Studies on the Synthesis of Hyoscyamine in *Atropa belladonna* L. and *Datura stramonium* L.

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In previous papers [Cromwell, 1933, 1937], experiments on the biosynthesis of the alkaloids berberine and hyoscyamine in *Berberis darwinii* and *Atropa belladonna* respectively led to the conclusion that these alkaloids are synthesized from products of carbohydrate and protein breakdown.

This work has been continued with the object of throwing further light on the nature of the intermediate reactions leading to the formation of hyoscyamine in *Atropa belladonna* and *Datura stramonium*.

METHODS

Material. Plants of *Atropa belladonna* and *Datura stramonium* were raised from seed and set out in plots or grown in pots.

Preparation of material for estimation of total alkaloid. Leaves were rapidly dried at a temperature of 60° in an oven and ground to fine powder. Samples of root ½ in. in diameter were detached from the crowns in the case of *Atropa*, washed thoroughly, cut into pieces and dried at 60°. Roots of *Datura* were also washed, and dried at 60°. When completely dry, the roots were ground to fine powder.

Estimation of total alkaloids. Leaf or root powder (5–10 g.) was exhaustively extracted with dry ethanol in a Soxhlet extractor. The extract was transferred to an evaporating basin and the ethanol removed on a water-bath at 60–70°. The resinous residue was extracted with 50 ml. 0·5N H_2SO_4 and subsequently washed twice with 25 ml. portions of acid. The combined extracts and washings were next shaken with 100 ml. of a mixture of 4 vol. of ether and 1 vol. of chloroform, to remove impurities. The acid extract was made alkaline with dilute ammonia and the bases shaken out with three 100 ml. portions of the ether-chloroform mixture. The three extracts were combined and the solvent distilled off. The residue was treated with two portions of 10 ml. ether which were completely removed by evaporation at 80°. Volatile bases and free ammonia were driven off by heating on the water-bath at 100° for 15 min. The residue was dissolved in 25 ml. N/50 H_2SO_4 and the excess titrated with N/50 NaOH with methyl red as indicator, and the results calculated in terms of hyoscyamine. (1 ml. of N/50 acid≡0·005784 g. hyoscyamine.)

Recovery of hyoscyamine as picrate gave proof that this base is chiefly responsible for the titration values obtained. The theoretical amount of aqueous picric acid was added to the alkaloid solution after neutralization, and the crystals (plates) purified by recrystallizing twice from water. The picrate obtained had m.p. 163–164°. (Found: N, 10·89%. Calc. for hyoscyamine picrate, N, 10·81%.) Pure hyoscyamine picrate has m.p. 165°.

The results obtained from four plants of *Atropa belladonna* are given in the following table:

	Wt. of powder (g.)	Titration value (ml. N/50 H_2SO_4)	Titration value (as g. hyoscyamine)	Theoretical wt. of picrate (g.)	Picrate found (g.)	% recovery
(1)	5 (leaf)	3·9	0·022	0·039	0·0242	62
	5 (root)	5·8	0·033	0·059	0·0386	65
(2)	10 (leaf)	8·1	0·046	0·082	0·0597	73
	10 (root)	10·3	0·059	0·105	0·0793	75
(3)	20 (leaf)	16·7	0·096	0·172	0·1446	84
	20 (root)	20·1	0·116	0·207	0·1782	86
(4)	50 (leaf)	43·2	0·249	0·447	0·4120	92
	50 (root)	54·3	0·314	0·562	0·5152	92

RESULTS

Alkaloid and carbohydrate content of Atropa belladonna

(1) *Normal plants (seasonal variation).* Samples of root, stem and leaves were taken from 2-year-old plants. The figures given in Table 1 represent the average content of six plants. Qualitative experiments on carbohydrate distribution are recorded in Table 2.

