A STUDY OF THE CLEARING OF ALCOHOLIC PLANT EXTRACTS*

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Clearing is generally a necessary part of the analytical procedure with plant materials preserved and extracted with 80 per cent. alcohol, as advocated in a previous paper (**3**). Clearing is equally necessary when samples, killed and preserved by drying, are not given a preliminary extraction with ether before extracting with alcohol. The work reported here suggests that this necessity is based more upon the mechanical removal of lipoids, waxes, and certain colloids not precipitated by 80 per cent. alcohol, than upon the chemical removal of copper reducing substances. It is probable that comparable, although somewhat high, reducing-sugar values may be obtained with certain alcoholic plant extracts without either preliminary ether extraction or clearing. On the other hand, the alcoholic extract from fresh mangel leaves so clogs the filter, when evaporated and taken up in water, as to be unfilterable by ordinary means. The same is true of a large number of leaf, green-fruit, and woody-stem extracts.

Correspondence with a number of physiological chemists indicated a lack of uniformity in clearing methods. A number of men were using neutral and basic lead acetate interchangeably although the work of BRYAN (**1**) indicates the danger of such a procedure. With these conditions in mind we have attempted to determine the limits of value and safety in the use of neutral- and basic-lead-acetate solutions for clearing plant extracts.

General procedure

Stock extracts of plant tissues, so chosen as to give a considerable range of clearing conditions, were prepared from fresh green material of known moisture content by dropping the sample into enough boiling 95 per cent. alcohol to give a final concentration of 80 per cent. After boiling for 2 or 3 minutes the samples were sealed and set aside 24 hours or longer. The extract was then decanted off, 2 ml. of 80 per cent. alcohol added for each gram of fresh material used, and the extraction repeated. The extracts were combined and preserved in glass containers. Samples of 100 to 300 ml. were taken for clearing and reduction, depending upon the reducing power of the extract. These were placed in pyrex beakers and the alcohol

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removed on a boiling water bath. Evaporating 200 ml. of a celery extract to 20 ml. when strong alcohol fumes were still given off gave a value of 28.0 mgs. dextrose per sample. Evaporating to 2 ml. which completely removed the alcohol gave a value of 29.0 mgs. dextrose. Since the difference of 1 mg. is within the error of weighing, the practice of evaporating to 3 to 5 ml. was adopted. One hundred milliliters of distilled water, less the quantity to be added in the lead solution, were added and heated to 80° C. to soften gummy precipitates and insure the solution of all reducing substances. As a further precaution the sides of the beaker were rubbed with a rubber police and insoluble masses were finely divided. After cooling to room temperature the desired quantity of lead solution was added and the samples filtered immediately into 200 ml. beakers to which had been added an estimated excess of potassium-oxalate crystals.[1]

The lead precipitate was allowed to drain on the filter and was washed with cold water until the filtrate no longer gave a precipitate in the oxalate solution below. The presence of an excess of oxalate was assured by testing with a drop of dilute lead-acetate solution. The deleaded solution, which was kept within a volume of 160 ml., was then filtered into a 200 ml. graduated flask, beaker and precipitate carefully washed, and the solution adjusted to 200 ml. at 20° C. in a water bath. Three 50 ml. portions were taken for copper reduction by the QUISUMBING and THOMAS procedure (**6**). Copper was determined by direct weighing of the cuprous-oxide precipitate. Several samples from each lot were retained and the purity of the precipitate determined by the official, sulphuric-nitric-acid, electrolytic method (**5**). Copper was deposited on a revolving platinum grid cathode by a current of 2 amperes for a period of 55 minutes. Calculations are based on the tables by QUISUMBING and THOMAS.

It was found practicable to hold the weights of the three copper precipitates to a maximum variation of 0.0015 gms. on the lighter and 0.0020 gms. on the heavier samples. This limits the error of any figure included in the data to three-tenths of a milligram of dextrose and requires differences of one to two milligrams for significance.

