cell sap.

THE TREATMENT OF BELLADONNA AND HYOSCYAMUS LEAVES UNDER VARIOUS CONDITIONS WHICH PRECLUDE ENZYME ACTION.

It has been shown that when conditions are arranged to favour enzyme action loss of alkaloid may occur. A further series of experiments have also been carried out under conditions which are unfavourable to enzyme action. In the first series, the fresh leaves were kept under the same conditions as those mentioned in the chapter on autolysis but at a temperature of 60 ' C. instead of 32' C. The leaves were kept in Bulloch jars as before but a hot water oven was used instead of a bacteriological incubator.

The effect on the alkaloidal content of fresh Belladonna leaves of continued heating at 60' C.

Table 8

A. Incubated at 60' C. for 240 hours.

Per cent alkaloid.....	0.651
.....	0.642
.....	0.638
.....	0.660
Average.....	0.648

B. Control dried in air without treatment.

Per cent alkaloid.....	0.651
.....	0.651
.....	0.651
Average.....	0.651

The effect on the alkaloidal content of fresh Belladonna leaves of continued heating at 60' C.

Table 9. Batch 7.

A. Heated at 60' C. for 72 hours.

Per cent alkaloid.....	0.434
.....	0.434
.....	0.441
Average.....	0.436

B. Heated at 60' C. for 240 hours

Per cent alkaloid.....	0.425
.....	0.441
.....	0.416
Average.....	0.427

C. Control dried in air without treatment.

Per cent alkaloid.....	0.425
.....	0.441
.....	0.407
Average.....	0.424

No loss of alkaloid resulted from this heating at an elevated temperature. This is taken as additional support to previous finding that the enzymes are the agents responsible for the loss of alkaloid as at this temperature they are unable to function.

Autolysis in the absence of air.

Among the early experiments conducted with a view to finding the autolytic changes taking place in Belladonna leaves were those in which the fresh leaves were packed down firmly one on the top of the other in a glass container with no provision for the entrance of air. The intention when this was done was to imitate the process which leads to the liquefaction of yeast when it is treated in this way.

The appearance of the leaf obtained by this method was entirely different from those autolysed with free access of air. They remained green and were rather mucilaginous to touch but dried quite well. The alkaloids suffered no diminution in amount even when the process was carried on for periods up to fifteen days.

As the other factors had remained unchanged, namely, a suitable temperature for the action of the enzymes, sufficiency of moisture and freedom from the attacks of microorganisms, it is concluded that the presence of air is necessary for the action of the enzymes which destroy the alkaloids.

On assay the results overleaf were obtained.

Autolysis of fresh Belladonna leaves at 32° C. for ten days in the absence of air.

Table IO. Batch 3.

A. Heated at 32° C. for ten days in the absence of air.

Per cent alkaloid	0.665
.....	0.637
.....	0.637
Average	0.646

B. Control dried in air.

Per cent alkaloid	0.637
.....	0.655
.....	0.646
Average	0.646

Table IO contd. Batch 4.

A. Heated at 32° C. for fifteen days in the absence of air.

Per cent alkaloid	0.425
.....	0.441
.....	0.441
Average	0.436

B. Control dried in air without treatment.

Per cent alkaloid	0.434
.....	0.441
.....	0.441
Average	0.439

To complete the series of experiments and also to prove that the inactivation of the enzymes prevents the loss of alkaloid if the leaves are kept at 32' C., stabilised leaves were dusted with powdered thymol and placed with free access of air in an incubator. The leaves were of course in the moist condition and had not been dried.

The effect of autolysis on leaves which have been treated with alcohol vapour under pressure.

Table II. Belladonna Leaves

Batch 7.

A. Stabilised at a pressure of one and a half atmospheres then heated at 32' C for ten days.

Per cent alkaloid.....0.407
..... 0.4I6
..... 0.4I6

Average..... 0.4I3

B. Stabilised as above then dried in air.

Per cent alkaloid..... 0.425
..... 0.434
..... 0.430

Average..... 0.430

Table II contd.

Batch 4. Hyoscyamus Leaves

A. Stabilised and heated for ten days as for Belladonna leaves above.

Per cent alkaloid	0.I35
.....	0.I43
.....	0.I47
Average	0.I4I

B. Control stabilised and dried without other treatment.

Per cent alkaloid	0.I39
.....	0.I47
.....	0.I47
Average	0.I44

THE DETERMINATION OF THE TYPE OF ENZYME CAUSING THE LOSS OF ALKALOID.

