CLIV. THE CONSTITUENTS OF THE SAP • OF THE VINE (*VITIS VINIFERA* L.).

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In a study of the sap exuding from the cut vine, Priestley and Armstead produced evidence in support of the view that besides mineral salts, there are present organic substances which are directly responsible for the production and maintenance of the necessary osmotic pressures. In view of the importance of these organic substances with regard to the alteration in physico-chemical equilibria and to the nutritive material supplied to the upper parts of the plant, it was considered desirable that a more exhaustive study of the constituents of the sap should be made, and for this purpose, a large quantity of the sap from bleeding vines was collected and examined as described below. The results obtained furnish information with regard to the nature of the organic and inorganic substances present, and in many cases those which are absent; they do not support the old idea that the sap is merely a dilute solution of inorganic salts.

Very little information could be obtained from previous investigations on the sap of plants, these investigations either having been carried out when the methods of chemical analysis were not sufficiently accurate to render the results conclusive, or else having been limited to the determination of the amounts of organic and inorganic matter present, and in some cases, the amounts of sugar exuded under varying conditions. In comparing the sap contents of the birch and maple, Schroeder [1869] estimated the percentage of sugar present, and indicated the variations in the amounts of calcium, magnesium, potassium and phosphoric acid present in different parts of the stem and roots. More recently, Moreau and Vinet [1923] have made analyses of the "bleedings" of the vine over varying lengths of time, and during and after periods of frost. These authors found that if the temperature is maintained at zero, or a little below, maximum richness results, though the volume exuded diminishes, and, over this period, the organic matter may form 94 % of the total solid matter, and of this organic matter 91.3 % is in the form of reducing substances. Thus they state that during a period of frost, followed by a rise of temperature, the sap yielded is very rich in organic material, and they refer to the fact that in Canada the sap of *Acer saccharinum* is collected in preference on a cold, frosty day, the liquid obtained being then much sweeter than at other times, and containing up to 35.7 g. of saccharose

per litre. This view, however, is opposed to that of Wiegand [1906], who states that the pressure and flow in the birch and the grape depend very little on the temperature of the air. Wiegand classifies trees into two groups, those which bleed early in the season, *e.g.* the maple, and those which, like the birch and grape, bleed later in the spring, and he attributes the flow of sap in the latter group entirely to root pressure. The later work of Moreau and Vinet shews that the vine behaves like the maple with regard to temperature variations, and this view is supported by the observations made during the collection of the vine sap for the work described here. The results of the present investigation are in entire disagreement with the statement made by Wiegand that the sap of the grape is almost pure water and contains no sugar.

From the "bleedings" of several vines, Moreau and Vinet obtained results which are expressed in tabular form as the mean volume collected in 24 hours, the dry matter at 100°, the ash, reducing substances (expressed as glucose) and the other organic matter. The figures given in this table prove that the view that the liquid contains 1-2 g. dry matter per litre, of which approximately two-thirds is organic and the rest mineral matter, is not strictly correct, and the authors conclude that the constituents vary between wide limits, the variations being related to a large extent to the external temperature. When, however, the sap is collected over a long period and the average figures for the whole period calculated, as was done in the work described in this paper, the older generalisation is found to be fairly accurate; the sap collected was found to contain 1.56 g. of solid matter per litre, of which 0.56 g. was inorganic and 1.00 g. organic. No distinction has been drawn between the sap yielded at the commencement of bleeding and that exuded later, and thus the results represent the total amounts of the various substances translocated by the xylem sap, while the percentages calculated represent the average figures for the whole period of bleeding. Quantitative tests, not recorded below, supported the results obtained by Priestley and Armstead, who found that the sap yielded in the later periods of bleeding contained less organic matter than the first bleedings. In this connection, it is interesting to note that Schroeder [1869] found that the amount of sugar present in the sap of the birch and maple decreased as bleeding proceeded.

From the physiological view-point, though not in a quantitative sense, the most important substances present appear to be the sugars, and a considerable time was spent in the estimations of the various carbohydrates. Priestley and Armstead [1922] found that hexoses and a much smaller amount of a disaccharide, presumably cane-sugar, were present, and this is confirmed by the results quoted below. Further, by the application of various methods of sugar estimation, it was found, as far as the methods adopted would allow, that the reducing sugars are glucose and fructose, the disaccharide cane-sugar, and that no other sugars are present with the possible exception of a trace of galactose. No polysaccharide could be detected, while disaccharides other than cane-sugar appear to be absent.