Table 1. *Seasonal variation of hyoscyamine content of* Atropa belladonna

Month	g. alkaloid/100 g. dry wt. Root	Stem	Leaf
Jan.	0·42	—	—
Feb.	0·40	—	—
Mar.	0·38	—	0·60 (shoots)
Apr.	0·42	—	0·51 (shoots)
May	0·56	—	0·39
June	0·59	0·12	0·45
July	0·62	0·16	0·48
Aug.	0·61	0·14	0·45
Sept.	0·60	0·10	0·43
Oct.	0·61	0·10	0·43
Nov.	0·56	—	—
Dec.	0·52	—	—

(2) *Etiolated plants.* At the beginning of March, before growth commenced, large plant pots were placed over the crowns of plants. Control samples were taken of roots. At the end of May further

Table 2. *Seasonal distribution of carbohydrates*

	Summer	Autumn	Winter
Roots:			
Reducing sugars	+	+	+
Non-reducing sugars	+	+	+
Starch	+ (wood and pith)	+ + +	+ + +
Stems:			
Reducing sugars	+	+	
Non-reducing sugars	+ +	+	
Starch	–	Trace	
Leaves:			
Reducing sugars	+	Trace	
Non-reducing sugars	+ +	+	
Starch	–	Trace	

Tannins were present in leaves, stems and roots in all seasons. Arginine was present in leaves and roots.

examination of roots was made and also of the etiolated shoots which had been formed. The results of this experiment are shown in Tables 3 and 4.

Table 3. *Hyoscyamine content of etiolated plants*

	g. alkaloid/100 g. dry wt.		
	Roots		
Plant	Control	Experimental	Etiolated shoots
A	0·40	1·10	0·43
B	0·37	0·98	0·45
C	0·41	1·20	0·41
D	0·37	0·88	0·45
E	0·45	1·51	0·47
F	0·42	1·52	0·47

Table 4. *Distribution of carbohydrates in etiolated plants*

	Roots	Etiolated shoots
Reducing sugars	–	+
Non-reducing sugars	+	+ +
Starch	+	–

Arginine was present in both roots and shoots.

The translocation of hyoscyamine in Atropa belladonna

The upward and downward movement of alkaloid was studied by two methods, (*a*) examination of the exudate from cut stems and (*b*) grafting experiments.

Exudate from stems. The shoots of 2-year-old plants were cut to within 3 in. of the crown, and stout rubber tubing, provided with a screw clip, immediately slipped over the stumps. Glass tubes were inserted and supported by retort stands. 10 ml. distilled water were run into the tubes and the level marked. After one week had elapsed the exudate from the stumps was collected and estimated for hyoscyamine. 20 ml. of the exudate from each of three plants gave respectively 1·1, 1·3 and 0·9 mg. hyoscyamine.

Grafting experiments. Experiments were carried out using *A. belladonna* roots as stocks and tomato shoots as scions and vice versa. Samples of root were removed from *A. belladonna* stocks before the experiment, and hyoscyamine estimated. After a period of 5 weeks the tomato scions were examined qualitatively and quantitatively for alkaloid. The Vitali test was applied to a water extract of one of twelve scions and gave a strongly positive reaction for hyoscyamine. The remaining scions were dried and estimated quantitatively. The results are shown in Table 5.

Table 5. *Hyoscyamine content of* A. belladonna *stocks and of tomato scions*

	g. alkaloid/100 g. dry wt.	
	Control	Experimental
(*a*) *A. belladonna* stock: A	0·52	0·51
B	0·56	0·53
C	0·52	0·50
D	0·54	0·51
(*b*) Tomato scions	0·12	—

The grafting of *A. belladonna* on tomato stocks proved unsatisfactory, only two scions out of twelve surviving. The stocks carrying these scions gave a trace of hyoscyamine by the Vitali test. These experiments show that upward movement of alkaloid from the root system of *A. belladonna* takes place freely, while downward movement from the leaves is very restricted.

The influence of the injection of various substances on the production of alkaloid

Solutions of potassium nitrate, ammonium sulphate, asparagine, urea, nitrogenous bases and amino-acids, alone and in association with glucose, were injected into plants of *Atropa* (2 years old) and *Datura*, with the object of observing the effect on the plants and on the production of alkaloid.

Petiolar and stem injections were first carried out but did not prove satisfactory. Injection through the stump of a stem proved the most rapid and reliable method of introducing solutions into *Atropa* plants, and branch injection in the case of *Datura*.

The solution was contained in a litre aspirator bottle placed 3 ft. from the ground and was conveyed to the cut end of the stump through stout rubber tubing provided with a screw clip. To avoid air locks the tubing was filled with solution by opening the screw clip before connexion was made. With good transpiration as much as 400 ml. of solution passed into *Atropa* plants in 3 days. 100 ml. was the average volume which passed into *Datura* plants. Samples of leaves and roots were taken before and after the injection period.