In this section experiments were carried out with:-

1... The juice of Belladonna leaves.

2... The residue remaining after expression of the juice.

3... The enzyme peroxidase obtained from spring turnips.

4... The enzyme tyrosinase from the Meal Worm (Tenebrio molitor).

The freshly expressed juice of Belladonna leaves was preserved by adding a layer of toluene. As more accurate assays of the alkaloid atropine can be made if there is a fair concentration of the alkaloid in the substance to be assayed, the juice was fortified by the addition of a solution of atropine. This addition of atropine, at first led to the view that the juice of the leaves was incapable of causing the disappearance of the alkaloid but this view was subsequently modified.

Strong solutions of atropine seem to inhibit the action of the enzymes. This inhibiting action has been noted by Rona (51) who has demonstrated the possibility of differentiating between liver and pancreatic lipase

by the inhibiting action of the alkaloid quinine on the liver lipase. Later experiments without the additional atropine were more successful.

The fresh juice was divided into two portions after the addition of the atropine solution. One was placed in an electrically cooled chamber at 4' C. and the other in the incubator at 32' C. This method adopted for the control was chosen as in the first trials the inactivation of the enzymes of the juice by heating on the water bath caused precipitation of proteins which it was felt would render the assay inaccurate. In addition the effect of heating the alkaloid on a water bath was considered better avoided.

After fifteen days both solutions were assayed as follows:- 25 c.c. were taken and 75 c.c. of 90% alcohol added. The precipitated matter was filtered off, the filter washed with alcohol and the filtrate evaporated at a low temperature on a water bath. The aqueous solution remaining after removal of the alcohol was transferred to a separating funnel and the alkaloids extracted and estimated in the usual way by extraction with chloroform and titration with dilute acid.

The effect of maintaining the juice of Belladonna leaves with the addition of atropine at 32' C.

Belladonna juice from the fresh leaves.

75 c.c. of juice with 25 c.c. of a solution containing the equivalent of two per cent of atropine.

25 c.c portions of the above gave	0.II0 gm. of atropine
	0.II3
	0.II5
Average	0.II2

The control.

25 c.c. portions gave	0.II7 gm. of atropine
	0.II8 gm.
	0.I20
average.....	0.II8

Further experiments were carried out with the fresh juice without the additional atropine. On assay it was found that the juice contained 0.040% of alkaloids calculated as atropine. About one litre of the juice was expressed and preserved by the addition of a little chloroform. Toluene had been used in the previous experiments but had a tendency to become entangled in the juice and lead to difficulty in measuring.

The following results were obtained using 200 c.c. portions of the fresh juice and maintaining at a temperature

of 32' C. for 25 days.

200 cc. of the fresh juice gave 0.047 gm of alkaloid.
0.041
0.038

average. 0.042

Control.

200 c.c. of the fresh juice gave 0.079 gm. of alkaloid
0.077

Average. 0.078

The juice of Hyoscyamus leaves has a very low content of alkaloid and was not used.

The leaf residue after the removal of the juice still contains alkaloid. To some of the expressed residue, thymol was added as a preservative and it was then maintained at the usual temperature for periods up to twenty days. No loss of alkaloid took place even on repetition of the heating. The leaf pulp had the tendency, previously mentioned, to become slimy due to the packing down of the soft mass with the result that air was unable to penetrate the mass. This, it is considered, is the explanation of there being no loss.

The mass of moist leaf did not turn brown. It will be remembered that the same retention of the green colour was found to occur when the leaves were autolysed in the absence of air.

The effect of maintaining the residue of Belladonna leaves at 32' C. for twenty days.

The moist mass was dried in air and then assayed.

A. Heated at 32' C. for twenty days.

Per cent alkaloid..... 0.325
..... 0.323
..... 0.291

Average..... 0.313

B. Control dried.

Per cent alkaloid..... 0.343
..... 0.358
..... 0.356

Average..... 0.352

On repeating the process the following figures were obtained.

A. Heated at 32' C. for twenty days.

Per cent alkaloid..... 0.307
..... 0.307
..... 0.311

Average..... 0.308

B. Control dried.

Per cent alkaloid..... 0.316
..... 0.321
..... 0.323

Average..... 0.320

The effect of the enzymes peroxidase and tyrosinase on solutions of atropine.