Experimental results

CONDITIONS AFFECTING THE DESTRUCTION OF REDUCING SUBSTANCES BY LEAD COMPOUNDS

When the preliminary experiments indicated considerable loss in the reducing activity of plant extracts treated with excesses of basic lead acetate,

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[1] Potassium oxalate was chosen as a deleading reagent on the basis of the work of SAWYER (7), EYNON and LANE (2), MEAD and HARRIS (4) and experimental data to be presented in a later paper. We have found potassium oxalate both safe and convenient when used in excess and the precipitate allowed to crystalize before filtration. The practice of filtering the cleared solution onto the oxalate crystals allows both of these conditions to be met.

it became important to determine the conditions under which these losses could be reduced to a minimum. One milliliter of 1.25 sp. gr. lead-acetate solution was sufficient to give a slight excess with the extract used for this experiment. Five milliliters were accordingly used for the comparisons. The data in table I indicate that increasing the quantity of neutral lead acetate used in clearing to 500 per cent. of the check had no effect on the reducing activity of the extract when the lead was removed at once. Basic lead gave a significant loss under the same conditions. Any standing before deleading, or heating with or without deleading (potassium oxalate does not remove all the lead), lowered the reducing activity, but the percentage reduction was always greater when basic lead was used.

TABLE I

THE RELATIONS OF TIME AND TEMPERATURE TO THE DESTRUCTION OF REDUCING SUBSTANCES BY LEAD COMPOUNDS

SOLUTIONS CLEARED WITH 5 TIMES THE QUANTITY OF LEAD NECESSARY TO GIVE A SLIGHT EXCESS AND DELEADED WITH POTASSIUM OXALATE AS INDICATED	REDUCING ACTIVITY OF MANGEL-LEAF EXTRACT AS A PERCENTAGE OF CHECK (1 ML. NEUTRAL)	
	Cleared with neutral lead acetate	Cleared with basic acetate
Deleaded at once at room temperature	99.3	86.5
Heated for 15 minutes after deleading	93.9	78.6
Heated for 15 minutes before deleading	71.5	47.5
Left at room temperature for 14 hours before deleading	88.6	77.6

A COMPARISON OF NEUTRAL AND BASIC CLEARING

The reducing values of a number of plant extracts cleared with minimum and excess quantities of neutral- and basic-lead-acetate solutions are compared in table II. The minimum dose of lead was taken as the quantity of 1.25 sp. gr. solution which gave a distinct, white precipitate when a drop of dilute potassium-oxalate solution was added to the extract-lead solution mixture. With the samples used, this varied between 1 and 2 ml. of solution. Five times the minimum dose was taken for the excess treatment. For ready comparison the reducing value of the minimum or low-neutral clearing is given in milligrams dextrose and the other treatments are calculated as a percentage of this value. Percentage differences which may be considered to be significant vary from 4 or 5 per cent. with the small reducing values to 1 or 2 per cent. with the larger. The data given in table

II show that, with the exception of the apple-wood extract, an excess of neutral lead acetate which was five times the quantity required for clearing had no effect upon the reducing power of the solutions. The minimum dose of basic lead acetate lowered the reducing power an average of 3 per cent. and this was increased to 11 per cent. by the excess treatment. In addition the variation between best and poorest recovery was about twelve times greater with the excess-basic than it was with the excess-neutral clearing. While a comparison of the absolute quantities of reducing substances in sweet potato roots and mangel leaves will rarely be required, that method which will most nearly give such values is most likely to give reliable results when comparing sweet potato roots of varying composition.

Although an excess of neutral lead decreased the reducing power of apple-wood extract this reduction was apparently due to an incomplete precipitation of reducing impurities when 2 ml. of lead solution were used. When 4 ml. was taken as the check value, high neutral gave a value of 98.4 per cent. which compares with the values obtained with the other extracts. The apple-wood extracts gave a very heavy precipitate with lead acetate and the first formation of a precipitate with potassium-oxalate solution apparently did not indicate sufficient excess of neutral lead to complete the removal of active impurities.

TABLE II

A COMPARISON OF THE REDUCING POWER OF EXTRACTS CLEARED WITH NEUTRAL AND BASIC LEAD ACETATE SOLUTIONS

CLEARING TREATMENT	LOW NEUTRAL AS MGS. DEXTROSE, OTHER TREATMENTS AS A PERCENTAGE OF THIS VALUE							
	Mangel leaf extract	Tomato leaf no. 1	Tomato leaf no. 2	Sweet potato roots	Apple wood no. 1	Apple wood no. 2	Celery stalks	Spinach leaves
Low neutral......	25.5	26.1	40.2	56.3	43.2	97.6	154.2	17.4
Excess neutral	98.8	99.6	99.8	98.6	94.9	94.0	100.0	....
Low basic...........	97.7	98.9	99.3	96.8	96.1	92.7	98.3	....
Excess basic......	79.6	85.4	87.8	96.3	85.9	87.4	93.5	88.5

