# METABOLIC PROCESSES OF POTATO DISCS UNDER CONDITIONS CONDUCIVE TO SALT ACCUMULATION[1]

F. C. Steward and G. Preston

(WITH NINE FIGURES)

## Introduction

Modern investigations (**11, 34**) show that vital activity plays an essential part in salt accumulation by plants. The ability to accumulate[2] demands properties peculiar to cells which are still able to grow and the rate at which these are utilized is causally related to the intensity of aerobic respiration. Salt accumulation is not merely the result of equilibria at membrane surfaces but demands some "dynamic machinery" in cells—to use a happy phrase which received prominence in a recent symposium. A similar concept is implied by the oft repeated assertion that during salt accumulation work is done and energy exchanges are involved. The identification of the "dynamic machinery" and the mechanism by which the energy stored in substances of high energy content is ultimately utilized in salt accumulation is one of the most challenging problems of modern plant physiology.

The earlier work, done on Nitella, Elodea, discs cut from storage organs, and roots, focussed attention upon those biochemical processes in the accumulating cells which involve carbohydrates and suggested that these represent an important part of the machinery of accumulation. In a brief review of available evidence (**33**) it was concluded that the biochemistry of salt absorption represented a virgin field for investigation and this inspired much of the work to be described in this and ensuing papers.

The relationship between salt accumulation and metabolism has been most investigated with reference to respiration as measured by carbon dioxide output. The conclusion to be drawn (**11, 33, 34**) is that the relation between salt uptake and respiration is not a simple and direct one; it concerns the aerobic phases of the respiratory process but it is the oxidation which these involve, rather than the production of carbon dioxide *per se,* which is causally concerned in salt accumulation. The ultimate connection between respiration and salt accumulation must therefore involve some aspect of the oxidative breakdown of sugar—the ultimate source of carbon substrates—other than the mere evolution of the carbon dioxide which is finally produced. The energy thus released must be involved; not only because it supplies the relatively small amount of energy necessary for the actual process of salt uptake, (a process which tends to increase the free

[1] This is the first of a group of papers on the biochemistry of salt absorption.

[2] Especially that most general type of accumulation which involves the simultaneous uptake of anion and cation and which has been termed "primary absorption." Certain absorption processes in which metabolism is not involved are not in question here.

energy of the system concerned) but also because it maintains the cells at that general level of metabolic activity which is an invariable characteristic of systems capable of a high degree of salt accumulation. Hence the biochemistry of salt accumulation entails an account of the fate of the carbohydrates metabolized during salt accumulation—an account which must ultimately be tested by a balance sheet of carbohydrates and their energy values. This aspect will be considered in a subsequent paper (**36**).

The relation between salt uptake and metabolism cannot, however, be restricted even to the processes of carbohydrate metabolism for it demands also a general picture of all the metabolic processes of cells engaged in salt accumulation. Only by the effect of the variables which determine accumulation upon these metabolic processes can those which are causally connected with salt uptake be recognized. It has been emphasized (**37**) that the conditions conducive to salt accumulation should also cause not only an enhanced respiration (oxygen uptake and carbon dioxide production), but also protein synthesis at the expense of soluble nitrogen reserves of the storage tissue. These processes should also be accompanied by a considerable evolution of heat; in fact, by all those processes which eventually lead to cell division and are involved in a recrudescence of growth in cells which were previously dormant in the storage organ. At that time much had to be inferred from evidence cited from other investigations as actual quantitative measurements of some of the metabolic processes described had not then been made.

The new evidence establishes the reality of some of the metabolic processes of thin, aerated potato discs which was hitherto presumed upon less direct evidence. This paper indicates the scope of the survey of metabolism which the general problem demands and deals with methods suitable for this type of investigation. Justification for the experiments to be described does not rest alone upon the more complete account they give of the metabolites of cells engaged in salt accumulation; they may also be scrutinized from another and still more general standpoint. The true nature of the relation between respiration and concomitant metabolic processes is as obscure as that which respiration bears to salt accumulation. Since the experiments to be described in this and subsequent papers record the respiration and metabolism of tissue subject to rigorous control, the data are relevant to the more general problem. The ultimate objective is a synthesis of results into a scheme which explains the mutual relations of all those vital processes which are dependent upon respiration for energy. The experiments to be described in this and following papers contribute to this end.

For the problem as thus envisaged the cut discs of storage tissue used in the earlier work have special advantages. The experimental technique (**31**) devised for the investigation of respiration and salt uptake is also necessary for the investigation of metabolism since the variables thus controlled (oxygen supply, temperature, and disc thickness) also control metabolism. The

amount of tissue used in each culture vessel (not exceeding 60 standard discs of 0.75 mm. thickness and a total weight of 45 gm. or its equivalent surface) is adequate for detailed biochemical examination and, being random sampled from a large batch of discs, the variability between replicate cultures can be reduced until it becomes negligible for most purposes. The general effect of oxygen concentration, temperature, and disc thickness upon respiration and salt uptake of potato discs is known and this information may be used when arbitrary values must be assigned to these variables. Previous work has localized the most active cells which contribute the bulk of the total respiration of the discs to a thin, hollow, cylindrical shell of tissue—the dimensions of which are known for standard conditions—which is situated at the surface of the disc. The technique which has evaluated the respiration rate, or the salt accumulation, of cells in given situations within the disc could also be applied to other metabolic processes. Hence the physiological behavior of thin discs of storage tissue need no longer be interpreted on the mistaken assumption that all of the cells of the disc are identical; on the contrary this type of experimental material has its greatest utility when it is recognized that the degree of activity in the cells is determined by their position within the disc. Thus the correct emphasis may be given to the very active cells which constitute that thin shell of tissue in which the properties of growth and active metabolism reside. Relative to the larger problem which involves the relation between respiration and other metabolic processes there is special reason for selecting experimental material in which cells retain the capacity for growth and cell division and also environmental conditions which permit these properties to be exercised. Senescent cells which have lost the properties in question clearly lack some fundamental metabolic property and exhibit a relationship between respiration and oxygen concentration which differs from that encountered in cells which are still able to grow. For example, contrast the effects of oxygen concentration on the respiration of potato discs (**32**), carrot and artichoke (**35**), potato roots (**35**), barley roots (**11**), and various seedlings (**15, 16**) with the effects of oxygen on the respiration of apples (**21**), tomato fruits (**10**), etc. The relationship of respiration to metabolism, therefore, may be different in kind in senescent and growing cells and there is every reason to believe that the latter constitute the most general case for the investigation of such problems.

## Experimental methods and results

### Composition of potato discs and basis for the expression of analytical results

The basis for the calculation of the results of analyses which portray the course of metabolism should be some quantity which is not subject to change.

Neither the final fresh nor dry weight of the rinsed, surface-dried discs fulfil this requirement. The initial fresh weight of a known number of standard discs is free from objection since the initial composition of replicate batches of discs random sampled from a large stock can be accurately reproduced. Variation in the fresh weight of replicate batches of standard potato discs, whether cut at the same time or not, is small if the stock of tubers is uniform (table I).

TABLE I

MEAN FRESH WEIGHT OF 50 DISCS

| NO. OF REPLICATES | MEAN FRESH WEIGHT | STANDARD DEVIATION | STANDARD DEVIATION AS PERCENTAGE OF MEAN |
|---|---|---|---|
| | *gm.* | *gm.* | % |
| 8 | 38.5 | 0.49 | 1.27 |
| 8 | 38.2 | 0.32 | 0.85 |

The uniformity in size and fresh weight of the discs cut and then washed 24 hours in running tap water does not conceal a much greater disparity in their composition as shown by the data of table II.

TABLE II

THE DRY WEIGHT AND CARBON CONTENT OF POTATO DISCS

| NO. OF REPLICATES | FRESH WT. | DRY WT. | | | CARBON CONTENT | | |
|---|---|---|---|---|---|---|---|
| | MEAN | MEAN | STANDARD DEVIATION* AS PERCENTAGE OF MEAN | AS PERCENTAGE OF FRESH WT. | MEAN | STANDARD DEVIATION* AS PERCENTAGE OF MEAN | AS PERTENTAGE OF FRESH WT. |
| | *gm.* | *gm.* | % | % | *gm.* | *gm.* | % |
| 4 | 27.8 | 4.22 | 1.73 | 15.1 | 1.62 | 5.57 | 38.6 |

* Standard deviation of the mean as a percentage of the mean = ½ this amount.

Table III shows the total sugar and nitrogen content of standard, washed discs cut from the same stock at various times during one month of the rest period. It will be seen that both the differences between parallel samples (series C and D) and between the different time series (series A, B, C, D) are small relative to the order of the effects to be described later. Where comparisons between experiments not run concurrently are made in this work the experiments were carried out within the shortest possible time to eliminate effects due to changes during storage.

Even where known varietal differences and great disparity in the soil

## TABLE III

SUGAR AND NITROGEN CONTENT OF STANDARD DISCS

| SERIES | SUGAR PER GRAM FRESH WEIGHT | TOTAL NITROGEN PER GRAM FRESH WEIGHT | FRESH WEIGHT PER DISC |
|---|---|---|---|
| | *mg.* | *mg.* | *gm.* |
| A | 3.53 | 2.06 | 7.43 |
| B | 2.74 | 2.05 | 7.56 |
| C | 3.15 | 2.05 | 7.91 |
| | 3.24 | 2.08 | 7.90 |
| D | 2.99 | 2.06 | 7.86 |
| | 3.20 | 2.05 | 7.94 |

## TABLE IV

COMPOSITION OF DIFFERENT STOCKS OF POTATO DISCS

| STOCK | DRY WEIGHT PER 45 GRAMS FRESH WEIGHT | STARCH PER 45 GRAMS FRESH WEIGHT | SUGAR PER 45 GRAMS FRESH WEIGHT | PROTEIN N PER 45 GRAMS FRESH WEIGHT | SOLUBLE N PER 45 GRAMS FRESH WEIGHT |
|---|---|---|---|---|---|
| | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* |
| 1934 | 6.40 | 4.28 | 0.22 | 37.8 | 59.0 |
| 1935 | (a)5.78 | 4.04 | 0.16 | 34.6 | 56.7 |
| | (b)5.74 | 3.79 | 0.12 | 33.2 | 59.4 |
| | (c)5.43 | 4.03 | 0.14 | 30.6 | 61.6 |

conditions are involved the principal organic constituents of standard potato discs are remarkably constant. This is well shown by the analysis of the two principal stocks of tubers used in the work to be described in this and a later paper. The one (1934, experiments at Berkeley, California) was a variety grown in the Delta soils of California which are rich in salts. The other (1935) represents King Edward tubers grown in Lincolnshire, England; samples A, B, and C were taken at different times during the storage period when experiments were made. Despite the evident uniformity of the composition of potato tubers the comparisons were mainly made between parallel treatments applied to discs random sampled from a uniform batch cut at one time.

### METABOLIC PROCESSES INVESTIGATED AND METHODS USED

The processes chosen for investigation and the technique adopted are outlined below.

CHANGE IN FRESH WEIGHT.—As in earlier work, this very valuable index of the behavior of the tissue was obtained by weighing the discs after they

were rinsed, and the surface dried twice with blotting paper. When the final sample was subdivided for analysis the fresh weight of the various aliquot parts was recorded.

CHANGE IN DRY WEIGHT.—Dried samples were used for some of the determinations (*e.g.*, calorific value). The dry weights were determined as follows. The fresh tissue was heated *in vacuo* for one hour at 100° C. to destroy enzymes. Constant weights were obtained after 36 hours drying *in vacuo* at 60° C. The dried ground tissue was always redried *in vacuo* at 60° C. before aliquot parts were withdrawn for analysis.