Preliminary injections were carried out with eosin solution to determine the extent and distribution of injected fluid, and it was found that the dye reached the greater part of the root and shoot system of the plants. In these experiments 2% solutions of glucose and 0·25% of all other substances were used and the duration of injection was 7 days. None of the substances injected at these concentrations appeared to injure the plants.

Table 6. *Influence of the injection of various substances on the alkaloid content of the plants*

(Values given represent the mean of three injections on separate plants.)

Solution injected	g. alkaloid/100 g. dry wt. Leaf: Control	Leaf: Experimental	Root: Control	Root: Experimental
A. *Atropa belladonna*				
Potassium nitrate	0·37	0·36	0·55	0·56
Do. +glucose	0·45	0·47	0·64	0·63
Ammonium sulphate	0·43	0·47	0·53	0·55
Do. +glucose	0·47	0·49	0·56	0·60
Asparagine	0·43	0·44	0·60	0·62
Do. +glucose	0·42	0·46	0·59	0·62
Glycine	0·39	0·41	0·55	0·58
Do. +glucose	0·46	0·48	0·63	0·66
Arginine	0·45	0·48	0·65	0·67
Do. +glucose	0·44	0·52	0·58	0·61
Lysine	0·45	0·45	0·55	0·58
Do. +glucose	0·41	0·44	0·52	0·54
Proline	0·42	0·45	0·55	0·56
Do. +glucose	0·41	0·45	0·59	0·61
Putrescine (dihydrochloride)	0·44	0·67	0·62	0·65
Do. +glucose	0·44	0·70	0·60	0·67
Cadaverine (dihydrochloride)	0·39	0·40	0·60	0·62
Do. +glucose	0·41	0·41	0·63	0·64
Choline (chloride)	0·40	0·42	0·61	0·62
Do. +glucose	0·44	0·48	0·60	0·64
Betaine (hydrochloride)	0·45	0·45	0·69	0·65
Do. +glucose	0·39	0·41	0·61	0·62
Methylamine (hydrochloride)	0·42	0·43	0·58	0·60
Do. +glucose	0·38	0·42	0·66	0·69
Dimethylamine (hydrochloride)	0·45	0·45	0·62	0·64
Do. +glucose	0·41	0·45	0·64	0·65
Trimethylamine (hydrochloride)	0·43	0·42	0·57	0·58
Do. +glucose	0·49	0·52	0·59	0·59
Hexamethylene tetramine	0·47	0·49	0·72	0·72
Do. +glucose	0·42	0·47	0·58	0·61
Formamol (hexamethylene tetramine-anhydro-methylene citrate)	0·45	0·48	0·55	0·58
Do. +glucose	0·43	0·49	0·62	0·68
Succinimide	0·44	0·43	0·65	0·67
Do. +glucose	0·49	0·49	0·69	0·68
Urea	0·43	0·43	0·58	0·55
Do. +glucose	0·41	0·42	0·61	0·60
B. *Datura stramonium*				
Asparagine	0·31	0·34	0·25	0·26
Do. +glucose	0·32	0·36	0·26	0·29
Putrescine (dihydrochloride)	0·33	0·33	0·22	0·25
Do. +glucose	0·32	0·36	0·22	0·29
Glycine	0·29	0·31	0·27	0·30
Do. +glucose	0·33	0·36	0·25	0·29
Arginine	0·34	0·34	0·29	0·32
Do. +glucose	0·33	0·36	0·30	0·32
Formamol	0·31	0·35	0·28	0·30
Do. +glucose	0·36	0·39	0·27	0·31

The results of the injection experiments are shown in Table 6; an increase of 0·05 g. hyoscyamine/100 g. dry weight is considered significant.

Injections of arginine with glucose, putrescine alone and with glucose, hexamine with glucose and formamol with glucose gave substantial increases in the hyoscyamine content of *Atropa belladonna*. Putrescine with glucose and formamol alone gave the best results with *Datura stramonium*.

Material from a second experiment on the injection of putrescine was used to check the titration values obtained in the first experiment, and to endeavour to show, by isolation as picrate, that increase of hyoscyamine is responsible for the increased titration value after putrescine injection (Table 7).