A notable feature of the loss of alkaloid which took place was that the presence of air seems to be essential, indicating that oxidation is involved. The experiments have also indicated that the loss only takes place when the conditions are suitable for enzyme action. These facts led to the belief that the oxidising enzymes are responsible and experiments to test the accuracy of this were instituted. It will be remembered also, that the alkaloid atropine disappeared from the tissues of a variety of plants which cannot have special mechanism for dealing with the alkaloids. It would seem therefore that an enzyme present in all plants must be responsible for the disappearance; this again lends support to the view that the oxidising enzymes are responsible.

In the earlier part of this work the presence of the enzyme tyrosinase was proved in Belladonna and Hyoscyamus leaves. Tyrosinase is capable of bringing about the oxidation of tyrosine and other amino acids. The following experiments were carried out with tyrosinase more conveniently prepared from another source, and atropine.

The most convenient spurce of the enzyme is the

Meal-Worm, (Tenebrio molitor) . A supply of the enzyme was isolated from this source using the method suggested by Morrow (52) but alcohol was used to precipitate the enzyme instead of sulphate of ammonia.

An aqueous suspension of the enzyme in chloroform water was divided in two equal portions. One of these was heated on the water bath for fifteen minutes to inactivate the enzyme and the water lost compensated. To each solution was added atropine alkaloid in the proportion of 0.05 gm. to each I00 c.c. On keeping in the incubator for fifteen days at 32' C. the following amounts of the added alkaloid were recovered.

Tyrosinase and atropine kept at 32' C. for fifteen days.

A.

Grammes of alkaloid recovered..... 0.0969
..... 0.0957
..... 0.09I3

Average..... 0.0946

B. Control.

Grammes of alkaloid recovered..... 0.0957
..... 0.0934
..... 0.09I2

Average..... 0.0934

No loss of alkaloid. On repetition of the process a similar result was obtained.

Peroxidase and atropine.

Of the remaining oxidising enzymes of Belladonna and Hyoscyamus the most marked is peroxidase. This enzyme which transfers the oxygen of peroxides, (derived from the catechol-like phenols of the juice, either by autoxidation or by the action of the oxidases,) to easily oxidised substances, can be obtained free from other oxidising enzymes in the Turnip and Horse Radish root.

A supply of the enzyme was prepared as follows:- Fresh spring Turnips were minced in a mincing machine. The product was extracted with 80% alcohol and the alcohol decanted off. The residue was pressed free from excess of alcohol and then extracted with 40% alcohol for 48 hours. Excess of 96% alcohol was added to the filtrate to precipitate the enzyme which was filtered off and dissolved in thymol water.

Two portions of this solution were taken. One was boiled, cooled and adjusted to volume and then to each was added two per cent solution of atropine sulphate in the ratio of 25 c.c. of the alkaloidal solution to 75 c.c. of the enzyme solutions. Each day 0.I c.c. of a I% solution of hydrogen peroxide was added to both the test solution and also the control. At the end of fifteen

days both solutions were assayed and the following results obtained.

Solution of peroxidase 75 c.c. with solution of atropine 25 c.c. kept at 32° C. for fifteen days.

A.... Test solution.

Grammes of alkaloid recovered..... 0.II6
..... 0.II3
..... 0.II3

Average..... 0.II4

B.... Control.

Grammes of alkaloid recovered..... 0.I2I
..... 0.I22
..... 0.I24

Average..... 0.I22

25 c.c. portions were taken for assay.

As there is practically no loss the experiments were carried on using a much weaker solution. In this series 0.05 gm of atropine alkaloid was added to each I00 c.c. of the solution of the enzyme and the solution of the peroxide as above. The conditions were otherwise as in the previously described experiments.

The alkaloid atropine in solution of peroxidase.

A. The test solution.

Grammes of the alkaloid recovered.....	0.0798
.....	0.0786
.....	0.0752
Average.....	0.0779

B. Control.

Grammes of the alkaloid recovered.....	0.0866
.....	0.0923
.....	0.0969
Average.....	0.0919

On repetition of the process.