RESPIRATION.—Methods by which the respiration of potato discs immersed in aerated salt solutions can be measured have been fully described (**31**). Whenever the total carbon dioxide—in contrast to the average rate during a brief period—was required, the residual external solution was acidified, aspirated, and the carbon dioxide yield determined. This correction attains importance in nitrate and calcium solutions.

When effects of salts on respiration are involved the interaction between the salt effect and time is often of importance. Hence methods which yield data at shorter time periods were desired and for this purpose the respiration determination was modified as follows.

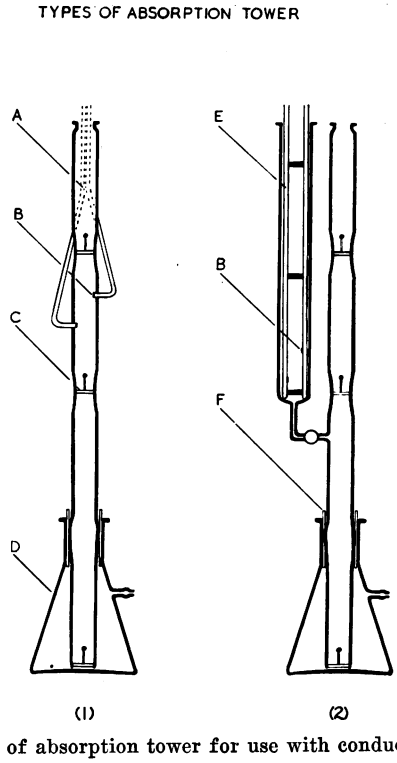


FIG. 1. Types of absorption tower for use with conductivity methods.

Into the modified Reiset absorption towers (fig. 1), of the type previously described (**31**) platinized-platinum electrodes [B, (1) fig. 1] with an area of 0.18 cm.$^2$ were inserted at a level between the two upper perforated silver discs. To increase electrical resistance in contact with a maximum concentration of 1.0 M caustic soda the electrodes were placed 2.0 cm. apart horizontally and 3.0 cm. apart in the vertical direction as in the method of NEWTON (**20**). External contact was made by means of tubes containing mercury [A, (1) fig. 1]. The gas stream was stopped during readings and the contents of the tower previously mixed by raising and lowering the liquid. It is sometimes convenient to insert before the tower a T-piece and a capillary resistance which can be opened at will, by a screw clip, to air. In this way the gas stream may be temporarily diverted and by operation of screw clips the absorption liquid can be rapidly raised and lowered. Others have shown (**4, 20**) that over a wide range the decrease in conductivity of a caustic alkali solution bears a linear relation to the carbon dioxide absorbed. The towers in question were calibrated for a fixed volume of alkali by carbon dioxide generated from known amounts of bicarbonate solution. Specimen curves for two such towers[3] are shown (fig. 2). The equation to the line shown was

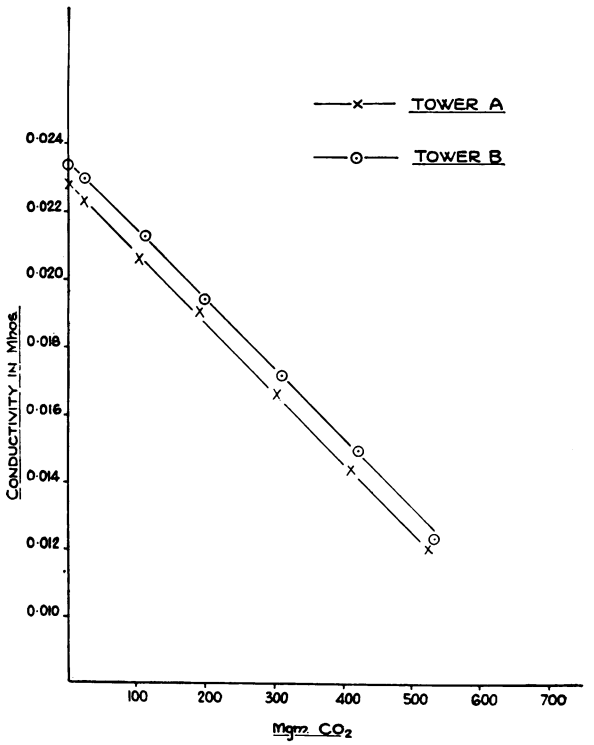


FIG. 2. Calibration curves of absorption towers.

[3] Charged with 50 ml. of MNaOH plus 100 ml. of water.

found by the method of least squares. For a constant volume of absorbing liquid (150 ml.), the constant of slope in the equation is characteristic of the tower and is unaffected within a narrow range, by the total concentration of alkali. Respiration rates were most conveniently expressed as mg. carbon dioxide per gm. initial fresh weight per hour.

Figure 1, (2) shows another modification which has the advantage that several towers may be used with one calibrated electrode and the platinized surfaces are only in intermittent contact with the alkali. A side arm communicates with the tower via a stopcock. During absorption the side arm is closed by a rubber stopper and the stopcock is shut. A rigid pattern of dipping electrode (E), with platinized platinum plates (B) is immersed in the absorbent which, for the purpose of reading, was transferred to the side arm by appropriate manipulation of the gas stream and stopcocks. Mixing was effected by raising and lowering the liquid as in the type (1). During

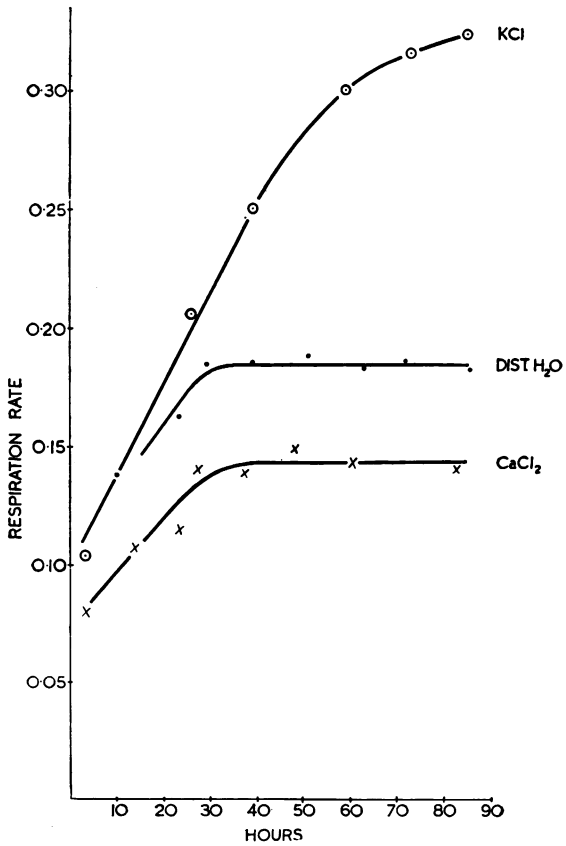


FIG. 3. Effect of salts (0.050 equivalents per liter) and of time on the respiration of potato discs at 23° C.

mixing the apparatus beyond the tower was isolated by a screw clip and the pressure before the tower released, by the slow passage of the accumulated gas to air, through capillary resistance again attached as a side arm by a T-piece.

### Effect of salts and of time on the respiration of potato discs

Using the above method for carbon dioxide determination the effect of time on respiration was determined for parallel cultures of 50 discs (average thickness approximately 0.75 mm.) placed in distilled water, 0.05 equivalent KCl, and 0.05 equivalent $CaCl_2$ respectively. Figure 3 shows clearly that after about 25 to 30 hours, during which a rising respiration rate obtained as the tissue became adjusted to the conditions of temperature and aeration, a steady level of respiration (0.186 mg. $CO_2$ per gm. per hour) was attained. Similar tissue in 0.05 equivalents potassium chloride per liter, and at the same oxygen concentration and temperature, finally attained a respiration rate 1.75 times as great as that in distilled water; whereas in calcium chloride of the same equivalent strength, the steady level attained was at 0.144 mg. $CO_2$ per gm. per hour. The behavior in time of discs in distilled water has long been a familiar feature of this work (**32, 37**) and, since all the factors affecting respiration were not known, it has been recognized that the plateau in the time curve was due to some factor, other than oxygen concentration, which was in relative minimum (**32**). It is clear from these curves that the essential factors concerned, which regulate the pace of the respiration of the tissue, are conditioned by the salt concentration in the external solution. The opposed action of potassium and calcium salts with a common anion indicates that it is the cations which are the causal agencies. The elucidation of this effect must await description of the other detailed metabolic effects caused by the salt treatment.

### Change in calorific value

The energy value of the processes of metabolism should be measured when the ability of cells to carry out processes which require energy is in question. Ideally this requires the measurement of both the total energy lost by the metabolizing cells in the form of heat and also of the change in the calorific value of the cells. Assuming that the cells do work the tissue should decrease in calorific value in excess of the energy which is lost merely in the form of heat and, if these measurements proved to be possible, a direct measurement of the energy converted into work by the tissue might be made. At the outset it must be recognized that this ideal is as yet unattainable but the experiments here described represent a first contribution towards this ultimate objective.

It is a well known fact that at a cut potato surface heat is produced in a quantity sufficient to raise the temperature of the superficial cells above that

of tissue which is deep seated and thus less active (**27**). Assuming that the bulk of the carbon dioxide production of these potato discs arises from the aerobic breakdown of sugar and that the energy value of this reaction appears in the form of heat in accordance with the equation

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O + 674{,}000 \text{ cal.}$$

then at a rate of respiration of 0.20 mg. $CO_2$ per gm. per hour—a rate which may be long maintained at 23° C.—one might anticipate a continuous heat production of the order of 22 cal. per hour for cultures of 60 discs (45 gm.). Thus stated the measurement of the respiration of potato discs by its heat production seems to be within the scope of the refined heat measurements which have been used in other types of investigations. The measurement of the heat production of nerve by DOWNING, GERARD, and HILL (**7**) involved the measurement of heat production of the order of $10 \times 10^{-6}$ cal. per gm. per second. The differential calorimeter of RANDALL and ROSSINI (**25**), equipped with multiple junction thermocouples and devices which measure a temperature rise as small as $21/5 \times 10^{-6}$ °C. in a calorimeter of heat capacity of the order of 65 cal. per degree, has been applied by FIFE (**9**) in an intensive investigation of the heat production and respiration of Azotobacter. FIFE was able to show that the heat produced by Azotobacter was exactly equal to the amount to be expected from the carbon dioxide released. In other words, within the limits of error, *all* of the energy released by those respiratory processes which culminate in carbon dioxide output was accounted for as heat and the source of the energy needed by the organism remained obscure. ALGERA (**1**) has used an automatic calorimeter (accurate to 1.4 per cent. on a heat production of 20 cal. per hour), in which the heat production of the organism (*Aspergillus niger*) is compensated by evaporation caused by forcing dry air into the system with a special pump. The change in the calorific value of the solution and the mycelium was measured as well as the heat and carbon dioxide evolved. The data led to the surprising conclusion that the energy derived from the respiration of glucose to carbon dioxide and water is not involved in the synthetic processes of the growing fungus but it is dissipated in the form of heat.

Direct attempts, however, to measure the continuous heat production of potato discs under the conditions conducive to salt accumulation were abandoned for the following reasons. Of necessity the tissue had to be immersed in a relatively large volume of solution and this increased the heat capacity of the culture vessel to such an extent that the most sensitive type of thermal measurement would be necessary. The correction imposed by the heat leak of the apparatus, a correction which even the best of large wide-mouthed Dewar flasks entail, was further complicated by the additional corrections due to the stirring system (the heating effect due to the mechanical stirrer and the conduction due to necessary metal parts) and also to the effect of the rapid air stream used for the supply of oxygen. It became apparent

that on a maintained heat production of the order of 22 cal. per hour, and with a calorimeter with a heat capacity of the order of 2 to 3 kgm. cal., the expected heat production was too near the order of the various corrections incidental to the technique and the apparatus to make this a profitable line of approach, at least in the early stages of the problem.

