

The Action of δ -Hexenolactone and Related
Compounds on the Metabolism of
Certain Plant Tissues.

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A B S T R A C T

Three methods for the synthesis of hexenolactone have been tested, and that of Haynes and Jones, based upon the condensation of propylene oxide and sodium acetylide is preferred. The uptake of glucose, galactose and arabinose by yeast cells under aerobic and anaerobic conditions has been studied, using three strains of Saccharomyces, namely S. carlbergensis nos. 74 and 355, S. cerevisiae no. 366. All three strains show the same type of response to hexenolactone, although no. 74 is most affected. Glucose uptake is inhibited, slightly during first 20 minutes of contact, but more markedly on longer contact with the lactone. Uptake of galactose or arabinose is unaffected, Uptake of inorganic phosphate by yeast cell is appreciably inhibited by hexenolactone. Hexenolactone has been shown to reduce the respiration (oxygen uptake) of mitochondria freshly prepared from cauliflower. Six members of the tricarboxylic acid cycle were used as substrate for respiration, and all were affected; but the inhibition was most marked with the most weakly-respiring systems (α -ketoglutarate, fumarate) and least with the most vigorous (succinate). Inhibition of the succinate system due to malonate was enhanced by hexenolactone, the degree of enhancement increasing with time of contact with the lactone. The phosphate-induced swelling of fresh mitochondria in the presence of a respiration-substrate is

inhibited by hexenolactone. Hexenolactone has been shown to produce changes in the ionisation pattern of certain proteins as shown by a shift in the iso-ionic point given by electrometric titration. The nitrogen distribution of sections of pea-stem tissue exposed to hexenolactone has been studied. The most notable effect of the lactone is to cause a very considerable exosmosis of the amino-acids of the tissue. Possible reasons for the observations made are discussed, and it is suggested that all are in agreement with the theory that the effect of hexenolactone is chiefly due to its influence on the permeability of tissue membranes. In this connection a comparison is made with certain fungistatic antibiotics.

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I N T R O D U C T I O N

It has been recognised for some time that certain simple unsaturated lactones possess distinct physiological activity, usually shown by their inhibitory effect on the growth or on the development of tissues. The subject was comprehensively reviewed by Haynes (1948) who listed five main types of activity exhibited by these compounds, namely,

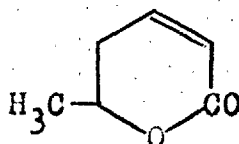
- 1) Inhibition of the growth of animal tissues.
- 2) Antibiotic activity.
- 3) Inhibition of the germination of seeds and of the growth of plant tissues.
- 4) Insecticidal and fish poison activity.
- 5) Cardiac activity.

The first three of these aspects have some bearing on the present work, and for this reason a summary of certain features thereof will be given as an introduction.

1) Inhibition of the growth of animal tissues:

Heaton (1926) showed that embryonic chick tissues could be cultured in vitro, in a suitable nutrient medium, and that in the presence of small amounts of malt extract the growth of connective tissue was markedly suppressed while epithelial tissue continued to grow freely; he concluded that the malt extract contained a "differential

growth inhibitor" of unspecified chemical nature. Heaton's observations were confirmed by Drew (1927) and Brues et al. (1936), while Medawar (1937) showed that a similar inhibitory effect was produced by extracts of wheat, oats, maize, barley, orange peel, etc. His experiments also suggested that the inhibitory action extended to all mesenchyme derivatives in animal tissues. Further investigations were undertaken by Medawar, Robinson and Robinson (1943) who isolated from malt extract, by vigorous steam distillation, an inhibitory substance, apparently an unsaturated lactone of the formula $C_6H_8O_2$. These authors pointed out that a natural lactone of this formula was already known, in the "parasorbic acid" isolated from the berries of mountain ash, Sorbus aucuparia, by Hofmann (1859). The identity of this compound with D- δ -hex-a-enolactone was suggested by Doebner (1894) and confirmed by Kuhn et al. (1943) who prepared the lactone synthetically from sorbic acid, and also demonstrated the inhibitory effect of their synthetic product. Medawar et al. also prepared in small



D-6-hex-a-enolactone

yield a synthetic lactone from the condensation of aldol and malonic ester in pyridine solution; its properties resembled those of the malt extract distillate, but its inhibitory activity was distinctly less than that of the natural product. Yet another method of synthesis was employed by Haynes and Jones (1945), namely semi-hydrogenation of the acetylenic hydroxyacid obtained by carboxylation of the ethynylcarbinol derived from propylene oxide (see under "Syntheses of lactones"); the lactone was obtained in good yield, and in pure form, but again its activity was appreciably less than that of the malt extract product. This diminished activity was apparently not due to the fact that the synthetic product is a DL-compound, since Kuhn et al. (1943) found the same degree of activity in the natural compound and their DL-compound, and it must be concluded that some other factor is present in the malt product. Nevertheless, synthetic hexenolactone does possess the differential inhibitory capacity, and this capacity is not common to all α -unsaturated lactones (Haynes and Jones, 1946).