These results agree in substance with those obtained by Dixon and Atkins [1916] who found that the sap of various trees contained both reducing sugars and cane-sugar, and that during the vernal mobilisation of the reserves, hexoses and maltose are present, while the latter is absent at other times. These authors attribute the whole of the rise in osmotic pressure, as the higher levels of the tree are reached, to the sugars present, while admitting that other organic matter may be present, and their results are generally in entire agreement with this view. In certain of the examples given, however, a rise in the osmotic pressure due to non-electrolytes is not accompanied by a parallel rise in sugar content, and here the factor concerned may be the "other" organic matter, for which no figures are given. The fact that cane-sugar is the most important sugar in translocation and that maltose is absent in the majority of cases, as in the vine, appears worthy of further consideration with a view to the study of the possibility of conversion of glucose into fructose and the subsequent formation of cane-sugar.

The other organic matter present consists mainly, if not entirely, of the dibasic acids, oxalic, tartaric, malic and succinic acids, present as their calcium salts, and the total amount of these acids present exceeds that of the sugars. Schroeder [1869] records the presence of malic acid in the sap of both maple and birch.

The inorganic matter was found to consist of the chlorides, sulphates, phosphates, nitrates and nitrites, and silicates of sodium, potassium, calcium, iron, magnesium, and to a less extent manganese and aluminium. This is in close agreement with the view of Jost that Cl, S, P, Si and the metals K, Na, Ca, Mg and Fe occur regularly in appreciable amounts in the ash of all plants and that Al and Mn are usually demonstrable. The iron was present in a readily dialysable form and thus is not in the form of a colloidal complex.

The whole of the nitrogen present appears to be in the form of nitrate and nitrite, with the exception of a trace of organic nitrogen associated with the enzymes. The most likely explanation of the absence of organic nitrogen is that the presence of nitrites and amino-acids in an acid solution is incompatible. Vine sap which had been stored in a coloured bottle in a dark cool cellar contained a very small amount of free amino nitrogen as measured by formaldehyde titration (equivalent to 0.45 cc. of *N*/10 NaOH per 100 cc. of sap).

No substance of fat-like nature could be detected in any appreciable quantity, though traces of a neutral fat and a fatty acid were isolated from the concentrated solution after removal of the salts, sugars, etc. by dialysis, and also from the precipitate obtained with lead acetate. The amounts obtained, however, were too small to allow of any tests other than those of solubility and staining, and they would appear to have no great significance in view of the extremely low concentration.

It was thought possible that certain "soluble" or fairly diffusible enzymes might be present and tests were carried out for some of them. Those found

to be present were peroxidase, diastase and a small amount of catalase, while those absent were invertase, maltase, lipase, glycerophosphatase, proteases and rennin. These results appear to agree in substance with those obtained by other workers on tree sap, and since the tests described below were carried out, it has been noted that Palladin and Manskaja [1923] have identified peroxidase and Votchal [1916] has discovered oxidases and diastase in tree sap. Priestley and Swarbrick [1924] have shown that peroxidase, diastase, and catalase are present in the sap of the sycamore, and have also identified invertase (unpublished observation). With regard to the oxidising enzyme present, it is interesting to note that no peroxide could be detected, since the vine juice would not blue guaiaconic acid or benzidine solutions, except on the addition of H_2O_2 , and this absence of peroxide could be demonstrated with solutions which had been preserved with toluene and exposed to the air for eight or nine months.

Method of collecting the sap and of its treatment prior to analysis.

Previous attempts to obtain a sterile juice had not proved entirely satisfactory, and it was considered desirable that more stringent precautions should be taken to ensure the absence of active bacterial organisms. The sap obtained by merely cutting the stem, attaching a piece of glass tubing to the latter by means of rubber tubing, and collecting the liquid under toluene, was found to turn cloudy after exposure to the light for several hours. This cloudiness, which was much more pronounced in the absence of toluene, was regarded as an indication of bacterial action, but, as is mentioned below, there is a phenomenon of preliminary opalescence which is not associated with ordinary bacterial action. Because of the tendency for bacterial action to take place, and as a considerable time elapses before the juice can be prepared for chemical analysis, the cut surface of the stem, the knife used for trimming the stem, and the different attachments were all carefully sterilised.

Each vine stem was sawn across about 1 to 2 ft. from the ground, the saw being previously sterilised in the flame, and the outside fibrous bark stripped off, great care being necessary to prevent injury to the phloem. The cut surface was then trimmed with a sterilised knife and the stem and the cut surface wiped with an alcoholic solution of K_2HgI_4 . The metal cap, a turned iron cylinder about 2 inches in length with a side tube attached to a short length of composition tubing, was sterilised by immersion in boiling water, and the rubber collar, a short length of the inner tube of a motor-cycle tyre, washed with alcoholic K_2HgI_4 . A pad of cotton wool soaked with toluene was fitted in the top of the metal cap. The rubber collar was then slid over the stem, and the whole bound on with wire and adhesive antiseptic surgical tape. The sap was collected in Winchester quart bottles previously sterilised in steam and containing 15–20 cc. of toluene, and as soon as one bottle became full, it was replaced by another containing toluene as before. The displaced air passed out of the bottle as the sap collected through a second

tube which was bent so that the free end pointed downwards, and was closed with cotton wool. The rubber stopper carrying the two tubes was in position when the bottle was sterilised in the steam steriliser. Fig. 1 illustrates the mode of collection of the sap.