Attention was therefore confined to the second part of the heat problem, namely, the measurement of the total heat changes as shown by the calorific value of the discs. If the theoretical amount of heat from the conversion of sugar to carbon dioxide and water is to be regarded as lost by the tissue to the surroundings (and the justification for this now rests mainly upon the work of FIFE and ALGERA) then comparison can be made between this quantity and the observed decrease in calorific value. Thus a first picture could be obtained of the energy exchanges involved in the metabolism of potato discs. Whereas the direct measurements of the continuous heat production of the immersed living discs would tax even the most refined technique, preliminary observations showed that the over-all change in calorific value caused by a period of 88.25 hours of metabolism at 23° C. was readily measurable, as shown by the data of table V obtained from discs treated under the standard conditions of the salt accumulation experiments.

These figures, which could be supplemented by many others of a similar nature, serve to demonstrate at once the very considerable magnitude of the heat changes which occur in the discs and also to present the problem that the loss of heat thus observed was *much in excess of that to be anticipated from the measured carbon dioxide production.* It seemed, therefore, that the determination of calorific value could be profitably applied to the problem of salt absorption and metabolism.

The measurements of calorific value in table V, were made using an

TABLE V

CHANGE IN CALORIFIC VALUE DURING METABOLISM OF POTATO DISCS*

| SAMPLE | CONDITIONS | FRESH WEIGHT 40 DISCS | VACUUM DRY WEIGHT 40 DISCS | CALORIES PER GRAM DRY WEIGHT | TOTAL CALORIES | CHANGES DURING METABOLISM | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | CALORIES OBSERVED | RESPIRATION | |
| | | | | | | | $CO_2$ | CALORIES CALCULATED |
| | | *gm.* | *gm.* | *cal.* | *cal.* | *cal.* | *mg.* | *cal.* |
| Initial | ..... | 32.50 | 5.296 | 4054 | 21,480 | ..... | ..... | ..... |
| Final | Distilled water | 34.90 | 4.456 | 4207 | 18,750 | 2730 | 687.2 | 1755 |
| Final | 0.001 N KBr | 35.95 | 4.539 | 4176 | 18,910 | 2570 | 730.2 | 1865 |

* 40 discs exposed to 2 liters of solution under standard conditions (33) at 23° C. for 88.25 hours. Solutions aerated by washed air at 15 liters per hour.

EMERSON calorimeter (a standard form of gold lined bomb calorimeter) with an oxygen pressure of 20 atmospheres. Thermocouple methods for the determination of the heating and cooling curves of the bath, before and after combustion, were not employed but the BECKMANN thermometer which was used was especially calibrated over the necessary part of the scale. An electrical vibrator activated at the time of each reading, prevented the meniscus from adhering to the walls. The determinations were made on amounts of approximately 1.0 gm. of dried, ground, and uniformly sieved potato tissue. Special attention had to be given in all of this work to the determination of dry weight and the redrying which must precede the removal of aliquot parts of dry matter for all analyses. For calorimetrical work the potato tissue was not pressed into a pellet but was mixed with sufficient magnesium oxide and ignited electrically on a small boat—thus the formation of small incompletely burnt pellets of carbon was prevented. The heat capacity of the calorimeter and bomb (2200 cal.) determined from the combustion of 1.0 gm. of sugar or benzoic acid, included a standard volume of water within the bomb which was sufficient to dissolve soluble products of combustion. The temperature of the water bath which contained the bomb was maintained uniform by a stirrer, the speed and operation of which were rigorously controlled. Special care was taken to procure symmetrical heating and cooling curves. The order of the expected temperature rise could be estimated in the light of experience and the weight of water in the bath and its initial temperature were accurately adjusted so that prior to ignition a smooth heating curve was obtained and finally a smooth cooling curve from a temperature approximately as much above room temperature as the bath temperature at the time of firing was below. These conditions permit the best results from the application of certain empirical constants in the calculation.

Prior to firing, the heating curve of the water bath was obtained by readings every half minute; the time of firing was noted and the course of the ensuing temperature rise and subsequent fall was similarly recorded. The observed maximum temperature increase was corrected for the thermometer error and also by a "radiation correction" calculated from the empirical formula suggested by SCOTT (**29**) in his description of the determination of the calorific value of coal. The calculated correction compensates for the errors due to radiation and the time lag in the response of the water bath. Unburned fuse wire, if any, was weighed and the appropriate correction applied. Under the best conditions combustions which involved the production of approximately 4000 cal. could be repeated on successive samples to within 50 cal.[4] In all cases where values are assigned to tissue samples in this and following papers the figure given is the mean of concordant, replicate determinations and its accuracy is of the order named.

[4] This accuracy was due principally to the care and time expended on these determinations by P. R. STOUT of the division of Plant Nutrition, University of California.

### CARBOHYDRATE AND NITROGEN METABOLISM

It was essential that the scheme of analysis should reveal the hydrolysis of starch to sugar and its utilization in the metabolism of the discs. Though ultimately desirable, a complete analysis of the various hexose sugars has not yet been attempted; the carbohydrate analyses were confined to the determination of starch and total sugar. Furthermore, to economize material and time it was necessary to make as many determinations as possible upon a single extract. The essential nitrogen and carbohydrate data were therefore obtained by the following scheme:

(1) Potato discs were killed, either by heating for 1 hour at 100° C. followed by drying to constant weight at 60° C. *in vacuo,* or by boiling on a water bath for 20 minutes in excess of alcohol at a final concentration of 70 per cent. by volume.

(2) Complete extraction of either alcohol killed discs or vacuum dry tissues by 70 per cent. alcohol was carried out on an electric water bath with reflux condenser.[5] The accumulated alcohol extracts were evaporated to dryness, the residue dissolved in water and made up to standard volume. On aliquot parts of this solution the total nitrogen (alcohol soluble N) was determined by micro-Kjeldahl methods (**24**) and, after clearing[6] with neutral lead acetate, deleading with oxalic acid (**17**), and acid (3 per cent. HCl) inversion, the total sugar was determined by the MUNSON-WALKER gravimetric method (**6**).

(3) The residue from alcohol extraction was dried, weighed, and used for the determination of alcohol insoluble nitrogen (protein N) and starch. The starch was digested with a salivary extract under toluene, alcohol extracted, the soluble products of hydrolysis inverted, the sugar determined by the MUNSON-WALKER method, and the result calculated in terms of glucose (**2, 3, 23, 42**).

A full record of the fate of the carbohydrates during the metabolism of potato discs will be given in a subsequent paper. It is necessary only to remark here that discs, cut from dormant tubers and washed in running tap water for 24 hours, commonly have a total sugar content of about 3.0 mg. per gm. fresh weight (table III) whereas after 72 hours in aerated distilled water the concentration of sugar increases to 8.0 mg. per gm. This confirms the evidence of starch hydrolysis which can be obtained microscopically.

The scheme needs further comment with reference to the nitrogen determinations. The alcohol-soluble nitrogen will be designated "soluble N"

[5] For special purposes (see under amide-nitrogen) a "cold alcohol extract" is desirable. This was made at about 5° C. in a refrigerator.

[6] In work in which the changes in sugar concentration, rather than absolute values, are involved the clearing technique can be omitted, as the amount of non-sugar material in potato determined by the MUNSON-WALKER method is small (order of 2 per cent. in "final" tissue).

and the alcohol-insoluble nitrogen will be identified as "protein N." This is subject to the reservation that the potato is relatively free from alcohol-soluble proteins and alcohol-insoluble non-protein nitrogen. It was anticipated that during the metabolism of the discs protein synthesis would occur and, therefore, that the alcohol-insoluble fraction would increase at the expense of the alcohol-soluble portion. A test of the recovery of the total nitrogen would be the constant sum of the two fractions. Difficulty was never encountered with the accurate recovery of total nitrogen, mainly because the discs do not lose nitrogen appreciably to aerated solutions.

Since many of the experiments to be described did show protein synthesis by the alcohol method the following evidence is quoted because it confirms that the *changes* in the alcohol-insoluble nitrogen really did measure the change in protein content. In table VI are given the changes in nitrogen

TABLE VI

PROTEIN SYNTHESIS BY POTATO DISCS. COMPARISON OF THE ALCOHOL AND TRICHLORACETIC ACID METHODS

| SERIES | SALT SOLUTION | SAMPLE | BY $CCl_3COOH$ METHOD | | BY ALCOHOL METHOD | |
|---|---|---|---|---|---|---|
| | | | PROTEIN N PER GRAM INITIAL FRESH WEIGHT | CHANGE IN PROTEIN N CONTENT | PROTEIN N PER GRAM INITIAL FRESH WEIGHT | CHANGE IN PROTEIN N CONTENT |
| | | | *mg.* | *mg.* | *mg.* | *mg.* |
| A | ..... | Initial | 0.66 | | 0.77 | |
| | 0.075 equiv. KBr | Final | 1.31 | + 0.65 | 1.42 | + 0.65 |
| B | ..... | | | | | |
| | 0.075 equiv. $CaBr_2$ | Initial | 0.66 | | 0.74 | |
| | | Final | 0.52 | − 0.14 | 0.64 | − 0.09 |

content of potato tissue which in series A had been exposed for 63.1 hours to aerated KBr solution and in series B had been exposed for 87.3 hours to aerated $CaBr_2$ solution at 23° C. At this stage these treatments need concern us only in that one produced a significant gain of protein and the other a slight loss. The samples were analysed both by the alcohol method and also by the use of trichloracetic acid as a protein precipitant and thus, by comparing the initial and final discs, independent estimates of the change of protein content could be calculated.

It will be seen from the table that the fact of protein synthesis in potato discs exposed to aerated solutions of potassium salts is established. This is not the place to enlarge upon the contrast between the results in the different salt solutions except to note that the effects of the salt on synthesis of protein are parallel to those already described on respiration.

Clearly the different methods yielded almost identical results. The discrepancy, though small, is real and the trichloracetic method yielded protein values which were lower than those by the alcohol method. The estimates of *protein synthesised* were unaffected, however, by the method of analysis (series A). It should be mentioned, however, that further difficulty has been encountered in the analysis of tissue rich in phosphate after absorption in strong phosphate solutions. In this case the alcohol method is unaccountably at fault and yields values which are too low; such difficulties may be overcome by the use of the trichloracetic acid procedure. The method adopted, then, is as follows. The dried, ground tissue is redried at 60° C. *in vacuo*. Aliquot parts of about 50.0 mg. are transferred to 50 ml. pyrex beakers and stirred with 20 ml. of 2.5 per cent. trichloracetic acid for about 15 minutes. The insoluble residue containing protein is then filtered, washed, and transferred on the paper to a Kjeldahl flask in which it is digested in the usual way.

## Nature of the compounds concerned in protein synthesis

The reality of protein synthesis in thin aerated potato discs under the standard conditions conducive to salt accumulation can be sufficiently established by reference to the fractions designated "soluble nitrogen" and "protein nitrogen" (table VI, series A). Later papers will be greatly concerned with the effect of different variables upon the process of synthesis. It is, therefore, necessary to specify in more detail the compounds which are involved. The soluble nitrogen fraction is a composite one and includes both amino acids and amides. Free ammonia is a negligible component of the soluble nitrogen except when the tissue has absorbed large amounts of nitrate, or has been so treated during analysis that ammonia arises from the breakdown of relatively unstable soluble nitrogen compounds (amides) which are normal constituents of the potato tissue.

The first point which arises, and one which assumes greater importance in the light of the relationship between respiration and nitrogen metabolism to be discussed later, is the relative contribution of amino acids and amides to the nitrogen used in protein synthesis.