The theoretical amount of picric acid was added to the combined neutralized solutions from three plants of *Atropa belladonna* to ensure a good recovery of the alkaloid picrate. 10 g. samples of leaf and root powder were extracted in each case.

Volatile base content of normal and etiolated plants of Atropa belladonna

The volatile bases pyridine, *N*-methyl pyrrolidine and *N*-methyl pyrroline are present in the leaves and roots of *Atropa belladonna* and *Datura stramonium*. Unfortunately a reliable method for separating and estimating these bases could not be devised, but it seemed desirable to examine in normal and etiolated plants the content of volatile bases extractable with ether-chloroform mixture. The procedure adopted was similar to that for the estimation of hyoscyamine with the exception that N NaOH was used for the liberation of the bases, which were then distilled into standard acid. The base content was calculated in terms of *N*-methyl pyrrolidine (Table 8).

DISCUSSION

As a preliminary to further investigations on the nature of the intermediates in the synthesis of hyoscyamine, it was considered desirable to obtain more information on the behaviour of the alkaloid in the plant organs throughout the entire year, and also to carry out translocation experiments with the object of locating the tissues in which synthesis takes place.

The results of experiments on the exudate from cut stems and on grafting indicate clearly that hyoscyamine moves in an upward direction from root to shoot system, and will pass into a scion which does not normally contain it. Although

Table 7. *The effect of injection of putrescine on the yield of hyoscyamine*

	Leaf		Root	
	Control	Experimental	Control	Experimental
(1) Putrescine (dihydrochloride):				
Combined titration value of 3 plants (g. hyoscyamine)	0·123	0·180	0·166	0·184
Theoretical weight of hyoscyamine picrate (g.)	0·220	0·322	0·297	0·329
Weight of picrate found (g.)	0·1871	0·2706	0·2583	0·2896
(2) Putrescine (dihydrochloride) + glucose:				
Combined titration value of 3 plants (g. hyoscyamine)	0·139	0·203	0·155	0·188
Theoretical weight of hyoscyamine picrate (g.)	0·249	0·363	0·277	0·336
Weight of picrate found (g.)	0·2138	0·3195	0·2383	0·2862

The picrates obtained had m.p. 163–164° (N content of sample 10·79%).

these experiments do not prove that the root system is the locus of synthesis, the evidence is strongly in favour of this view. In plants growing under normal conditions it is therefore probable that the alkaloid is formed principally in the root and moves upwards through the vessels into the stems, leaves, fruits and seeds. It would appear that downward movement is very restricted. The work of Dawson [1941] on the tobacco plant similarly leads to the conclusion that nicotine is synthesized mainly in the root and that it moves from the root system via the xylem to the leaves. Shmuck, Smirnow & Ilyin [1941] also conclude from their grafting experiments that the synthesis of nicotine by *Nicotiana* is intimately connected with the root system and the stem. Kerkis & Pigulevskaya [1941] found atropine in all parts of tomato scions grafted on *Datura stramonium*.

Table 8. *Volatile base content of normal and etiolated plants*

	Content of volatile base calculated as g. *N*-methyl pyrrolidine/100 g. dry wt.	
	(*a*) Normal plants	
	Leaves	Roots
Plant A	0·07	0·04
B	0·09	0·06
C	0·07	0·05
	(*b*) Etiolated plants	
	Shoots	Roots
Plant D	0·12	0·09
E	0·14	0·13
F	0·18	0·10

The leaves of *Atropa* plants growing in full sun generally give a higher yield of alkaloid than plants growing in shade, and it is possible that the greater transpiration occurring in such plants would tend to draw up more alkaloid from the root system. Sievers [1921], working with *Datura* spp., found that removal of flower buds brought about an increase in alkaloid content of leaves. This finding may be interpreted on the assumption that alkaloid which would normally reach the fruits and seeds would be diverted to the leaves.

The seasonal distribution of alkaloid in roots, stems and leaves justifies, in the main, conclusions drawn from translocation experiments. For leaf tissues a minimum occurred in May (sufficient fully developed leaf material was not available before May), and a maximum in July, after which there was a slight fall until October, when the plants were killed by frost. The low concentration in the stems and the comparatively high concentration in the leaves during autumn show that little downward movement had taken place and therefore, on the subsequent death of leaves and stems, a considerable amount of alkaloid is lost to the plant. The peak of concentration is coincident with full maturity of the plant.