Fig. 1. Apparatus in position.

The liquid collected in this way was removed from the glasshouse as soon as possible and stored in a dark cool cellar until required. Some of the samples, usually the first "bleedings," were water-clear, but others were distinctly opalescent, although no bacteria could be detected by the ordinary bacteriological tests. Priestley and Raper had both thought that light played an important rôle in this change, and the latter investigated the action of rays of sunlight on some of the clear solution, setting up a control of the same liquid in a tube covered with lead foil on the outside. The solution exposed to the light rapidly became cloudy, while the control remained unchanged.

Concentration of the sap.

The dilute sap was concentrated by distillation *in vacuo* from three one-litre glass distillation flasks at a temperature of 50–55° and a pressure of 20–30 mm. Whenever the distillation was stopped, toluene was added to the concentrated juice, and as a precautionary measure, the air bubbled through the liquid to assist distillation was filtered through cotton wool. As the liquid was concentrated, a brownish-grey deposit separated on the sides of the distilling flasks, and this increased in bulk as distillation proceeded. Finally, when 90,300 cc. of sap had been concentrated to about 1 litre, the distillations were stopped, the solutions filtered and the precipitate washed with a small

quantity of distilled water. Some of the precipitate which adhered to the sides of the flasks was dissolved in a small quantity of warm dilute hydrochloric acid and treated separately. The main bulk of the precipitate (ppt. 1) was then dried on a porous plate in a vacuum desiccator, weighed and examined later, while the solution, which had a volume of 1205 cc. and to which was added 15–20 cc. of toluene, was stored in an ice-chest during the whole period of examination. When this solution had stood for a few days, it was found to contain a small amount (0.45 g.) of a crystalline deposit (ppt. 2) which, after removal by filtration, was found to consist of colourless prisms. The filtrate from this deposit, henceforward referred to as the conc. vine juice, had a slight brownish-yellow colour, and prepared and stored in this way, was found to be free from active bacterial organisms.

Dr H. V. Phelon, of the Department of Bacteriology at this University, kindly examined the solution at intervals and found no vegetating organisms present at any time.

General method of procedure.

The distillate obtained during the concentration of the juice was tested for volatile acids, reducing substances, and alcohol. No precipitate could be obtained by adding lead acetate, silver nitrate, or barium chloride solution to the distillate, and no reducing substances could be detected. By redistilling about two litres of the distillate, collecting only the first tenth and distilling off the first tenth of this last distillate, a solution was obtained which contained a small amount of alcohol, but no reducing substances. The alcohol was identified by the formation of iodoform and ethyl benzoate, both detected by smell, and also by the reaction with nitric acid and potassium dichromate. The amount of alcohol present, even after concentration by this method was too small to be of any significance, and it is considered most probable that the source of it was the spirit used for sterilising the instruments, etc.

The various deposits, ppt. 1, the hydrochloric acid solution of part of the same ppt., and ppt. 2 were separately examined for inorganic and organic substances and their ash and phosphorus contents determined.

The main bulk of the concentrated solution was first examined for sugars by the methods described below. The solution was first cleared by the addition of basic lead acetate until maximum precipitation was produced. This method of clearing the solution and of removing organic acids which would affect the polarimetric readings, appears to be the only one available, but in spite of the statement to the contrary made by Davis and Daish [1913], it does not appear to be entirely satisfactory. Adsorption of sugars by the precipitate does take place to some extent and the adsorbed sugar cannot be removed by washing with water. Thus the precipitate obtained with basic lead acetate was found to contain fructose and possibly glucose, even after repeated washing with water, and although the amounts were not considered sufficient to vitiate the results obtained, preferential adsorption of one sugar may in some cases

lead to erroneous views. Qualitative tests such as the preparation of the osazone, reactions with Seliwanoff's reagent, etc., were carried out with the solution and quantitative estimations of the reducing power and polarimetric value were made. The disaccharides were estimated by the alteration in reducing power and rotation by hydrolysis with HCl, citric acid, and also a solution of invertase. The results, which were almost identical by the three methods, indicated the presence of a small amount of cane-sugar, and the absence of other hydrolysable carbohydrates. The other methods used for the various sugar estimations are described later.