This question may be answered directly by reference to the results of the further fractionation of the soluble nitrogen fraction and, indirectly, by reference to the changes caused in protein synthesis in the titration curve of the sap extracted from the frozen tissue. Before dealing with the type of nitrogen compound concerned in protein synthesis, however, the existence in the actively metabolizing potato discs of unstable nitrogen compounds and their effect upon the fractionation of the "soluble nitrogen" must be appreciated.

Unstable nitrogen compounds.—The soluble nitrogen fraction obtained by alcoholic extraction of the fresh discs contained nitrogen compounds of

very different degrees of stability. It was observed that the evaporated alcoholic extracts contained unexpectedly large amounts of ammonia which can be attributed to "easily hydrolyzable amides" of the kind which VICKERY (**43**) has shown may be hydrolyzed at pH 7.0 by boiling for two hours. The following evidence establishes that unstable amides are real components of potato tissue and also shows their relation to protein synthesis.

Extracts of the soluble nitrogen of potato tissue were made in two ways: a "hot extract" made from the fresh tissue by boiling in 70 per cent. alcohol (pH 5.8 to 6.0), and a "cold 70 per cent. alcoholic extract" made at 5° C. from the dried tissue. The amino-nitrogen and ammonia-nitrogen content of these extracts calculated to the initial fresh weight of the discs (table VII) clearly show that the method of hot extraction released ammonia from some component of the soluble nitrogen fraction which, though stable in cold 70 per cent. alcohol, decomposed on boiling for several hours.

Table VII shows that the unstable substance which released ammonia in the hot extracts was amide since the amide content of the cold extracts (by hydrolysis with 6N HCl for 3 hours suitably corrected for the free ammonia obtained by distillation with magnesia) exceeded that of the hot extracts by an amount equivalent to the extra ammonia the latter contained. VAN SLYKE determinations made on the hot and cold extracts showed, however, that there was also an apparent loss of amino-nitrogen in the hot extracts. The "apparent" amino-nitrogen which decomposed as a result of prolonged, hot (100° C.) extraction at the pH of the tissue extract (5.8 to 6.0) corresponded to 80 to 90 per cent. of the unstable nitrogen present and *did not reappear as ammonia,* since the latter could be ascribed entirely to easily hydrolyzable amide. These results can be explained in the light of the work of VICKERY, CHIBNALL *et al.* (**44**) on the hydrolysis of glutamine. It has been shown that the amide group of glutamine yields ammonia quantitatively (over 98 per cent.) after 2 hours hydrolysis at pH 6 to 7) and moreover that about 80 to 90 per cent. of this amide group also contributes to the "apparent amino nitrogen" which is determined by the VAN SLYKE method. Further, during the hydrolysis of the amide glutamine, there is a concomitant loss of the amino group without the formation of ammonia so that for glutamine the decrease in "apparent amino nitrogen" tended to be double the ammonia released from the amide group. The combined effect of these processes is that the quantitative liberation of ammonia from the amide group of glutamine, which occurs in weakly acid or neutral solutions in two hours, is accompanied by a decrease in the "apparent amino nitrogen" content. VICKERY, CHIBNALL, *et al.* (**44**) emphasize that although a simultaneous loss of "apparent amino nitrogen" accompanying the quantitative hydrolysis of the easily hydrolyzable amide is strong quantitative evidence that the amide concerned is glutamine (or same other substance ca-

## TABLE VII

EFFECT OF SALTS AND AERATION ON THE NITROGEN FRACTIONS OF POTATO DISCS. ALL QUANTITIES OF NITROGEN IN MG. PER GRAM OF INITIAL FRESH TISSUE

| EXPERIMENTAL TREATMENT OF DISCS | PROTEIN N | SOL. N | AMINO N | | | AMIDE N | | | AMMONIA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | HOT EXT. | COLD EXT. | APPARENT "UNSTABLE" | HOT EXT. | COLD EXT. | EASILY HYDROLYZABLE | HOT EXT. | COLD EXT. | $NH_4 \equiv$ TO UNSTABLE AMIDE |
| | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| 52.5 hours in aerated 0.00075 M KCl at 23° C. | 0.92 | 1.15 | 0.90 | 1.04 | 0.14 | 0.129 | 0.305 | 0.176 | 0.178 | 0.002 | 0.176 |
| 53.0 hours in aerated 0.075 M KCl at 23° C. | 1.21 | 0.85 | 0.57 | 0.675 | 0.105 | 0.156 | 0.270 | 0.114 | 0.120 | 0.002 | 0.118 |
| Original washed discs | 0.68 | 1.38 | 1.09 | | | 0.208 | 0.348 | 0.140 | 0.144 | 0.002 | 0.142 |

pable of forming a pyrrolidone ring) the value of the ratio between these two quantities is too variable to be of use for quantitative measurement. The ratio of the decrease in apparent amino nitrogen to the decrease in easily hydrolyzable amide was observed by VICKERY, CHIBNALL, *et al.* (**44**) to vary from 0.5 to 1.24 for different derivatives of glutamic acid.

Table VIII shows that the "easily hydrolyzable amide" which was de-

TABLE VIII

CONTENT OF UNSTABLE NITROGEN COMPOUNDS IN POTATO DISCS. ALL QUANTITIES OF NITROGEN IN MG. PER GRAM OF INITIAL FRESH WEIGHT

| EXPERIMENTAL TREATMENT OF DISCS | APPARENT UNSTABLE AMINO-N | EASILY HYDROLYZABLE AMIDE | $NH_3$ FROM UNSTABLE N COMPOUNDS | APPARENT UNSTABLE AMINO N ×100 / EASILY HYDROLYZABLE AMIDE |
|---|---|---|---|---|
| | *mg.* | *mg.* | *mg.* | *mg.* |
| 52.5 hours in aerated 0.00075 M KCl at 23° C. | 0.14 | 0.176 | 0.176 | 79.5 |
| 53.0 hours in aerated 0.075 M KCl at 23° C. | 0.105 | 0.114 | 0.118 | 92.0 |

composed during alcoholic extraction yielded ammonia quantitatively but that the parallel loss of VAN SLYKE amino nitrogen corresponded to only 80 to 90 per cent. of this and therefore only to the amount reasonably expected from an unstable amide group, such as that contained in glutamine, which also reacts in the VAN SLYKE method. With this reservation and in the light of the statement of CHIBNALL and VICKERY referred to, these data establish a strong presumption that the unstable amide is either glutamine, or a closely related compound. The isolation and complete identification of this unstable amide present in actively metabolizing potato discs will be a necessary part of future investigations. If the "unstable amide" were contained in glutamine, the true amino nitrogen of the hot extracts would be less than that of the cold extracts and the nitrogen concerned in this change would not be estimated in any of the fractions determined. The sum of the amino-nitrogen (of the hot extracts), however, the stable, and the unstable amide, do account for the total soluble nitrogen. It appears, therefore, that the amino groups, if any, of this glutamine-like amide are too stable for this substance to be glutamine itself.

DETERMINATION OF AMIDE AND AMINO NITROGEN IN POTATO DISCS.—Clearly,

therefore, the true measure of the *total amide* content of potato discs is obtained only by the determination of this group by acid hydrolysis at pH 0 for 3 hours on the cold extract of tissue dried *in vacuo* at 60° C. This hydrolysis was appropriately corrected for the free ammonia obtainable by distillation with magnesia. The difference between the figure for total amide and that obtained by hydrolysis at pH 6 for 2 hours (which equals the gain in ammonia content in the hot extracts) is a measure of the easily hydrolyzable amide. The free ammonia content of the discs is obtained from either the cold extract or by direct distillation of the dry powder with magnesia. A measure of the amino nitrogen[7] (free from interference due to the unstable amides) is obtained by the application of the VAN SLYKE method to the hot alcoholic extract. Table IX shows that these fractions

## TABLE IX

SOLUBLE NITROGEN OF POTATO DISCS. ALL QUANTITIES IN MG. PER GRAM OF INITIAL FRESH WEIGHT

| EXPERIMENTAL TREATMENT OF DISCS | TOTAL SOL. N | AMINO N | TOTAL AMIDE | $NH_4$ | AMINO + AMIDE + $NH_4OH$ NITROGEN |
|---|---|---|---|---|---|
| | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| 52.5 hours in aerated 0.00075 M KCl at 23° C. | 1.15 | 0.90 | 0.305 | 0.002 | 1.207 |
| 53.0 hours in aerated 0.075 M KCl at 23° C. | 0.85 | 0.57 | 0.270 | 0.002 | 0.842 |
| Original washed discs | 1.38 | 1.09 | 0.350 | 0.002 | 1.442 |

account accurately for the total soluble nitrogen of the tissue. Since the amide fraction, which is stable in neutral or weakly acid extracts, is probably asparagine a probable figure may be derived for the "non-asparagine amino nitrogen" by deducting from the total amino nitrogen (determined as above) an amount equivalent to the stable amide fraction. This is probably the best measure available from these data of the amino content of the free amino acids.

SOURCE OF THE NITROGEN INVOLVED IN PROTEIN SYNTHESIS.—Table X compares the changes in the soluble nitrogen and its component fractions with the gain of protein nitrogen per gram of initial fresh weight which occurred in potato discs during experiments in which they were exposed to dilute salt solutions and the standard conditions conducive to salt accumulation and

[7] Excluding the amino group of glutamine, if present, but including that of asparagine.

## TABLE X

NITROGEN FRACTIONS INVOLVED IN PROTEIN SYNTHESIS IN POTATO DISCS. ALL AMOUNTS OF NITROGEN IN MG. EQUIVALENTS PER GRAM INITIAL FRESH WEIGHT

| EXPERIMENTAL SOLUTION | | HOURS | GAIN PROTEIN N | LOSS SOLUBLE N | LOSS OF $NH_2$-N | | LOSS OF NON-ASPARAGINE AMINO NITROGEN | | LOSS OF TOTAL AMIDE | | LOSS OF STABLE AMIDE (ASPARAGINE?) | | GAIN OF UNSTABLE AMIDE | | LOSS OF $NH_2$-N + TOTAL AMIDE | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SALT | CONCENTRATION EQUIVALENTS PER LITER | | | | AMT. | AS PER CENT. OF LOSS OF SOL. N | AMT. | AS PER CENT. OF LOSS OF SOL. N | AMT. | AS PER CENT. OF LOSS OF SOL. N | AMT. | AS PER CENT. OF LOSS OF SOL. N | AMT. | AS PER CENT. OF LOSS OF SOL. N | AMT. | AS PER CENT. OF LOSS OF SOL. N |
| | | | *mg.* | *mg.* | *mg.* | % | *mg.* | % | *mg.* | % | *mg.* | % | *mg.* | % | *mg.* | % |
| $CaCl_2$ | 0.00075 | 53.1 | 0.18 | 0.18 | 0.14 | 78 | 0.10 | 55.6 | 0.008 | 4.5 | 0.041 | 22.8 | 0.033 | 18.3 | 0.15 | 83 |
| KCl | 0.00075 | 52.5 | 0.24 | 0.23 | 0.19 | 83 | 0.12 | 52.3 | 0.029 | 12.6 | 0.074 | 32.1 | 0.045 | 19.5 | 0.22 | 96 |
| KCl | 0.075 | 53.0 | 0.55 | 0.53 | 0.52 | 98 | 0.47 | 90.6 | 0.078 | 14.7 | 0.052 | 9.8 | −0.03 | −5.6 | 0.60 | 112 |
| $CaBr_2$ | 0.00075 | 88.0 | 0.35 | 0.39 | 0.36 | 92 | 0.21 | 53.9 | 0.082 | 21.0 | 0.15 | 38.5 | 0.068 | 17.4 | 0.44 | 113 |
| KBr | 0.00075 | 63.5 | 0.28 | 0.29 | 0.31 | 107 | 0.21 | 72.5 | 0.021 | 7.3 | 0.10 | 34.5 | 0.079 | 27.2 | 0.33 | 114 |
| KBr | 0.075 | 63.0 | 0.65 | 0.61 | 0.56 | 92 | 0.45 | 73.7 | 0.118 | 19.4 | 0.11 | 18.0 | −0.08 | 13.1 | 0.68 | 111 |

high respiration. The agreement between the gain of protein nitrogen and the decrease of soluble nitrogen is close. The table shows (column 6) that almost the entire amount of the soluble nitrogen used in protein synthesis was derived from the amino nitrogen fraction and when the maximum[8] allowance is made for that part of the amino nitrogen which might have been derived from the asparagine metabolized (column 7) it is clear that amino compounds other than asparagine, which in the light of the titration curve data must be amino acids, contribute most of the nitrogen used in the synthesis of protein by the potato cells. The table also shows certain specific effects of the salts. The stronger potassium chloride solution increased the protein synthesis. This confirms a result which has already been noted (table VII). The table, however, shows that the effect of the salt (KCl) on protein synthesis could have been entirely at the expense of the reserve of non-asparagine amino (amino acid) nitrogen. In the presence of dilute calcium solutions the non-asparagine amino nitrogen contributed much less of the total used in protein synthesis than when the tissue was in contact with the stronger aerated potassium salts (column 7).