In normal seasons buds develop on the crowns during late autumn and remain dormant throughout the winter. Mild winters, however, may induce considerable growth of these buds, which may assume the form of shoots in February.

Analysis of entire shoots made in early March reveals a substantially high alkaloid content, but leaves detached in May from strongly growing shoots give yields which are low for leaf tissues. In the root a minimum occurs in March and a maximum in July, and from July until November the concentration is well maintained. From November to March an appreciable loss takes place which may be correlated with the development of buds and shoots at this period. From conclusions reached regarding translocation it would appear that loss of alkaloid takes place from root to shoot system. Later work, however, has shown that synthesis may take place to some extent in shoots developing in absence of light, and this was previously found

to be true for leaves from which light was excluded [Cromwell, 1937].

The distribution of carbohydrates follows the course usual for the majority of perennial plants. Starch is the principal carbohydrate stored in the roots during winter and is mobilized in the spring. During the summer months a trace of starch is present in the leaves, whereas mobile sugars are abundant in both leaves and stems. The high concentration of alkaloid found in the roots of plants from which light had been excluded during initial development is particularly significant. Non-reducing sugars and arginine also are present in roots and etiolated shoots, and therefore conditions for synthesis would be optimum, on the assumption that products of carbohydrate breakdown and amino-acids are the essential raw materials. On the other hand, it could be argued that the high content of alkaloid in the roots of these plants is the result of little translocation having taken place into the poorly developed etiolated shoots. Subsequent work has shown, moreover, that etiolated shoots from plants receiving similar treatment are able to synthesize alkaloid to a small extent. The yield of volatile bases is also stimulated by subjecting the plants to continued darkness for 3 months at the beginning of the growing season; hence it may also be assumed that conditions leading to accumulation of amino-acids and carbohydrate derivatives favour synthesis of these bases.

In the injection experiments an increase in alkaloid content of 0·05% over that of the controls is considered significant, although, if limiting factors are operating, it would not necessarily follow that values below this figure rule out the possibility that the substances in question act as intermediates in alkaloid synthesis. It is to be noted that, after injection, the increase of alkaloid in the leaves is usually more marked than in roots, but if synthesis is taking place in the root and the alkaloid moves to the leaves large variations in the root would not be expected. In many instances the stimulating effect of glucose is apparent and is probably due to the provision on breakdown of a ready source of energy and also of intermediates for hyoscyamine production.

Before discussion of the effects of specific substances on injection, it will be convenient to refer to laboratory syntheses of derivatives and near relatives of hyoscyamine. Robinson [1917] synthesized tropinone from succindialdehyde, methylamine and acetone-dicarboxylic acid, highly reactive substances which might occur in the plant. Robinson considers that succindialdehyde and methylamine may arise from the interaction of formaldehyde and ornithine, and acetone-dicarboxylic acid from citric acid. Schöpf & Lehmann [1935] showed that pH affected the yield of tropinone, and obtained at pH 7 a yield of 73% and at pH 5 90%. Schöpf [1937] synthesized teloidine from mesotartaric dialdehyde, methylamine and acetone-dicarboxylic acid.

Of the various amines and amino-acids injected into plants of *Atropa belladonna* and *Datura stramonium*, putrescine, arginine, hexamine and formamol are responsible for the greatest increase in the yield of alkaloid. The effect of putrescine, alone and in association with glucose, is especially marked and suggests that this diamine is an intermediate in the synthesis of hyoscyamine. Arginine injected in association with glucose likewise increases the content of alkaloid substantially. It is therefore inferred that arginine occupies a key position in the synthesis of hyoscyamine as the precursor of putrescine. In processes of bacterial putrefaction, putrescine is generally regarded as arising from arginine via ornithine, and in this connexion Gale [1940] has shown that a mixture of washed suspensions of *Streptococcus faecalis* Sargent and *Escherichia coli* growing in symbiosis produces putrescine from arginine by way of ornithine. The subsequent fate of putrescine is the subject of a later paper in which further experiments will be discussed.