The precipitate produced on adding basic lead acetate to the concentrated vine juice was washed with water, dried on porous plates in a vacuum desiccator and weighed. After being ground up to a paste with water, it was decomposed with H_2S , and the filtrate from the precipitated PbS examined for various organic and inorganic acids and polysaccharides, and both filtrate and precipitate were examined for fat-like substances.

About 100 cc. of the concentrated vine juice contained in a very permeable collodion sac were dialysed under a slight pressure against running water and the non-dialysable portion examined for proteins, carbohydrates and fats. As was expected, practically all the solid matter passed through the membrane and the solution left in the sac contained merely the enzymes, together with a very small quantity of both a neutral fat and a fatty acid.

For the different enzyme tests described later, various solutions were used, these being the ordinary dilute sap, the concentrated juice, dialysed dilute sap, and dialysed concentrated juice, and whenever it was thought desirable, suitable co-enzyme solutions were added to the dialysed solutions in order to replace any lost by dialysis.

Tests for organic nitrogen by the fusion with sodium method were carried out with all the precipitates and also with the residues obtained by heating to dryness on a water-bath the dilute sap, and also the concentrated vine juice. In some cases, the reactions were very faintly positive, but were thought to be due to the enzymic substances present, and at other times to reactions between nitrites and nitrates and the organic matter.

Estimations of the solid content of the concentrated juice were made by heating on a water-bath to dryness, drying at 100° and weighing, while the ash contents of this residue, and of the other precipitates, were determined by heating in an electric furnace at $500\text{--}550^\circ$ until constant weight was attained.

First deposit (ppt. 1).

This deposit which, together with that which had to be dissolved in dilute HCl, contained 40.1 g. of solid matter, had a total ash content of 14.55 g. in the form of the phosphates and oxides of calcium and iron, and to a less extent magnesium. Small amounts of silica and alumina, and a trace of manganese were also present. Over half the precipitate was soluble in dilute acetic acid, and this acetic acid solution was examined for the calcium salts of certain

organic acids. The acids present proved to be tartaric, malic and succinic, the first being precipitated as its calcium salt on neutralisation of the solution, and the last two as calcium salts on addition of excess of alcohol to the filtrate. Tartaric acid was identified by the colour reaction of Pinerua [1897] with β -naphthol and conc. H_2SO_4 , by the reaction with conc. H_2SO_4 and resorcinol, by the formation of potassium bitartrate, and by a m.p. determination. Similarly the succinate and malate were decomposed with acid, but in these cases the acids were extracted by continuous ether extraction and identified as in the lead acetate ppt.

That portion of ppt. 1 not soluble in dilute acetic acid was extracted with dilute HCl , when over half the remainder dissolved and could be reprecipitated by neutralisation with NaOH . This ppt. proved to be calcium oxalate and the acid was isolated by decomposition of the ppt. with the calculated amount of H_2SO_4 and also by ether extraction of an acid solution of the ppt.

After extraction with dilute acetic acid and dilute HCl there still remained some insoluble matter in ppt. 1, and this was found to be a mixture of silicates, and a small amount of coagulated protein matter presumably from the enzyme matter destroyed during part of the distillation process. Thus ppt. 1 consists of the oxalates, tartrates, malates, succinates, phosphates and silicates of calcium, iron, magnesium, and aluminium, with a very small amount of coagulated protein matter. The bulk of the ppt., at least 80 %, consists of the salts of the organic acids with Ca and Fe , and the acids are present in roughly equal quantities. From an acid solution of ppt. 1, a mixture of organic acids was obtained by continuous ether extraction, and from this mixture an acid with a melting-point of $161-2^\circ$ was isolated. The sharpness of the m.p. indicated that the substance was fairly pure and presumably not tartaric acid, but sufficient was not available for identification. This acid was very soluble in water, but only slightly soluble in ether.

Second deposit (ppt. 2).

This small deposit of 0.45 g. was found to consist of calcium tartrate, together with a very small amount of either malate or succinate. Almost the whole of the precipitate consisted, however, of the tartrate, and this acid was identified by the methods described in the previous chapter.

The precipitate with basic lead acetate.

The precipitate obtained by adding excess of basic lead acetate to 200 cc. of concentrated vine juice was washed well with water, dried on porous plates *in vacuo*, and weighed 24.2 g.