COMPLETENESS OF CLEARING.—When a small but distinct excess of neutral lead acetate was used the color of the solutions so cleared compared favorably with that of the best clearing obtained. Low basic was almost invariably less satisfactory than either of the neutral clearings from the standpoint of color and ease of handling. High basic was normally lightest in color, but showed a distinct tendency to darken if allowed to stand after deleading. Water-clear solutions were obtained with all of the extracts used, by deleading with hydrogen sulphide after adding an excess of neutral lead acetate. Although hydrogen sulphide left the solutions acid to methyl red while they were boiled to expel the excess of the gas, such a treatment gave very close checks with potassium-oxalate deleading on an unhydrolyzed celery extract which contained approximately twice as much non-reducing as reducing sugars. Neutral-lead-acetate-hydrogen-sulphide clearing is recommended for trial when complete removal of coloring matter is required. It has no advantage when reducing sugars are to be estimated with Fehling's solution, and is less convenient. The precipitation of a large excess of lead with hydrogen sulphide will lower the reducing power of the solution, presumably by co-precipitation of reducing substances.

NEUTRAL AND BASIC CLEARING OF PURE REDUCING-SUGAR SOLUTIONS.—Extensive tests were made of the effect of neutral and basic lead acetate on the reducing power of pure sugar solutions and on the purity of the reduced cuprous oxide. The data given in table III are typical of the results obtained and agree with those presented in table II. The low-neutral clearing with potassium-oxalate deleading, which was used as a standard in this work, is shown to give reliable results on pure sugars. Also, a large excess of neutral lead acetate is much less destructive of reducing sugars than is a corresponding excess of basic acetate. There is less difference in the stability of the two sugars than would have been expected from the work of BRYAN (**1**). The same results were obtained, however, when known quantities of levulose and dextrose were added to plant extracts of known reducing value. When 22 mgs. of dextrose were added to an apple-wood extract, 98.2 per cent. was recovered from the high-neutral and 90.4 per cent. from the high-basic clearing. Of 18 mgs. of

TABLE III

THE EFFECT OF CLEARING ON PURE SUGAR SOLUTIONS

TREATMENT SOLUTIONS CLEARED AND DE-LEADED AS ABOVE—1 AND 5 ML. OF THE LEAD SOLUTIONS WERE ADDED	REDUCING SUGAR FOUND—CALCULATED AS MGS. AND AS PER CENT. OF CHECKS (UNCLEANED)			
	LEVULOSE		DEXTROSE	
	Mgs. sugar found	Recovery as per cent. of check	Mgs. sugar found	Recovery as per cent. of check
Pure solutions (check)	182.9	....	132.2	....
Low-neutral clearing	183.0	100.06	132.1	99.92
High-neutral clearing	182.4	99.73	130.8	98.94
Low-basic clearing	179.3	98.03	131.3	99.32
High-basic clearing	159.5	87.21	122.5	92.66

levulose added to parallel samples, 96.1 per cent. was recovered from the high-neutral and 86.6 per cent. from the high-basic clearing. A third series of extracts receiving the same clearing treatments was used as the basis of comparison.

A STUDY OF THE LOSS OF REDUCING SUBSTANCES IN BASIC CLEARING

The consistently lower reducing value of solutions cleared with basic lead may be assumed to be due to one of the following causes or to a combination of them: (1) The loss may be due to the co-precipitation or reducing substances. (2) It may be due to the formation of an insoluble, sugar-lead compound or to the formation of a slightly soluble compound which is co-precipitated with, or adsorbed by the clearing precipitates. Or (3), it may be due to the removal of organic substances which are precipitated and weighed as cuprous oxide when clearing with neutral lead acetate. The last supposition is not in accord with the uniform losses obtained with plant extracts and pure sugars. The uniformly higher dry weights of the excess-basic-clearing precipitates, approximately three times the weight of the excess-neutral precipitates, lends color to the co-precipitation theory.

RELATION OF QUANTITY OF PRECIPITATE TO LOSS OF REDUCING SUBSTANCES.—If the loss of reducing substances in basic clearing is due to co-precipitation alone, then the loss should be proportional to the volume of precipitate and independent of the precipitating reagent. Apple-wood extract was used for an experiment on this question because it gave the heaviest precipitate of any of the materials available. Four samples containing respectively 200, 100, 50, and 25 ml. of extract were made up to 200 ml. with 80 per cent. alcohol, the alcohol driven off and the residue taken up in water and cleared with 2 ml. of neutral-lead-acetate solution. The 100 and 50 ml. samples were best cleared by this quantity of lead and gave slightly lower results.