A. Test solution.

Grammes of the alkaloid recovered.....	0.0880
.....	0.0863
.....	0.0854
Average.....	0.0866

B. Control.

Grammes of alkaloid recovered.....	0.0981
.....	0.0940
.....	0.0929
Average.....	0.0950

200 c.c. portions taken for assay.

CONCLUSIONS.

Only enzymes such as are commonly present in foliage leaves have been detected in the leaves examined. These have no obvious connection with the alkaloids of the leaves, yet, under conditions which favour the action of the enzymes, loss of alkaloid has taken place. This loss has not been repeated when the conditions were unfavourable to enzyme action.

The loss of alkaloid during drying, noted by other workers, has not been repeated in the experiments with Belladonna and Hyoscyamus leaves until the time taken in drying has been very much extended. This agrees with the known differences in the respective rates of oxidation of the alkaloids concerned. Under conditions which cause simple oxidation, the volatile alkaloids are much more readily oxidised than the non-volatile.

In the light of the present work, the findings of a number of workers that the alkaloids can be absorbed by many plants and then seem to be utilised, would indicate that a type of enzyme commonly present in plants and not directly concerned in the formation or the decomposition of these bodies, causes their disappearance from the tissues.

Air is necessary for the reactions which lead to the disappearance of the alkaloids. This at once leads to the view that the oxidising enzymes are concerned at some stage in the process and also accords well with the idea expressed above that a commonly occurring enzyme is responsible. The enzymes as a rule act only on specific substrates. The oxidising enzymes, some of which liberate atomic oxygen from peroxides, are not so restricted in their action and are able to act on many easily oxidisable substances. In the plant experiments, the alkaloids which disappeared most easily were those which undergo oxidation most readily. Morphine and nicotine are examples. Atropine, less easily oxidised, disappeared, but more slowly.

Whether, in the growing plant, the course of the reactions which ultimately lead to the disappearance of the alkaloid follows this course is another matter, but, as has been pointed out above, strong support for such a possibility is lent by the disappearance of alkaloid from a number of plants of different species and which have no direct connection with the alkaloids.

There are several points which are not easy to explain at present, the relation between nitrates and the alkaloids, (Carr, page I2), the assimilation of

the nitrates of alkaloids which are, as a rule, rather toxic (page I4) and the stimulation of the rate of respiration by quinine. It is interesting to note the fact that nitrates have a high oxygen content. All these points might be taken to emphasise the connection between the oxidisng enzymes and the alkaloids.

The increase in the amount of morphine in extracts of papaveraceous plants by oxidising agents is rather difficult to reconcile with the above, yet, in stored Opium an increase has been claimed to take place which at times gave way to a decrease. The enzymes are generally credited with the ability to catalyse reactions in both directions. There is much still to be explained here.

The utilisation of the alkaloids by the plants which have absorbed them and the beneficial results which have followed, might be taken to indicate that the products resulting from the oxidation of the alkaloid are a suitable form of nitrogen for assimilation by the plant. This would support the theory that the alkaloids are a form of nitrogen reserve, produced in times of active synthesis and oxidised and absorbed when conditions are less favourable for the formation of the various nitrogenous complexes required for the elaboration of proteins.

In plant culture experiments strong solutions of alkaloids had a poisoning effect. In this work strong solutions of the alkaloid seemed to inhibit the action of the enzymes.

Regarding the alkaloids from the point of view of waste metabolites, it would have been reasoable to expect an increase in amount during autolysis when all changes are degradative, such an increase did not occur.

The obvious physical changes in the appearance and other properties of the constituents of the cell are of interest as they seem to indicate that much of the change taking place when the plant dries is the result of the action of the enzymes as they are not repeated when the enzymes are inactivated. The possibilty of simple oxidation taking place in leaves so treated is equal to that in the ordinary leaf yet it retains many of the properties of the fresh leaf.

The change of colour from green to brown is an example. The products of hydrolysis of chlorophyll are still green. The development of the brown colour masks this green colour and the browning is the result of the oxidation of the phenolic substances of the cell sap by the oxidising enzymes.

The change in colloidal properties which was the cause of great difficulty in the assay processes is another feature which does not seem to have attracted attention.

It is concluded that:-

I. The alkaloids of Belladonna and Hyoscyamus leaves decrease in amount under the influence of the oxidising enzymes in the tissues.