The bulk of this precipitate was made into a paste with a small quantity of distilled water, more water added, and the lead salts decomposed by H_2S . After removal of the precipitated PbS by filtration, the solution was evaporated to a small bulk under reduced pressure. This concentrated solution containing

traces of phosphoric, sulphuric and hydrochloric acids, and a large amount of organic acids, was then shaken up six times with an equal volume of ether. The mixed ethereal extracts and the aqueous solution remaining after ether extraction were examined separately for organic acids, using, in the first place, the differences in the solubilities of the various calcium salts in hot and cold water, and alcohol. The free acids were then separated from their calcium salts by decomposition with dilute H_2SO_4 , or whenever possible by ether extraction from a phosphoric acid solution of the calcium salt.

The ethereal extract of the concentrated solution of the acids was found to contain malic acid and a smaller amount of succinic acid. Various solvents were used, but it was found impossible to obtain more than a small amount of malic acid in the crystalline state. This was found to melt at the correct temperature (100°). In view of the difficulty of obtaining malic acid in the pure crystalline condition, and owing to the absence of distinctive colour reactions, it is realised that the criticism levelled by Franzen and Keyssner [1923] against the methods used for identifying malic acid in plants, and against the view that this acid is widely distributed, is quite justifiable, and whenever possible, tests should be carried out to exclude other acids, such as succinic acid, whose calcium salts have properties very similar to those of calcium malate. In the present work, not enough of the precipitate was available to permit use of the method recommended by these authors, and so recourse had to be made to other methods. A small amount of the acid was obtained in the crystalline condition and found to melt at the correct temperature, while the acid could be extracted from an acid solution of the calcium salt by extracting a few times with ether, whereas succinic acid is only very slightly soluble in ether. Finally, the colour reaction with β -naphthol and conc. H_2SO_4 suggested by Pinerua [1897] was obtained by using some of the free acid. This test, used in conjunction with controls in which the same weights of succinic, tartaric, malic, citric, and malonic acids were employed, proved extremely satisfactory and the importance of this test as a confirmatory test, when compared with suitable controls, does not appear to have been realised sufficiently.

The acids not extracted by the ether were found to be succinic acid, a smaller amount of malic acid, and a trace of tartaric acid, together with the inorganic acids, HCl , H_2SO_4 and H_3PO_4 . To the neutralised solution, calcium chloride solution was added in excess, and a slight precipitate separated on long standing. This precipitate was identified as calcium tartrate by the tests adopted in connection with the first deposit (ppt. 1). The filtrate, on boiling, gave a further small amount of precipitate which, however, was not calcium citrate but a mixture of the malate and succinate. The solution was filtered and excess of alcohol added, when a bulky white precipitate separated out. This precipitate proved to be calcium succinate with a smaller amount of calcium malate, the two acids being separated by their different solubilities in ether. By this method, a sample of crystalline succinic acid was isolated

which melted at 184–185° (M.P. succinic acid 185°), and had the same melting point when mixed with an equal amount of pure succinic acid. The acid also gave an insoluble iron salt from neutral solutions and this ferric salt on decomposition with dilute NH_4OH and removal of excess of ammonia by boiling, yielded a crystalline precipitate, presumably barium succinate, with 5 % barium chloride solution.

The basic lead acetate precipitate was thought at one time to contain lipins, and possibly the lead salts of the higher fatty acids, but no lipin could be isolated. A very small amount of nitrogen was found to be present by the fusion with sodium method, but this was attributed to traces of adsorbed nitrite and nitrate, for these could be detected in the precipitate. Traces of a fat-like substance could, however, be isolated from the lead acetate precipitate by extraction over an extended period with alcohol, ether or chloroform. The residue obtained by evaporating off all the solvent used was found to be insoluble in water, soluble in all the fat-solvents, and could be stained with Nile-Blue, Sudan III and Scharlach R. Not enough of the fatty substance was available for a M.P. determination, but by means of light petroleum the residue was divided into two portions. That portion more soluble in light petroleum behaved as a neutral fat, and the other as a fatty acid towards Nile blue. No stain was produced with osmic acid.

Lactic acid among other acids was shown to be absent, and no other organic acid could be detected. The solution obtained by decomposition of the lead salt with H_2S was tested for inulin and other polysaccharides, but without any success.

Thus the lead acetate precipitate consists of the lead salts of malic and succinic acids, with much smaller amounts of the salts of tartaric, phosphoric, sulphuric, and hydrochloric acids.

Sugars.

In general, the method adopted for the estimation of the various sugars is that advocated by Davis and Daish [1913] with certain modifications and additions. Basic lead acetate was added to 200 cc. of the concentrated vine juice until maximum precipitation was effected. The solution was then filtered, the precipitate washed several times with distilled water, and the whole of the filtrate freed from lead by the addition of solid Na_2CO_3 until no more precipitate was produced. The filtrate from the lead carbonate was then made up to 500 cc. with distilled water, and this water-clear solution used for all the sugar-estimations (solution *S*). These estimations were all carried out on the day of preparation of this solution and the fermentation experiments were started on the same day, but were not completed, of course, until about one month later.