The conclusion, therefore, is that the nitrogen involved in protein synthesis is drawn principally from amino compounds (amino acids) other than asparagine or glutamine but also that the salts present affect not only the total amount of protein synthesized but also the source from which the nitrogen is obtained. Increased concentrations of potassium salts, which increase the total synthesis, also increase the relative utilization of the amino acids whereas the calcium salts, which depress the total synthesis, also decrease the utilization of the amino acids relative to other compounds.

The status of the amide fraction of the soluble nitrogen is complicated by the formation during the period of active metabolism of easily hydrolyzable amides. Protein synthesis (table X) was also accompanied by a decrease in the total amide fraction and this, together with the observed loss of amino nitrogen, accounts for the entire change in soluble nitrogen with an error which is probably not excessive in view of the complications in these determinations. The change in the total amide, however, conceals a much greater utilization of the more stable amides (asparagine) which is offset by the accumulation, in the actively metabolizing tissue, of easily hydrolyzable amides. In the light of the evident connection between respiration and nitrogen metabolism, the extreme reactivity of this substance, its apparent increase when the conditions of oxygen and temperature permit rapid metabolism (*e.g.* in dilute salt solutions), and its further depletion when, in strong potassium salts, protein synthesis is stimulated, are all features which might suggest that this substance may be a reactive intermediary between the stable reserves of amino acid amide on the one hand and protein on the

[8] If any of the disappearance of "stable amides" is accounted for by the conversion of asparagine to aspartic acid, which remains as such, this correction is too great.

other. These considerations arouse an interest in the nature and reactions of this glutamine-like component of the soluble nitrogen fraction which is not overshadowed by the greater abundance in the tissue of the more stable nitrogenous substances.

### Titration curves of potato sap

The determination of titration curves on the expressed sap of plants reveals the effect of experimental treatments on the constituents of the buffer complex. Since the components of the buffer system include certain substances which are not readily determined quantitatively (*e.g.* amino acids and organic acids) this determination has considerable value. For this purpose, however, the buffering in the region of the pH of the sap—with which most investigations have been concerned—is not especially instructive and the titration must be extended far beyond this to reactions both more alkaline and more acid. See Small (**32**, pp. 265–291) for a discussion of the buffering of potato sap between the limits pH 4.5 to 7.0. Dunne (**8**) has utilized this method of approach in connection with problems of mineral nutrition, and Hoagland and his collaborators have freely utilized it as an indirect means of investigating the chemical changes which accompany the uptake of salt in barley roots. The authors were familiar with the latter investigations and hence applied this technique in the case of potato discs.

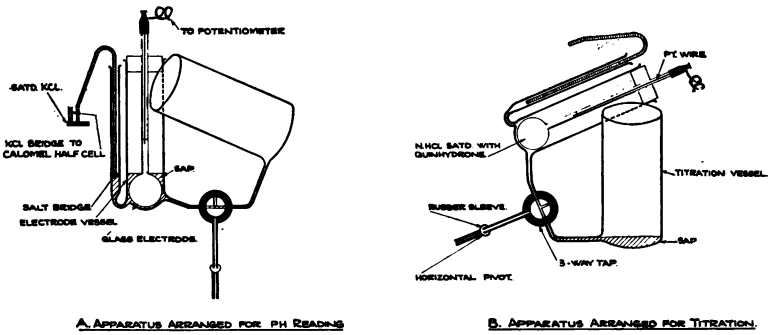


Fig. 4. Rocking electrode and titration vessel for the determination of titration curves of sap.

The titration curves were carried out on aliquots of potato sap freshly expressed from discs which had been frozen and stored at −20° C. after the conclusion of the experimental treatment they received. The change of pH with the addition of 0.20 N HCl, between the pH of the sap and about pH 2.5, was recorded; for the alkaline range to pH 11.5 the treatment was repeated on another aliquot of the sap using 0.20 N NaOH. The amount of acid or alkali added was arbitrarily adjusted according to the shift in pH obtained in the region concerned. The earlier curves (figs. 6 and 7) were

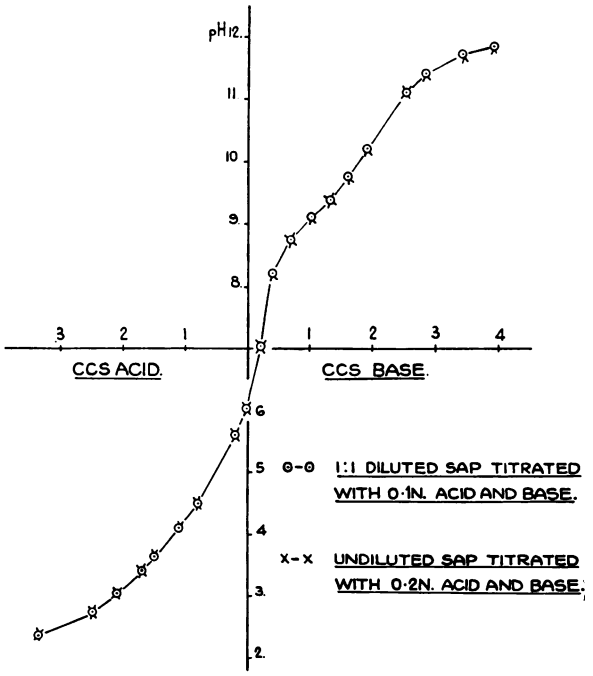


FIG. 5. Titration curve of potato sap.

made from readings obtained using a hydrogen electrode by A. ULRICH[9] although subsequent determinations made with a glass electrode show that the type of curve obtained is not dependent upon a particular method of recording the E.M.F.

The titration curve of potato sap can be more rapidly and effectively explored by the use of a glass electrode system in conjunction with a potentiometer of the Electrometer Valve pattern of the Cambridge (England) Instrument Co. This instrument permits readings to be obtained which are automatically corrected for temperature and which can be stated in E.M.F. or pH units with an accuracy which corresponds to 0.01 pH unit. A convenient form of combined electrode and titration vessel is that described in figure 4 A and B. The necessary additions of acid or alkali are made (from burettes reading to 0.01 ml.) to the sap sample which is contained in the titration vessel, placed for the purpose in the position of figure 4 A. In the position shown in figure 4 B the liquid undergoing titration flows by gravity into the glass electrode chamber and covers the bulb of the electrode. The combined electrode and titration vessel rocks from the position of figure 4A to that of figure 4B on an axis provided by a horizontal portion of the outlet tube. Repeated passage of the liquid from the electrode chamber to the

[9] Division of Plant Nutrition, Berkeley, Cal.

titration vessel insures efficient mixing. The glass electrode bulb contains N HCl, saturated with quinhydrone into which dips a platinum wire. The electrical connection between the glass electrode vessel and the calomel half cell is made (in the position of fig. 4B) by a salt bridge and a saturated potassium chloride solution.

The combined rocking electrode and titration vessel mounted on a hard wood base was so designed that it worked with a minimum of 4 ml. of liquid. To economize material, 2 ml. of sap plus 2 ml. of water were used for each of the titrations with acid and base. It was verified experimentally that dilu-

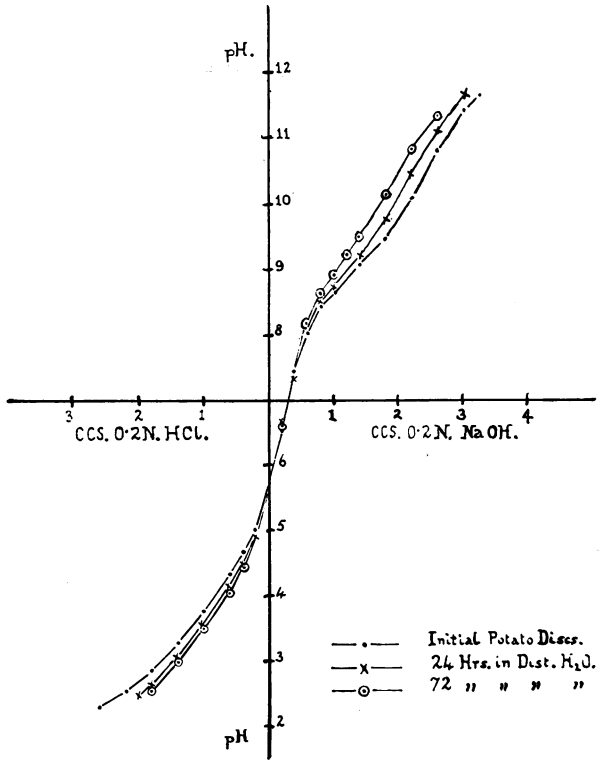


FIG. 6. Changes in the buffer system of potato discs during metabolism at 23° C. in aerated distilled water—the effect of time.

tion in the ratio of 1:1 did not change the nature of the buffer system of the sap. This is evident from figure 5 which shows the titration curves of undiluted potato sap using 0.2 N acid base and also of sap diluted 1:1 with 0.1 N acid and base.

### EFFECT OF SALTS AND METABOLISM ON THE BUFFER SYSTEM OF POTATO

The effect on the titration curve of potato sap of the metabolic processes which occur in the discs in distilled water, and under the conditions of

temperature and aeration conducive to maximum respiration and salt accumulation was determined. In figure 6 can be seen the titration curves of the sap which was expressed after freezing from the initial discs, prior to their period of intense metabolism, and also after periods of 24 and 72 hours, respectively, of high respiration.