2. That the presence of air is necessary before this loss takes place.

3. That strong solutions of the alkaloids are able to inhibit the action of the enzymes.

4. That there is no evidence of any other type of enzyme being concerned in the elaboration of the alkaloids from simpler substances.

Bibliography.

I. Baly, Heilbron and Hudson. J.C.S. I922. I. I079

2. Gadamer. Ber. deut. pharm. Geselsch. I9I4. 24, 35.

3. Pictet.Arch. Sci. Phys. Nat. I905. IV. I9, 329. and Ber. deut. pharm. Geselsch.I907. 40. 377I.

4. Pictet and Rotschy. Arch. Sci. Phys. Nat. I90I. I2. 209.

5. Pictet and Court. Bull. Soc. chim. de Fr. IV ser. '07. I. I00I.

6. Robinson. J.C.S. I9I7. III. 762, 876.

7. Raper. Address to Brit. Ass. Adv. Sc. (Physiology) I930.

8. Chevalier. Bull. gén. de Thérap. I906. I52, 28I.

9. Carr. Chemist and Druggist. I9I2. 8I. 432.

I0. Deluard. Thèse. Paris. I922.

II. Palladin. " Plant Physiology" 228.

I2. Knop and Wolf. Through Goris : La localisation et rôle des alcaloides et des glucosides chez les végétaux.

I3. Reveil. through Goris. (loc. cit.)

I4. De Varigny. Rev. gén. bot., I892. 4. 407, 420.

I5. Comère. Bull. Soc. bot., I9I0. 57. 277.

~~I6.~~

I7. Bourquelot. Jour. de Pharm et de Chem. I896. 48I.

I8. Debourdeaux. Jour. de Pharm et de Chem. I9I2. VI. 49I.

20. Annet and Singh. Pharm.Jour. (4). LXIII. 304.

2I. Heinrici. J.C.S.I. I9II. XXX. 649.

22. Wokes and Elphick. Quart. Jour. Pharm and Pharmacol. I930. 59.

23. Catillon. Jour. Pharm. et Chem. I9I2. V. 268.

24. Blau. Year Book of Pharmacy I904. 66.

25. De Jean. Jour. Pharm et Chem. I908. XXIX. 279.

26. Grier. Proc. British Pharm. Conference. 1923.

27. Leseur. Jour. Pharm. et Chem. I909. XXX. 49.

28. Tunman and Jenzer. Through Goris. (loc.cit.)

29. Griffiths. Quart. Jour. Pharm. and Pharmacol. I930, 52.

30. Ribaut. Bull. Sci. pharm. I908. I5. 495.

3I. Fricotel. Bull. Sci. pharm. I908. I5. 687.

32. Rosenthaler and Meyer. Archiv. Pharm. I908. XXVIII. 247.

33. Lepinois. Year Book of Pharmacy. I899. I39.

34. Onslow. "Practical Plant Biochemistry." p. I23.

35. Carles. Repert. de Pharm., through Y.B.P. I907.

36. Golaz. Schweiz. Apoth. Zeit. I923. 6I. I27

37. Arnould Goris. Bull. Sci. pharm. I907. XIV. I59.

38. Bourquelot. C.R. Ac. Sc. I90I. I. I33. 690, 692.

39. Perrot and Goris. Jour. Pharm. et Chem. I909. XXX. I86.

40. Dhers and Pugol. Bull. Sci. pharm. I922. 29. I54.

4I. Aurousseau. Bull. Sci. pharmacol. I9I0. I7. 320.

42. Chevalier. Bull. gén. de Thérap. I906. I52. 28I

43. Auenmuller. Schweiz. Apoth. Zeit. I920. 58. 434.

44. Panchaud. Schweiz. Woch. fur Chem und Pharm. I903, 4I.

45. Clayton. " Emulsions and emulsification."

46. Larsonneau. "Researches on the volatile alkaloids of Belladonna" Thèse. Paris. I922.

47. Markwell and Walker. Chemist and Druggist. I928. 525.

48. Tschirch. Compte Rend. XIme Congres Inter. de Pharm.

49. Squire." Companion to the British Pharmacopoeia." p.24I.

50. Henry. " The Plant Alkaloids." I9I3. p.60.

5I. Rona. through Waldschnidt " Enzyme Actions and Properties." p. 69.

52. Morrow. " Biochemical Laboratory Methods."