Portions of the solution were hydrolysed with citric acid, an invertase solution, and also with dilute HCl , and the reducing power, estimated by Bertrand's method, and the polarimetric value of the solution before and after

hydrolysis determined. The three methods gave results which were in very close agreement with each other and the results showed that with the exception of a small amount of cane-sugar, no disaccharide was present.

The absence of maltose was confirmed by the fermentation experiments suggested by Davis and Daish. A portion of solution *S* was freed absolutely of lead by treatment with H_2S , filtration and the subsequent removal of excess of H_2S by passing a current of air for two hours, and was then diluted with water. Aliquot portions of this solution were autoclaved, inoculated with *S. exiguus* (a maltase-free yeast) and incubated for 28 days. An equal number of controls were set up with ordinary brewer's yeast as the fermenting agent. At the end of the incubation period, the reducing powers of the solutions were determined and the differences between the experiments and their controls found to be extremely small and within the limits of experimental error. Thus the amount of maltose present is negligible. When estimating the reducing power of solution *S*, the hydrolysed solutions, etc., a small correction has been made to allow for the reducing action of the small amount of non-fermentable reducing substances present. An attempt was made to determine the nature of these non-fermentable reducing substances by fermenting more of the solution with *S. exiguus*, precipitating the protein matter with alumina cream, and testing the filtrate for pentoses. This solution on concentration had a slight reducing action towards Fehling's solution, gave a positive phloroglucinol test, but a negative Bial's orcinol reaction, and it is suggested that the substance concerned may be galactose. Some support of this view was obtained by the isolation of a very small amount of a crystalline substance on oxidation with conc. HNO_3 , for this crystalline deposit appeared to be identical with mucic acid as far as solubility and crystalline form are concerned.

With regard to the monosaccharides, these appear to consist entirely of glucose and fructose, but the only method of calculating the amounts of each present which can be applied here, is that based on the relationship between the reducing power and the polarimetric value, after allowances have been made for the cane-sugar and non-fermentable substances present.

Preparations of the osazones from solution *S* gave phenylosazones which in crystalline form and in m.p. were identical with glucosazone. Repeated attempts were made to separate other osazones by making use of their different solubilities in hot and cold water, methyl alcohol and ethyl alcohol, but no other osazone crystals could be detected under the microscope. In addition the absence of galactose, and lactose, in any appreciable quantities was confirmed by the inability to obtain more than a trace of mucic acid from solution *S* on oxidation with conc. HNO_3 .

Experimental details.

- S.* Filtrate from the precipitate with basic lead acetate, delead and made up to 500 cc.
- A.* 30 cc. solution *S* diluted to 100 cc. and the reducing sugars estimated by Bertrand's method.
- B.* 60 cc. solution *S* rendered just acid to methyl orange with conc. H_2SO_4 , 4 cc. of a conc. invertase solution added and incubated for 72 hours. The solution made up to 100 cc. and the polarimetric and reducing values determined. A control was set up with 60 cc. H_2O , 4 cc. invertase solution and 2 cc. toluene.
- C.* 60 cc. solution *S* + conc. H_2SO_4 until just acid to methyl orange and 6 g. citric acid boiled for 10 minutes, neutralised with NaOH and made up to 100 cc. The reducing power and polarimetric value after decolorisation with animal charcoal determined as for solution *B*.
- C'*. As in *C*, except that HCl was used as the hydrolytic agent instead of citric acid. The results obtained were almost identical with those of *C* and so are not recorded below.
- D.* H_2S was passed through 40 cc. of solution *S* to delead completely. No precipitate could be observed, but the solution was filtered, excess of H_2S removed by a current of air passed through for 2 hours, and the solution made up to 100 cc. Six samples of 10 cc. each were autoclaved, three of these inoculated with *S. exiguus* and to the others small quantities of brewer's yeast added, and all incubated for 28 days. After the addition of alumina cream, each sample was filtered, diluted to 25 cc. and the reducing power determined. The differences between those fermented by the two yeasts proved to be within the limits of experimental error.

Polarimetric values	Solution <i>S</i>	Solution <i>B</i>	Solution <i>C</i>
Rotation calculated for original solution <i>S</i>	0.715°	0.675°	0.670°
Thus on inversion the rotation in 20 cm. tube is lowered by 0.0425° at 16° C.			

Percentages of sugars in the concentrated juice.

<i>Cane-sugar</i>	By polarimeter	=0.060 %
	By reduction	=0.062
	∴ Average cane-sugar	=0.061
<i>Hexoses</i>	By reduction	=2.46

Amount of glucose and fructose by polarimeter.