Normally the sap of the potato is most strongly buffered between pH 8.5 to 9.5 (figs. 5 and 6), the region in which the buffer effect of amino acids is at its maximum.[10] Progressively with time the metabolic processes in the

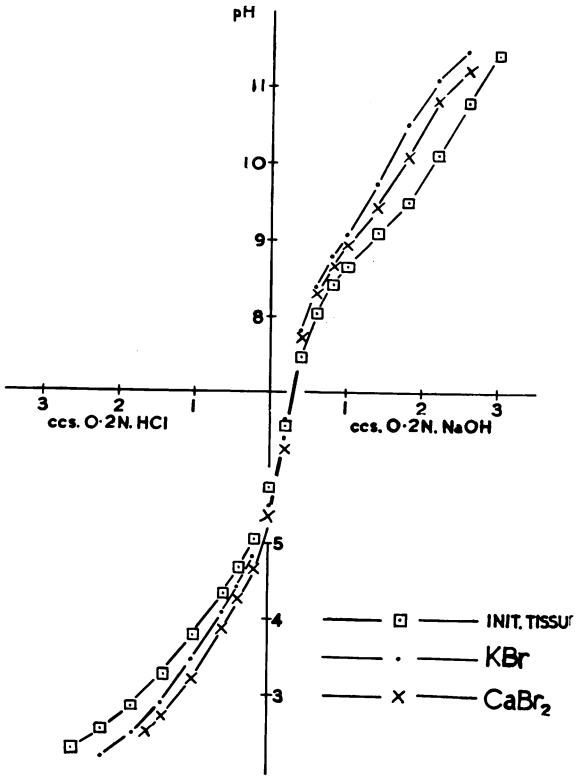


FIG. 7. Changes in the buffer system of potato discs during metabolism at 23° C. in aerated solutions—the effect of salts (KBr, $CaBr_2$).

thin, aerated discs in water cause a decrease in these buffer components which is to be correlated with their conversion to substances with little buffer value, *e.g.*, protein. As figure 7 shows the decrease of constituents which buffer between pH 8 and 10 is greater when the tissue is in contact at 23° C. with aerated solutions of potassium salts than calcium salts—a result which is in agreement with the finding that the former accentuate and the latter

[10] The pKa values for some typical amino acids are as follows: glycine 9.7, aspartic acid 9.9, glutamic acid 9.8, tyrosine 9.4, phenylalanine 8.6.

depress protein synthesis from the stored amino compounds of the potato tissue.

Since the changes which occur in that portion of the titration curve which lies between the limits pH 8.0 to 10 are closely related to the metabolism of the cells, it is desirable to specify the substances involved. Further proof that amino acids are responsible is provided by the data in figure 8,

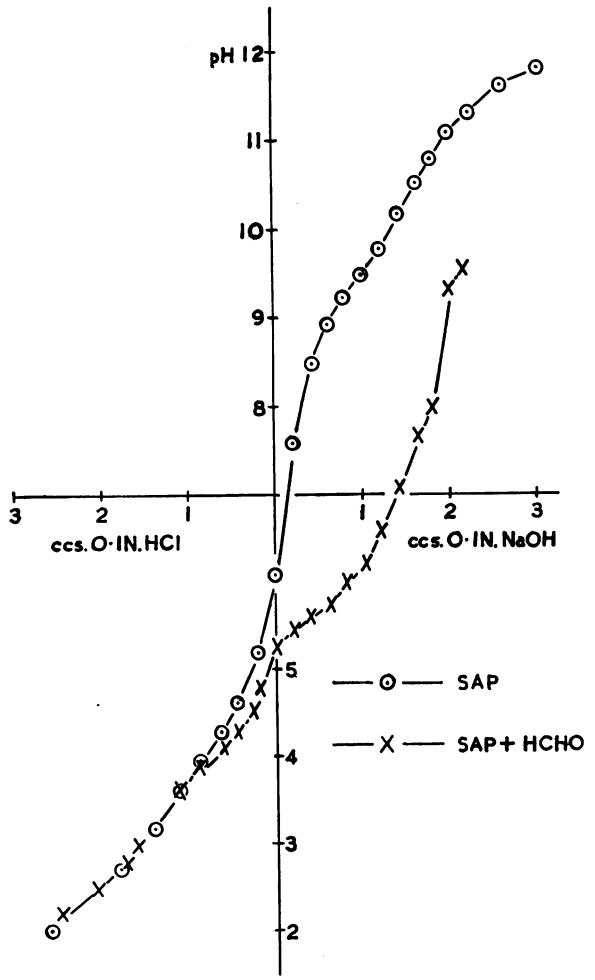


FIG. 8. Effect of formaldehyde on the titration curve of potato sap.

which shows the titration curve of diluted potato sap (2 ml. sap plus 2 ml. $H_2O$) with and without the addition of neutralized formaldehyde (4 ml. of 44 per cent.). In the acid reactions (more acid than pH 4.0) the two curves coincide, but in presence of formaldehyde the buffer capacity normally found

in the region pH 8 to 10 is displaced to the region pH 5.0 to 6.5—a region in which the sap is normally only weakly buffered. The compounds involved must therefore be amino acids which in presence of formaldehyde form methylene-imino derivatives which, being stronger acids, have their maximum buffer effect at a lower pH (pKa values of the methylene-imino derivatives of common amino acids range from 5.4 to 6.9).

It still remains to determine whether the amino-acid moiety (pKa = 8.87) of the asparagine present in potato tissue could account for the changes which occur in the buffer value in the region of pH 8 to 10 during metabolism. SMALL (30) estimated that asparagine contributed only 5 per cent. of the buffer value of potato sap in the region pH 6.7. Figure 9, which

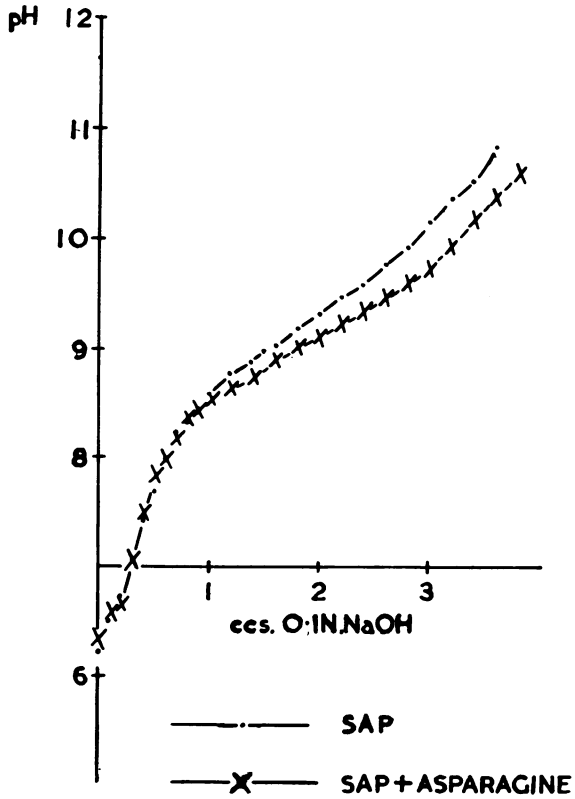


FIG. 9. Effect of added asparagine on the titration curve of potato sap.

shows the titration curve of 2 ml. of potato sap, with and without the addition of an amount of asparagine such that its amino-N content was equal to the total soluble nitrogen of the normal sap. It is evident that *per unit of soluble nitrogen* the normal sap has much greater buffer value than the amino-acid moiety of the asparagine which it contains.

The conclusion, therefore, is that the substances which buffer the normal sap strongly in the region of pH 8 to 10 are amino acids other than asparagine and that the changes induced in this part of the titration curve due to the combined effects of alkali halides and metabolism reflect the effect of these treatments upon the amino acid content of the tissues. This conclusion is in agreement with that obtained by the direct determination of the components of the soluble nitrogen.

At reactions more acid than the natural sap there is a considerable degree of buffering which must be attributed to organic acids. According to SMALL (**30**) citrates and phosphates might contribute 60 per cent. of the buffering between pH 6 and 7 where, however, the buffering is weak; but at reactions more acid, their contribution is much less *e.g.*, 19 per cent. at pH 4.5. SMALL was led to the conclusion that even at reactions as near neutral as pH 5.0 other organic acids were involved and he demonstrated the presence of ether soluble organic acids. From the curves given in figures 5, 6, and 7 it seems clear that the strongly buffered sap at a pH more acid than 5.0 must owe this property to organic acids and their identification is an imperative task for the future.

The progressive metabolism which occurs in aerated distilled water is accompanied by a continual decrease in the buffer value of the sap at reactions between pH 2.0 and 6.0, and this must mean that organic acids are being consumed. An effect of salts on the organic metabolism of the potato discs is suggested by the greater decrease of the buffer value between pH 2 and 6.0, which occurs when the tissue is exposed to aerated calcium bromide, than when it is respiring in aerated potassium bromide. This is an example of a phenomenon observed frequently by HOAGLAND[11] and associates on barley roots. Both potato and barley results are consistent with the view that the bromide ion is absorbed from calcium bromide solutions mainly unaccompanied by the cation so that organic anions disappear from the sap and bicar-

TABLE XI

EFFECT OF DRYING ON ASCORBIC ACID CONTENT OF POTATO DISCS

| SERIES | FRESH WEIGHT OF SAMPLE | STATE OF TISSUE WHEN EXTRACTED | ASCORBIC ACID PER GRAM FRESH WEIGHT |
|---|---|---|---|
| | *gm.* | | *mg.* |
| I | 7.50 | Fresh + NaCN | 0.164 |
| | 7.80 | Dry | 0.188 |
| II | 7.50 | Fresh | 0.170 |
| | 7.50 | Dry | 0.172 |

[11] Results awaiting publication by ULRICH and by HOAGLAND and BROYER privately communicated to the authors.

bonate ions accumulate in the external solution (**36**) which also becomes more alkaline. The titration curves, therefore, not only extend and confirm the conclusions on the nature of the nitrogen metabolism which were obtained from direct determinations but they also suggest that the potato cells metabolize organic acids in amounts which are determined by time and the nature of the salt present. With reference to the rôle of organic acids, the conclusions from the titration curves stand alone, as direct analyses of organic acids have not been made. Clearly the above account does not distinguish between the buffer effect attributable to nitrogen-free organic acids and to organic acid radicles contained in polybasic amino acids.

### Behavior of potato oxidase, ascorbic acid, and glutathione in the actively metabolizing discs

Under the conditions conducive to salt accumulation potato discs turn slightly brown owing to the activity of the familiar oxidase system of the potato cells which acts upon phenolic compounds (including tyrosine) stored therein. This behavior, noted earlier (**34, 36, 39**) is sensitive to the oxygen pressures which also affect respiration. It is, therefore, a result of no little interest that the effects induced by salts upon respiration are paralleled by visible effects on the oxidation of phenolic compounds—potassium salts increase and calcium salts decrease the browning reaction. The specific effects of the salt are also increased with concentration. It is clear that the cellular oxidation of phenols and respiratory substrates is similarly effected by salts and oxygen even if the same catalytic mechanism is not involved.

The effect of neutral salts, other than those which contribute to buffer solutions, on the activity of the aerobic oxidase preparations isolated by Raper and his colleagues from potato tissue does not appear to have been studied in detail. Samisch (**28**) made an intensive investigation of the effect of salts on the oxidase which causes darkening of fruits (apricots). However, the experiments of Samisch on the extracted enzyme reveal no sign of a contrasted effect of potassium and calcium salts such as the organized living cells of potato reveal. In his work, on the contrary, increased concentrations of alkali (*e.g.*, Na) halides *decreased* the activity of oxidase extracts in presence of catechol.

Szent Gyorgyi (**39, 40**) showed that the substance now known as ascorbic acid could reduce the oxidized products produced by potato oxidase from those substances which contain the catechol group, and believed that ascorbic acid could provide for the reversibility of this catalytic system. The first products (quinones) of the oxidation of the phenolic substances of potato are reduced to the phenolic condition by ascorbic acid, which is in turn oxidized. Johnson and Zilva (**13**) found that the enzyme (phenolase) does not act upon ascorbic acid directly but that this substance is oxidized by those inter-

mediates (orthoquinones) which also deaminate amino acids. From the work of KEILIN and MANN (**14**) on purified polyphenol oxidase, which is apparently a copper-protein compound, it is also clear that the oxidase oxidizes ascorbic acid only in the presence of catechol.