Hexamine and formamol in acid solution both give rise to formaldehyde and ammonium salts, formamol in addition yielding citrate, and hexamine, on reduction, methylamine. These compounds are potential sources of probable intermediates in the synthesis of alkaloids of the tropane group and their effect on injection bears out this conclusion. None of the remaining substances injected gives a significant increase of alkaloid content. Urea appears to inhibit the production of alkaloid, and this observation was also previously made in experiments on urea feeding [Cromwell, 1937]. It is considered that accumulation of urea in the tissues may influence adversely the formation of ornithine from arginine, thus inhibiting the production of putrescine.

Methylamine, dimethylamine, trimethylamine and choline are each responsible for stimulation of alkaloid production when injected with glucose. Ackermann & Schütze [1910, 1911] found methylamine and trimethylamine as the result of bacterial action on choline, and if methylamine is an intermediate in hyoscyamine synthesis it may be derived from choline, which is known to be present in the root of *Atropa belladonna* [Kunz, 1885]. Glycine, however, may provide methylamine on decarboxylation, and this amino-acid also causes a slight increase of alkaloid on injection. Lysine and proline both show a stimulating effect. Ammonium sulphate and asparagine give a positive result which may be due to their indirect effect on amino-acid production. Cadaverine, betaine, succinimide and potassium nitrate are virtually without effect.

SUMMARY

1. The seasonal distribution of hyoscyamine in the leaves, stems and roots of *Atropa belladonna* has been investigated. The maximum concentration in leaves, stems and roots occurs in July, the minimum in leaves during the early part of May and in roots in March. Little downward movement takes place from the leaves in autumn.

2. Translocation experiments lead to the view that the root system is the principal seat of alkaloid synthesis. The alkaloid moves as such from the root system to stems, leaves, fruits and seeds.

3. An approximate estimate of the seasonal distribution of carbohydrates has been made in plants growing under normal conditions and in plants deprived of light.

4. The effects of injection of various amines and amino-acids have been studied. Putrescine, arginine, hexamine and formamol stimulate alkaloid synthesis considerably. Putrescine is regarded as an intermediate in the synthesis of hyoscyamine.

5. Plants sprouting in darkness give a high yield of alkaloid and volatile bases in both roots and etiolated shoots.

6. The significance of these results is discussed.

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The Role of Putrescine in the Synthesis of Hyoscyamine

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When putrescine (tetramethylenediamine) is introduced directly by injection into plants of *Atropa belladonna* and *Datura stramonium*, a considerable increase in the content of hyoscyamine has been found to occur. This result has been interpreted as an indication that putrescine is an intermediate in the synthesis of hyoscyamine [Cromwell, 1943]. If this interpretation is correct it becomes of interest to attempt to discover the manner in which putrescine provides its contribution to the alkaloid molecule. If one bears in mind the use of succindialdehyde in the synthesis of tropinone by Robinson [1917] and Schöpf & Lehmann [1935], the most probable conclusion would be that putrescine first gives rise to succindialdehyde as the result of oxidation. Alternatively, putrescine may undergo partial oxidation with the formation of an aminoaldehyde (γ-aminobutyraldehyde).

The occurrence of a diamine-oxidase system in animal tissues has been reported by Best & McHenry [1930], Zeller [1938, 1940], Zeller, Birkhauser, Mislin & Wenk [1939], and in bacteria by Gale [1942]. Zeller [1938] showed that this enzyme brings about deamination of histamine, putrescine, cadaverine and agmatine, and Gale [1942], working with washed suspensions of *Ps. pyocyanea*, found that at pH 7·5 the oxidation of putrescine, cadaverine and agmatine proceeds to completion. Zeller [1940] reported inhibition of the diamine-oxidase system by cyanide, semicarbazide, choline, monoamines, and over-optimal concentrations of substrate. It was thought that an enzyme bringing about oxidation of putrescine might exist in the tissues of *Atropa*, and accordingly an attempt was made to prepare an active extract from this plant.

EXPERIMENTAL

(1) *Preparation of crude extract from* Atropa *roots, leaves and etiolated shoots.* Freshly gathered material (500 g.) was washed and finely minced with silver sand in a mincing machine. The pulp was transferred to a muslin bag and pressed out in a strong hand-press. Sufficient glycerol was added to the press-juice to make 5% and the press-cake treated with the glycerol solution for ½ hr. The extract was again filtered through muslin and the residue pressed out. The combined press-juice and filtrate was centrifuged