Rotation of solution <i>S</i> due to cane-sugar	=0.032°.
∴ Rotation of solution <i>S</i> due to glucose and fructose	=0.683° for a 20 cm. tube.
Glucose and fructose in solution <i>S</i> by reduction	=0.984 %.
∴ Solution <i>S</i> contains 0.865 % glucose and 0.12 % fructose.	
Concentrated vine-juice contains 2.16 % glucose and 0.30 % fructose.	

Sugars in the original sap.

0.0288 % glucose, 0.0040 % fructose, and 0.00081 % cane-sugar.
 Total amounts in 90,300 cc.: 29.64 g. hexoses (26.03 g. glucose and 3.61 g. fructose) and 0.73 g. cane-sugar.

Nitrate and Nitrite.

No organic nitrogen could be detected either in the conc. vine juice or in the two deposits, with the exception of a small amount of nitrogenous matter which was probably associated with the enzymes, and thus the whole of the nitrogen present appears to be in the form of nitrate and nitrite. The presence of nitrite, observed by Priestley and Armstead [1922], was confirmed by using the starch iodide test and also the specific Griess-Ilosvay reagent (α -naphthylamine and sulphanilic acid in acetic acid solutions). The estimation of the amounts of nitrogen present in the form of inorganic salts was carried out according to the method advocated by Strowd [1920] and the results expressed as total inorganic N present. The relative amounts of the two acids concerned is not of much significance in view of the prolonged treatment prior to analysis, and of the fact that alterations in the ratio appear to take place to an extent which cannot be controlled. Indeed samples of ordinary vine juice which had

been stored in coloured bottles in a dark cellar, and to which plenty of toluene had been added, had not the same nitrite-nitrate ratio, and therefore the results given below are only of value as a measure of the total inorganic nitrogen present and as quantitative evidence that both nitrate and nitrite are present.

Nitrate and nitrite. 5 cc. of conc. vine juice were diluted to 250 cc. and subjected to the action of 1 g. of Devarda's alloy in alkaline solution. The ammonia produced was distilled over into standard H_2SO_4 and titrated with NaOH; methyl red was found to be more suitable as the indicator than sodium alizarine sulphonate. A control was set up without the alloy and treated in exactly the same way.

Amounts of $N/28$ NaOH used (average of 2) with alloy = 27.50 cc.
 " " " without " = 45.02 cc.
 $\therefore$ Nitrate and nitrite N in 5 cc. concentrated vine juice = 8.76 mg.

Nitrate. The nitrate N in 5 cc. of conc. juice was determined as above after the decomposition of the nitrite by heating with about 0.5 g. aspartic acid.

$N/28$ NaOH used with alloy 28.62 cc.
 without " 44.82 cc.

Thus 5 cc. conc. vine juice contain 8.10 mg. nitrate N and 0.66 mg. nitrite N.

Total inorganic N in 90,300 cc. ordinary vine sap = 2.11 g.

In the conc. juice, 1.95 g. of this N is in the form of nitrate and 0.16 g. as nitrite.

Enzymes.

In testing for enzymes, various solutions were used according to the nature of the enzyme concerned. In certain cases the natural sap could be used, while in others the conc. vine juice was found to be more suitable. Both solutions were also dialysed in a very permeable collodion sac against running water for 4–6 days until no sugar could be detected in the sac contents, and these dialysed solutions used for the tests. Another solution employed was a water solution of the precipitate obtained by adding excess of alcohol to 100 cc. of the dialysed conc. vine juice, and all the tests described below were carried out in duplicate or triplicate, different enzyme solutions being used in each case. Toluene was added to all the solutions. Whenever it was thought necessary, the solutions were buffered and also suitable co-enzyme solutions were added to replace any lost by dialysis, while control tests were always carried out, using the boiled sap or enzyme solution.

Diastase. This enzyme was found to be present and could be detected in the dilute sap, conc. vine juice and the two dialysed solutions.

Invertase. None of the sugar-free solutions had any hydrolysing action on cane-sugar, even in the presence of a boiled invertase solution, added to supply any heat-stable co-enzymes necessary. In confirmation, cane-sugar was added to the dilute sap and the reducing power of the solution

determined at intervals. No alteration in the amount of reducing sugars present occurred.

Maltase. The dialysed solutions were added to maltose solutions buffered at p_H 6.7 and incubated for eight days, together with controls. No alteration in reduction took place during the whole of the period of incubation, and attempts to prepare glucosazone at different times all proved to be unsuccessful. Thus maltase is not present.