Accordingly attention was turned to the reducing substances to be found in cells and particularly to two substances (ascorbic acid and glutathione) both of which are commonly supposed to bear a close relation to metabolic activity, to be capable of reversible oxidation and reduction, and which have been freely incorporated into the proposed catalytic systems of cells. The expectation was that increased amounts of the brown, oxidized products might be attributed to, or accompanied by, a decreased concentration of the reduced form of the substances in question. It can be stated at once that no clear relationship of this kind between the browning effects and the behavior of either ascorbic acid or glutathione in the living cells has yet emerged. The observations made, however, present yet another feature of the metabolism of potato discs which is of particular interest since the substances involved are supposed to be connected with, if they are not causal agents for, vital activity.

### THE DETERMINATION OF ASCORBIC ACID IN POTATO DISCS

The familiar 2:6 dichlorophenolindophenol titration was applied. The dye was used in solutions of 0.10 gm. in 200 ml. of solution buffered at pH 6.8 with a phosphate buffer. The TILLMANN reagent was standardized by a solution freshly prepared from lemon juice assuming that all of the reducing value (by iodine) of this extract was due to ascorbic acid.

As others have found, the difficulty is to standardize the extraction technique. Discrepancies between different workers and methods have led to much controversy in the literature which cannot be dealt with here. Only a brief statement of the method applied and the results obtained will be attempted.

If potato tissue is extracted for ascorbic acid as directed by BESSEY and KING (5) in 8 per cent. acetic acid, 90 per cent. of the initial activity is lost in a period as short as 20 minutes. Fresh tissue, however, ground in 8 per cent. acetic acid + M/10 NaCN gave a constant ascorbic acid titre during a period of two hours after the extraction and, after standing overnight exposed to air, 84 per cent. of this original activity still remained. If the extraction technique was preceded by vacuum drying for ¾ hours at 100° C. slightly higher (up to 20 per cent. greater) values were obtained than when extraction took place from the fresh tissue in presence of cyanid (table XI). Some (**19**) in this sort of difference evidence for combined and uncombined ascorbic acid whereas others (**18, 38**) regard these differences as caused by the more effective inactivation of enzymes in the heated tissue. Since such small differences have not yet been correlated with metabolism, only that method

which gave the best determination of the reduced ascorbic acid was applied (extraction of tissue, vacuum dried, in presence of cyanide). The ascorbic acid exists in washed, living discs *only* in the reduced form. If extracts were made as above, saturated with hydrogen sulphide, and the latter removed in a vacuum in the presence of cyanide, the titrations were the same as for the extract untreated with hydrogen sulphide.

### Effect of time on the ascorbic acid content of potato discs in aerated salt solution

In thin potato discs in aerated solutions at 23° C. a progressive *increase* in the content of ascorbic acid occurs (see two series of table XII). This

TABLE XII

Ascorbic acid content of potato discs in aerated salt solutions at 23° C.

| Series | Hours | Ascorbic acid per gram fresh weight |
|---|---|---|
| | | *mg.* |
| KCl (0.001 equivalents, per liter) | 0.0 | 0.094 |
| | 75.5 | 0.161 |
| | 125.0 | 0.198 |
| $CaCl_2$ (0.001 equivalents, per liter) | 0.0 | 0.094 |
| | 75.5 | 0.155 |
| | 125.0 | 0.188 |

occurs in tissue in contact with aerated distilled water at 23° C. and is yet another symptom (there is no reason to believe that it is a cause) of the increased metabolism which, although it can proceed independently of salt absorption, is a prominent feature of the system which is capable of absorp-

TABLE XIII

Effect of aerated salt solutions and time on the content of reduced ascorbic acid and glutathione in potato discs

| Composition | External solution. Salt concentration per liter | Sample | Period hours | Ascorbic acid per gram initial fresh weight | Glutathione per gram initial fresh weight |
|---|---|---|---|---|---|
| | | | | *mg.* | *mg.* |
| ..... | ..... | Initial | 0.0 | 0.104 | 0.064 |
| ..... | ..... | Initial | 0.0 | 0.102 | 0.059 |
| $H_2O$ ..... | ..... | Final | 70.0 | 0.151 | 0.059 |
| KCl solution ..... | 0.05 | Final | 70.0 | 0.163 | 0.056 |
| $CaCl_2$ solution ..... | 0.05 | Final | 70.0 | 0.162 | 0.060 |
| $H_2O$ ..... | ..... | Final | 115.5 | 0.189 | 0.071 |
| KCl solution ..... | 0.05 | Final | 115.5 | 0.199 | 0.067 |
| $CaCl_2$ solution ..... | 0.05 | Final | 115.5 | 0.213 | 0.065 |

tion. The salts which do affect respiration (KCl which increases it, and $CaCl_2$ which decreases it) have only a little, and barely significant effect on the final, total ascorbic acid content (compare the data for the KCl and $CaCl_2$ series at the same time periods in tables XII and XIII). If—against the weight of evidence—the total ascorbic acid content does include a component which is either combined or in intimate association with the protoplasm it is still possible that the salt treatments affect the combined fraction more significantly than these determinations of total ascorbic acid content indicate.

### Effect of time and salt solutions on reduced ascorbic acid and glutathione in potato discs

The simultaneous effects of the salt and aeration treatment upon the glutathione and ascorbic acid content of potato discs was examined (table XIII). Glutathione, to which a special rôle in biological oxidation has been ascribed, is now known to bear a definite relationship to the oxidation of ascorbic acid by the so-called "hexo-oxidase" of Szent-Gyorgyi (**41**). Hexo-oxidase is a specific enzyme of the expressed juice of cabbage, which Zilva (**45**) thought was present in apple and which oxidizes ascorbic acid (hexuronic acid) to dehydro-ascorbic acid. According to Hopkins and Morgan (**12**) the enzyme hexo-oxidase does not oxidise glutathione directly, but in mixtures of glutathione and ascorbic acid the former is oxidized at the rate at which the latter would have disappeared if the glutathione had been absent. In other words the ascorbic acid is oxidized and the glutathione promptly reduces it, so that ascorbic acid behaves like a co-enzyme in the oxidation of glutathione by "hexo-oxidase" and itself remains in the reduced condition.

After the treatments indicated in table XIII the potato discs were vacuum dried and extracted with acetic acid. In the one case (determination of ascorbic acid) the extract was titrated when standardized ascorbic acid reagent (2 : 6 dichlorophenolindophenol) and in the other, for the determination of total reducing value (ascorbic acid + glutathione), with 0.05 N iodine according to the method of Hopkins and Morgan (**12**).

The data (table XIII) show again that, at 23° C., in thin discs in aerated solutions, the content of ascorbic acid rises in a manner which bears an approximately linear relationship to time. Comparison of tables XII and XIII with figure 3 verifies that the change in the ascorbic acid content of the discs with time does not follow the same course as the respiratory time drift, (*e.g.*, tissue in distilled water) neither is there an outstanding effect which may be attributed to the presence of salts. Glutathione (reduced) contributes only a small part of the total reducing value and the quantity present in the discs does not change significantly when they are exposed to aerated salt solutions.

These data show that the total ascorbic acid content of the living cells is not the factor which directly determines either the browning reaction referred to or the respiratory rate of the cells. The salt effects upon respiration, protein synthesis, and oxidase action in the living cells must either be localized in centers distinct from the milieu in which the ascorbic acid occurs or else the ascorbic acid is protected against oxidation by other unknown substances. The progressive browning of discs with time is not compatible with the continued increase of their reduced ascorbic acid content and, neither in its relation to time nor to salts is the ascorbic acid content of living potato discs affected in a manner similar to respiration. Future work must determine the nature of the substance, or circumstances, which prevents the ascorbic acid in the living cells from being oxidized by the enzyme present. The now familiar rôle of ascorbic acid as a potential carrier of oxidation as, *e.g.*, between hexo-oxidase and glutathione, suggests that it may enable phenolase to activate a much wider range of substances than its appraised specificity would indicate. Hence, although the quantity of ascorbic acid cannot as yet be correlated with the respiration rate, or even the browning reaction, its presence in actively metabolizing potato discs may yet prove to be essential to the processes they carry out.

A possible relationship between the sugar and ascorbic acid content of potato discs is suggested by the fact that the increase of ascorbic acid occurs during a period of starch hydrolysis, and this requires further investigation. Such relationships, however, like the recognition that ascorbic acid is more concentrated in cells which show vital activity, *e.g.*, the apical regions of roots (**26**), or the young shoots on resting potato tubers (**22**), does not yet justify the supposed regulatory rôle of this substance in the metabolic processes which involve oxidation since it does not distinguish between ascorbic acid as a product and as a primary cause of the vital activity in question.

One can only repeat that the facts are that salts (of K and Ca) do influence the metabolism of potato discs. They exert an effect in the cells upon the activity of the enzyme system which catalyzes the oxidation of phenolic compounds and *in vitro* produces oxidative deamination of amino acids; the suggestion is that the latter processes provide the link with the rate of aerobic respiration and protein synthesis. As yet, however, similar effects of salts have not been demonstrated *in vitro* in preparations which lack the organization of the living cells; and therefore the mechanism of the salt and oxygen effects described cannot be ascribed solely to the oxidase system of the cells. It also involves the more intimate part of the living system which, in conjunction with the system which catalyzes oxidation, brings about protein synthesis. A large number of experiments have been made to investigate the effect of potassium and calcium salts on the browning of tissue extracts, on the oxidation of catechol and of ascorbic acid by crude

potato enzyme preparations, and on the autoxidation (catalyzed by copper) of ascorbic acid. It is clear from these that the work must be continued with purified enzyme preparations.

## Summary

The theoretical implications of the data have been elaborated in the text. It remains only to summarize and recapitulate the essential results and conclusions.

The variability in the composition of replicate batches of 40 to 60 standard potato discs, cut from a uniform stock of tubers and washed for 24 hours in running tap water, is small and negligible relative to the changes which occur due to metabolism during periods of the order of 72 hours under the conditions conducive to salt absorption (40 to 60 discs in 2 liters of aerated solution at 23° C.).

Since the fresh and dry weight of the discs changes during the course of metabolism the best basis for the calculation of the results is the initial fresh weight of a known number (40 or 60) of standard discs of known weight (30 or 45 gm.).

The application of conductivity methods to the determination of absorbed carbon dioxide is described. By this means the effect of salts on the time drift in the respiration of potato discs has been investigated.

In relatively strong (0.05 equivalent per liter) potassium salt (KCl) the tissue approaches a steady respiratory rate at a level which far exceeds that of tissue in distilled water and, conversely, in a calcium solution ($CaCl_2$) of the same equivalent strength the steady level (attained after 30 hours) is much lower than that for distilled water. The contrast in the effect of potassium and calcium salts is one which reappears in many aspects of the metabolic behavior of the tissue.

The direct measurement of the heat evolved by potato discs during their metabolism under the required conditions is beset with technical difficulties. The over-all change in the caloric value of the discs, as a result of their metabolism, can be measured by the use of standard bomb calorimetry. The discs decrease in calorific value by amounts much in excess of expectations on the basis of their respiration.

A scheme for the analysis of potato discs has been described. This reveals the changes which occur in the dry weight, fresh weight, starch, sugar, soluble nitrogen and protein content of the discs. Two independent methods show that the estimate of protein synthesized is not appreciably affected by the peculiarities of a single method.

Discs of potato in aerated distilled water or potassium salt solutions synthesize protein. The synthesis is greater in relatively strong potassium salts (KCl) than in water and it can be suppressed in relatively strong (0.075 equivalents per liter) calcium solutions ($CaCl_2$ or $CaBr_2$). The nitrogen for synthesis is drawn from the soluble nitrogen fraction.

The soluble nitrogen fraction of dormant potato tubers consists of both amides and amino compounds and only negligible amounts of free ammonia. The total amide contains two components: the one, probably asparagine, which is stable under hot alcoholic extraction and only hydrolyzed by 6N HCl under reflux condenser for 3 hours and the other, similar to glutamine, which is unstable under hot alcoholic extraction and is hydrolyzed by 2 hours at pH 6 to 7. The accompanying changes in the VAN SLYKE amino nitrogen fraction show that the easily hydrolyzable amide groups behave like that in glutamine. Recognizing these complications, methods are described which permit the determination of the stable amide, the "heat unstable amide" and the amino-nitrogen fraction (free from confusion with the amide group of glutamine-like substances). The fractions determined account quantitatively for the soluble nitrogen content of the tissue.