TABLE IV

THE EFFECT OF A NEUTRAL-LEAD PRECIPITATE ON THE RECOVERY OF REDUCING SUBSTANCES FROM APPLE-WOOD EXTRACT

QUANTITY OF EXTRACT IN SAMPLE, MILLILITERS	WEIGHT OF LEAD PRECIPITATE		REDUCING SUBSTANCES AS DEXTROSE	
	Mgs. per sample	Gms. per liter extract	Mgs. per sample	Gms. per liter extract
Two hundred	887	4.435	88.0	1.760
One hundred	467	4.670	43.1	1.724
Fifty	243	4.860	21.6	1.728
Twenty-five	109	4.360	10.9	1.744

In general, however, the reducing-sugar values agree closely through a variation of 800 per cent. in weight of precipitate. In another experiment, 5 ml. of basic lead gave a precipitate weighing 890 mgs. per sample and a loss of 9.2 per cent. of the reducing value of the solution. In this case, with a larger sample, 2 ml. of neutral lead gave a precipitate of 887 mgs. in the same volume and no measurable loss of reducing substances. Apparently co-precipitation alone will not account for the observed losses.

RECOVERY OF REDUCING SUBSTANCES PRECIPITATED BY BASIC-LEAD CLEARING.—When it became apparent that loss of reducing substances in lead clearing is associated with the presence of lead oxide in the solution it seemed probable that an insoluble or difficultly soluble, lead oxide-reducing sugar compound was being formed. To determine, if possible, the fate of the lost reducing substances, five 200 ml. samples of a mangel-leaf extract were cleared with 10 ml. portions of basic-lead-acetate solution. After thorough washing with cold water the precipitates were washed from the filters with approximately 100 ml. of boiling water and refiltered. The combined hot-water extract was deleaded with hydrogen sulphide and reduced on a water bath to 150 cc., transferred to a volumetric flask and made to 200 ml. for a determination of reducing substances. The extracted precipitates were then combined, suspended in water and treated with hydrogen sulphide. The water-clear filtrate was made to volume and tested for reducing substances after removing the excess of hydrogen sulphide. The results are given in table V.

TABLE V

THE RECOVERY OF REDUCING SUBSTANCES PRECIPITATED BY BASIC LEAD ACETATE

SAMPLES	REDUCING SUBSTANCES IN MGS.		LOSS OR RECOVERY AS A PERCENTAGE
	Per sample	Per liter extract	
Check (low neutral)	39.2	784	0.0
High basic	33.9	678	....
Loss	5.3	106	13.5
Hot water extraction	9.1	36.4	34.3
H_2S extraction	11.7	46.8	44.2
Recovery		83.2	78.5

With the clearing given in this experiment there was a loss of 13.5 per cent. of the reducing substances or a total of 106 mgs. for the liter used. Of this loss 34 per cent. was recovered by one extraction with hot water and an additional 44 per cent. was recovered by treating with hydrogen sulphide. The 23 mgs. not accounted for may to a large extent be assumed to have been held by the very heavy lead-sulphide precipitate which is always washed with difficulty. The completeness of the recovery and the fact that the reducing substances were apparently unchanged strongly suggested the formation of lead oxide-reducing sugar combinations which are either less soluble than the reacting substances or which are more strongly adsorbed by various precipitates. The formation of a lead compound, particularly of levulose, has been frequently mentioned in the literature. Although our data on this point are not complete enough for final conclusions, we suggest that lead oxide is the active portion of basic lead and that both ketose and aldose sugars form lead combinations, although the former perhaps more readily.

THE RELATION OF CLEARING TO THE PURITY OF THE CUPROUS-OXIDE PRECIPITATE

When reducing substances are to be estimated from the weight of cupric copper reduced, it is commonly supposed that clearing is necessary to remove, as completely as possible, precipitable organic impurities which would otherwise be retained on the filter and weighed as cuprous oxide. Investigators who have used basic lead as a clearing reagent have justified themselves on this score. We have shown that basic-lead clearing lowers the reducing value of plant extracts or pure sugar solutions from 3 to 15 or more per cent., that the loss may vary considerably from one sample to the next, and that it is roughly proportional to the degree of clearing. The losses obtained when the clearing operation was carried out on pure sugar solutions would preclude all of the difference between neutral and basic clearing being due to a lower percentage of organic impurity in the latter case, but they do not insure the efficiency of neutral clearing. The percentages of copper in the cuprous-oxide precipitates from the low-neutral or check clearing, and the high-basic or complete clearing, are assembled in table VI. Copper was deposited on a revolving grid cathode from a mixture of nitric and sulphuric acids as previously described.