Oxidases. All solutions were rich in peroxidase, though no satisfactory proof of the presence of a complete oxidase system could be obtained. With benzidine and α -naphthol the solutions gave only the peroxidase action, but with guaiaconic acid, a strong peroxidase and a very weak complete oxidase reaction after about 6 hrs were obtained. The blueing of guaiaconic acid in the absence of H_2O_2 is probably due to the nitrites present, because this action still persists to some extent when the solution is boiled, while the peroxidase action is destroyed by heat. The dialysed solutions and the solution of the precipitate with alcohol gave only the peroxidase action.

Catalase. The ordinary sap was found to contain an exceedingly small amount, and the conc. vine juice a larger amount of the enzyme catalase.

Lipase. This enzyme was tested for qualitatively and quantitatively by using as substrate a suspension of olive oil, and in other cases milk. In no case did any fat hydrolysis take place.

Glycerophosphatase. The method used in the tests for this enzyme is essentially that used by Plimmer [1913]. 400 cc. of ordinary vine juice were added to 100 cc. of 5% sod. glycerophosphate solution, toluene added, and this solution, together with a control in which boiled vine juice was used, incubated for three weeks. The inorganic phosphate content of each solution was estimated at intervals by the uranium acetate method and also by that used by Plimmer, the gravimetric method. The figures obtained were constant throughout the experiment, indicating that no glycerophosphatase was present.

Inorganic phosphorus present (as P_2O_5) in the whole of the solution.

	Solution A	0.056 g.	0.058	0.052
	Solution B (control)	0.056	0.055	0.054
Total phosphorus (as P_2O_5) = 1.79 g.	Time (days)	0	5	21

The experiment was repeated later with conc. vine juice with the same result.

Proteases. Ordinary vine sap and the conc. juice were incubated for 7-14 days with small amounts of fibrin, and suitable controls were set up. No digestion was apparent and the solutions and controls gave identical amino-acid titrations (formaldehyde method) after seven days. Similar tests were carried out, using Witte's peptone and testing for free tryptophan, with negative results. Thus proteases of similar nature to pepsin and erepsin appear to be absent.

Of the enzymes for which tests were made, diastase, peroxidase, and a trace of catalase were present in the sap, and maltase, invertase, lipase, proteases, glycerophosphatase and rennin were absent.

GENERAL SUMMARY OF THE QUANTITATIVE RESULTS.

Twelve vines yielded about 110 litres of sap, of which 90,300 cc. were used for analysis. The figures given below represent the total amounts present in the various precipitates and solutions.

	Total g.	Amount in g. per litre of natural sap
Ash	50.3	0.56
Total solid	140.7	1.56
P ₂ O ₅	2.92	0.032
Reducing sugars	29.64	0.33
Glucose	26.03	0.29
Fructose	3.61	0.04
Cane-sugar	0.73	0.008
Inorganic N	2.11	0.023
Organic acids (oxalic, tartaric, malic and succinic) approx.	50	0.56

In conclusion, I should like to express my indebtedness to Professor Priestley for having supplied the sap, and my heartiest thanks to Professors Priestley and Raper for much helpful advice given to me during this investigation.

SUMMARY AND CONCLUSIONS.

1. The sap obtained from the bleeding vine is a dilute solution of inorganic and organic substances, containing 1.56 g. of solid matter per litre, of which approximately one-third is inorganic and the remainder organic. These figures are the average figures for a large volume of the sap collected over an extended period of bleeding.

2. The organic constituents are chiefly sugars and organic acids. The sugars present are glucose, fructose, and a very small amount of cane-sugar.

3. The organic acids present are oxalic, tartaric, malic and succinic acids, and the total quantity of these organic acids exceeds that of the sugars present. A small amount of an acid m.p. 161–2° C. was isolated, but could not be identified.

4. The mineral salts present are the chlorides, sulphates, nitrites, nitrates, silicates and phosphates of sodium, potassium, calcium, iron, magnesium, and to a lesser extent manganese and aluminium. The iron present is readily diffusible.

5. Only a very small amount of organic nitrogen is present and this may possibly be attributed to the nitrogenous portion of the enzymes. Thus the whole of the nitrogen appears to be in the form of nitrates and nitrites, though the possibility of amino-acids being present, and of their decomposition with the nitrous acid must not be overlooked.

6. No lipins could be detected in the concentrated sap. A mere trace of a fat-like substance was isolated, but in quantities which did not allow of any tests other than those of solubility and staining. The latter tests suggest that the substance isolated was a mixture of neutral fat and free fatty acid.

7. The enzymes diastase, peroxidase, and to a small extent catalase, are present in the sap, while maltase, invertase, lipase, proteases, glycerophosphatase and rennin were found to be absent.

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