During protein synthesis nitrogen is drawn from both the stable amide fraction and the amino nitrogen fraction. The bulk of the nitrogen converted to protein is derived from amino compounds other than asparagine. Salts (KCl and $CaCl_2$) influence both the total synthesis of protein and the relative utilization of amino acids and other compounds. Potassium salts increase, and calcium salts decrease, the relative utilization of the amino acids.

Under the conditions of active metabolism and protein synthesis the unstable, glutamine-like amide, increases in the tissue. It appears to be a reactive intermediary and its subsequent utilization is accelerated by the salt conditions which stimulate synthesis.

The metabolic processes described above are reflected in changes in the buffer system of potato. Methods suitable for the rapid electrometric titration of potato sap are described. Titration curves show a strong buffering (pH 8.0–10.0) which cannot be attributed to asparagine; it is shown to be due to amino acids. The amino acid buffering decreases as synthesis of protein occurs. The salts which affect synthesis also affect the titration curve of the sap. Simultaneous effects upon that portion of the titration curve in which organic acid radicals are effective suggest that these also diappear during metabolism in distilled water and their utilization is still greater in calcium bromide solutions.

The salt and oxygen treatments (aerated KCl solutions) which stimulate the respiration of potato discs also increase the superficial browning which is due to the oxidation of phenolic compounds by the aerobic oxidase of potato. The converse effect is observed in aerated calcium chloride solutions. It is believed that the parallel effects of salts on the activity of the oxidase and protein synthesis are to be ascribed to the deaminating action of substances (ortho-quinones) which are intermediates in the oxidation of the phenolic compounds.

The bulk of the reducing (by iodine) action of potato extracts is due to

ascorbic acid. Special precautions are described which nullify the effect of the tissue oxidase on the ascorbic acid in extracts and the determination of the reduced form of ascorbic acid in the potato discs is described. During the rapid metabolism which occurs in tissue in aerated distilled water the reduced ascorbic acid content of potato discs increases progressively with time. Neither in its relation to time, nor to salts, does the ascorbic acid content of potato discs appear to be a causal agent in the respiration or the browning reaction of the living cells. The content of reduced glutathione in the tissue is not significantly affected by the conditions conducive to salt absorption or by the nature of the salt supplied. The possible rôle of ascorbic acid as a carrier of oxygen between the phenolase and non-phenolic substrates is recognized.

*The outstanding metabolic processes of potato discs under the conditions conducive to salt accumulation are those which are conditioned by oxygen and the nature of the salts supplied and these are mutually inter-related. Of these the synthesis of protein, the utilization of amino-acids and stable amides, the formation of unstable amides, the use of organic acid radicals and the oxidation phenomenon shown by the browning reaction in the living cells, are all linked with the rate of aerobic respiration and must contribute to the metabolic machinery which renders salt accumulation possible.* Only future work can tell which, if any, of these diverse processes is more intimately concerned than all the rest.

This paper is based on work which has been in progress since 1933 and which, started in the Division of Plant Nutrition, University of California, has been continued in the Department of Botany, Birkbeck College, University of London. Collaboration with C. PRESTON was confined to the work at Birkbeck College. Our grateful appreciation of the support which the work received from both laboratories is here acknowledged, and our thanks are accorded to Prof. HOAGLAND for reading the proofs. A grant from the publication fund of Birkbeck College assisted the preparation and publication of figures.

The conductivity apparatus used was provided by a grant from the DIXON Fund of the University of London.

The effective continuation of the work at Birkbeck College, upon which this and subsequent papers are based, was made possible by grants for which separate acknowledgment has been made elsewhere.

BIRKBECK COLLEGE
UNIVERSITY OF LONDON

## LITERATURE CITED

1. ALGERA, L. Energiemessungen bei *Aspergillus niger* mit helfe eines automatischen Mikro-Kompensations-calorimeters. Rec. des Trav. bot. néer. **29**: 47–163. 1932.

2. ARCHBOLD, H. K. Physiological studies in plant nutrition. VII. The rôle of fructosans in the carbohydrate metabolism of the barley plant. I. Materials used and methods of sugar analysis employed. Ann. Bot. N.S. **2**: 183–202. 1938.
3. BARKER, J. Analytic studies in plant respiration. VI. The relation of the respiration of potatoes to the concentration of sugars and to the accumulation of a depressant at low temperatures. Part 3. The relation of the respiration to the concentration of sucrose. Proc. Roy. Soc. London B **119**: 453–473. 1936.
4. BAYLISS, L. E. Conductivity method for the determination of carbon dioxide. Biochem. Jour. **21**: 662–664. 1927.
5. BESSEY, O. A., and KING, C. G. The distribution of vitamin C in plant and animal tissues, and its determination. Jour. Biol. Chem. **103**: 687–698. 1933.
6. BROWNE, C. A. A handbook of sugar analysis. London. 1912.
7. DOWNING, A. C., GERARD, R. W., and HILL, A. V. Heat production of nerve. Proc. Roy. Soc. London B **100**: 223–251. 1926.
8. DUNNE, T. C. Plant buffer systems in relation to the absorption of bases by plants. Hilgardia **7**: 207–234. 1932.
9. FIFE, J. M. The physiology of Azotobacter. Unpublished manuscript. Library of Citrus Experimental Station, Riverside, California.
10. GUSTAFSON, F. G. Intramolecular respiration of tomato fruits. Amer. Jour. Bot. **17**: 1011–1027. 1930.
11. HOAGLAND, D. R., and BROYER, T. C. General nature and the process of salt accumulation by roots with description of experimental methods. Plant Physiol. **11**: 471–507. 1936.
12. HOPKINS, F. G., and MORGAN, E. J. Some relations between ascorbic acid and glutathione. Biochem. Jour. **30**: 1447–1462. 1936.
13. JOHNSON, S. S., and ZILVA, S. S. The oxidation of l-ascorbic acid by plant enzymes. Biochem. Jour. **31**: 438–453. 1937.
14. KEILIN, D., and MANN, T. Polyphenol oxidase purification, nature and properties. Proc. Roy. Soc. London B **125**: 187–204. 1938.
15. LEACH, W. Researches on plant respiration. IV. The relation between the respiration in air and in nitrogen of certain seeds during germination. (b) Seeds in which carbohydrates constitute the chief food reserve. Proc. Roy. Soc. London B **119**: 507–521. 1936.
16. LEACH, W., and DENT, K. W. Researches on plant respiration. III. The relationship between respiration in air and in nitrogen of certain seeds during germination. (a) Seeds in which fats constitute the chief food reserve. Proc. Roy. Soc. London B **116**: 150–169. 1934.
17. LOOMIS, W. E. A study of the clearing of alcoholic plant extracts. Plant Physiol. **1**: 179–189. 1926.

18. MACK, G. L. The state of ascorbic acid in plant tissues. Nature **138**: 505–506. 1936.
19. MCHENRY, E. W., and GRAHAM, M. L. Estimation of ascorbic acid by titration. Nature **135**: 871–872. 1935.
20. NEWTON, R. G. An improved electrical conductivity method for the estimation of carbon dioxide and other reactive gases. Ann. Bot. **49**: 381–398. 1935.
21. PARIJA, P. Analytic studies in plant respiration. II. The respiration of apples in nitrogen and its relation to respiration in air. Proc. Roy. Soc. London B **103**: 446–490. 1928.
22. PETT, L. B. Changes in the ascorbic acid and glutathione contents of stored and sprouting potatoes. Biochem. Jour. **30**: 1228–1232. 1936.
23. PHILLIS, E., and MASON, T. G. Studies on the transport of carbohydrates in the cotton plant. III. The polar distribution of sugar in the foliage leaf. Ann. Bot. **47**: 585–634. 1933.
24. PREGL, F. Quantitative organic microanalysis. Transl. by FYLEMAN. London. 1930.
25. RANDALL, M., and ROSSINI, F. D. Heat capacities in aqueous salt solutions. Jour. Amer. Chem. Soc. **51**: 323–345. 1929.
26. REID, MARY E. Localization of ascorbic acid in the cowpea plant at different periods of development. Amer. Jour. Bot. **24**: 445–447. 1937.
27. RICHARDS, H. M. The evolution of heat by wounded plants. Ann. Bot. **11**: 29–63. 1897.
28. SAMISCH, R. The measurement of phenolase activity. Jour. Biol. Chem. **110**: 643–654. 1935.
29. SCOTT, W. W. Standard methods of chemical analysis. London. 1922.
30. SMALL, J. Hydrogen-ion concentration in plant cells and tissues. Protoplasma-monographien II. Berlin. 1929.
31. STEWARD, F. C. The absorption and accumulation of solutes by living plant cells. II. A technique for the study of respiration and salt absorption in storage tissue under controlled environmental conditions. Protoplasma **15**: 497–516. 1932.
32. ————. The absorption and accumulation of solutes by living plant cells. V. Observations upon the effects of time, oxygen, and salt concentration upon absorption and respiration by storage tissue. Protoplasma **18**: 208–242. 1933.
33. ————. Mineral nutrition of plants. Ann. Rev. Biochem. **4**: 521–544. 1935.
34. ————. Salt accumulation by plants—the rôle of growth and metabolism. Trans. Faraday Soc. **33**: 1006–1023. 1937.
35. ————, BERRY, W. E., and BROYER, T. C. The absorption and

accumulation of solutes by living plant cells. VIII. The effect of oxygen upon respiration and salt accumulation. Ann. Bot. **50**: 345–366. 1936.

36. ————————, STOUT, P. R., and PRESTON, C. The biochemistry of salt absorption by plants. II. The balance sheet of metabolites for potato discs showing the effect of salts and dissolved oxygen on metabolism at 23° C. (In press.)
37. ————————, WRIGHT, R., and BERRY, W. E. The absorption and accumulation of solutes by living plant cells. III. The respiration of cut discs of potato tuber in air and immersed in water, with observations on surface: volume effects and salt accumulation. Protoplasma **16**: 576–611. 1932.
38. STONE, W. Ascorbic acid oxidase and the state of ascorbic acid in vegetable tissues. Biochem. Jour. **31**: 508–512. 1937.
39. SZENT-GYORGYI, A. v. Zellatmung. IV. Über den Oxydationmechanismus der Kartoffeln. Biochem. Zeits. **162**: 399–412. 1925.
40. ————————. Observations on the function of peroxidase systems and the chemistry of the adrenal cortex. Description of a new carbohydrate derivative. Biochem. Jour. **22**: 1387–1409. 1928.
41. ————————. On the function of hexuronic acid in the respiration of the cabbage leaf. Jour. Biol. Chem. **90**: 385–393. 1931.
42. VAN DER PLANK, J. E. The estimation of sugars in the leaf of the mangold ( *Beta vulgaris*). Biochem. Jour. **30**: 457–483. 1936.
43. VICKERY, H. B., PUCHER, G. W., LEAVENWORTH, C. S., WAKEMAN, A. J., and NOLAN, L. S. Chemical investigations of the tobacco plant. V. Chemical changes that occur during growth. Connecticut Agr. Exp. Sta. Bull. 374. 1935.
44. ————————, ————————, CLARK, H. E., CHIBNALL, A. C., and WESTALL, R. G. The determination of glutamine in the presence of asparagine. Biochem. Jour. **29**: 2710–2720. 1935.
45. ZILVA, S. S. Reversible enzymatic oxidation of vitamin C. Biochem. Jour. **28**: 663–666. 1934.