All of the low-neutral solutions showed some color and most of the apple extracts were so dark as to entirely obscure the blue of the Fehling's solution. In every case the complete clearing gave a water clear solution but the tendency was for the percentage of copper in the cuprous-oxide precipitate to be lowered rather than increased by this clearing. The question of the presence of organic impurities is not closed by these data, although we were unable to detect any traces of carbon in the solutions remaining after the copper had been removed by electrolysis. If such impurities are concerned,

TABLE VI

PURITY OF CUPROUS-OXIDE PRECIPITATE FROM EXTRACTS CLEARED WITH NEUTRAL AND BASIC LEAD ACETATE

EXTRACT	PERCENTAGE OF COPPER	
	Check clearing (low neutral)	Complete clearing (high basic)
Mangel	88.18	87.81
Apple wood no. 1	87.01	86.39
Apple wood no. 2	87.61	87.30
Apple wood no. 3	87.67	87.69
Tomato	86.82	84.52
Sweet potato	87.42	88.04
Celery	86.03	85.60
Average	87.25	86.76
Dextrose solution	88.62	88.78

however, they do not belong to the group of coloring compounds which are removed by complete clearing with basic lead acetate. The percentages of copper in the dextrose precipitates were obtained under the same conditions and indicate the error due to oxidation in drying, and loss in handling. The highest value is within 0.04 per cent. of the theoretical yield of 88.82 per cent. copper, and the lower yield is probably within the error of the procedure employed.

Three points appear significant in connection with these data. (1) The percentage of copper in the cuprous-oxide precipitate of plant extracts was lower than in the precipitate of sugar solutions—both the direction and magnitude of the difference are in accord with previous observations. (2) The copper percentage value was independent of the clearing treatment and did not vary significantly between no clearing and complete clearing, although the reducing value was considerably lowered by the latter treatments. This is contrary to the reason frequently given for the need of clearing. (3) Although the percentage of copper in the plant-extract precipitates was low, the difference was small and consistent, being of the order of 2 per cent. or less. In physiological studies a constant error of 2 per cent. will not affect the conclusions to be drawn, and the gravimetric determination of reducing sugars should be satisfactory when sufficiently large quantities of cuprous oxide can be weighed to minimize fluctuations in the weights of the crucibles used. The use of basic lead acetate, on the other hand, introduces a variable error and has no advantages when reducing substances are to be determined by their action on cupric copper.

Summary and conclusions

A procedure which has given consistent results on a variety of plant extracts is recommended for the clearing of solutions to be used in the determination of reducing substances. After removing all but the last traces of alcohol from the extract the residue is taken up in warm water, allowed to cool to room temperature and sufficient neutral-lead-acetate solution, sp. gr. 1.25, added to just form a faint, white precipitate with a drop of dilute potassium-oxalate solution. Twice this quantity is used for clearing the solution tested, to insure precipitation of reducing impurities. Without standing for more than a few minutes the cleared solution is filtered onto an excess of potassium-oxalate crystals or powder. Only cold water is used for washing the lead precipitate. This method is convenient, rapid, and gives a definite end point in reducing value.

Any heating or standing in contact with a lead solution causes a rapid destruction of reducing substances. Basic lead acetate is more destructive than neutral and gives a variable loss even in cold solutions, the magnitude of which depends upon the completeness of clearing. This makes it unreliable as a clearing reagent.

The loss of reducing substances in basic clearing appears to be due to the formation of a lead oxide-sugar combination which is less soluble than the original substances and is more readily adsorbed by the clearing or deleading precipitates. The sugars may be recovered with their reducing properties unchanged by removing the lead with hydrogen sulphide.

The completeness of clearing has not affected the purity of the cuprous-oxide precipitate. In all cases where a plant extract or a mixture of extract and sugar solution was used, the percentage of copper has been approximately 87 per cent. instead of the theoretical 88.8 per cent. No good explanation for this difference is at hand. It is pointed out, however, that the magnitude and consistency of the error makes it possible to use a correction factor for gravimetric results on plant extracts or to follow the usual calculation procedures with reasonable certainty that results so obtained will be comparable, although varying perhaps 2 per cent. from the absolute values.

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