Some important observations were made by Hauschka and his colleagues (1944, 1945) on the effect of hexenolactone on the flatworm Dugesia tigrina. When this aquatic

invertebrate is exposed to the lactone (5×10^{-5} M. soln.) for 12 to 20 hours a very typical degeneration of the animal takes place. The first visible effect is a partial evisceration followed by disintegration of the three branches of the gut, and finally damage is caused to the external wall overlying the gut. This effect was not produced by a number of other substances tested, including some closely similar in structure to hexenolactone. Hauschka also found that this rather specific action of hexenolactone was not shown in the presence of cysteine, glutathione, α -alanine or β -alanine, also in concentrations of the order of 5×10^{-5} M., and from this and other studies he concluded that hexenolactone owed its activity to its ability to condense with, and thereby inactivate, certain thiol groups of essential enzymes; but as Haynes (1948) has pointed out, reactivity with SH groups is a property common to most if not all $\alpha\beta$ -unsaturated lactones, and since there is a marked structural specificity in the growth-inhibitory activity shown by these compounds it seems unlikely that this simple explanation can be considered adequate; this point is discussed in more detail later.

In a further paper, Hauschka (1946) seems to tend towards the opinion that direct interaction with SH groups may not be the only channel through which hexenolactone affects animal tissues. He again calls attention to the

remarkable specificity of the effect on Dugesia tigrina; this effect is apparently unique to hexenolactone, whereas eighteen other growth inhibitors tested produced only what he terms the "classical anterior-posterior gradient syndrome", i.e., disintegration from the head downwards. He concludes that "the hexenolactone-induced lesion pattern in D. tigrina may be explained both on the basis of differential penetration and tissues-differential susceptibility", possibly conditioned by the different distribution of enzyme complexes dependent on free SH groups. Beatty (1951) also ascribed a major role to the combination of hexenolactone with SH. He studied the effect of known SH inhibitors on the development of amphibian eggs and in an analysis of the so-called "blocked SH syndrome" in these eggs he distinguished eleven steps of which at least seven were affected by hexenolactone as well as by the well-known SH inhibitor iodoacetic acid. Iodoacetate itself would seem to have a certain capacity for differential inhibition at least in high concentrations, since dorsal portions of frog embryo tissues were found by Rapkine and Brachet (1951) to be much more susceptible to its action than the ventral portions.

Among other investigation into the action of hexenolactone on animal tissues the work of Briggs (1946)

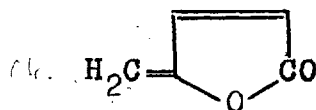
may be mentioned. She found that the sensitivity of the embryo of Rana pipiens was greatest at the beginning of development, when an appreciable retardation was produced by 5×10^{-5} M lactone. Sensitivity diminished until the post-neurula stage, when it again increased, but was confined to certain organs which had by then become differentiated - a further example of selective inhibition. The nuclear mechanism was apparently undamaged.

It will be clear from the account given above that while various features of the differential inhibition of animal tissues have been recorded in some detail, little positive advance has been made from these studies as to the chemical mechanism underlying the phenomena. Apart from the lactone - SH relation proposed by Hauschka, the only proposal has been that made by Medawar et al. (1943), namely that hexenolactone interferes with the metabolism of pantothenic acid; but Kuhn et al. (1943), Hauschka et al. (1945) and Roy (1948) found no evidence to support this theory.

2) Inhibition of the growth of micro-organisms:

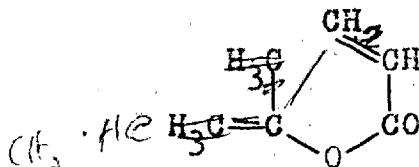
Quite a number of simple unsaturated lactones have been found to possess rather feeble antibacterial activity. Probably the simplest to show this activity is

protoanemonin, the lactone of β -acetylacrylic acid.



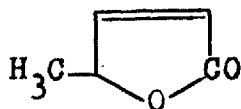
Protoanemonin

This acid is stated by Baer et al. (1946) to show no antibacterial activity, but Moffat et al. (1946) claim that its homologue, β -propionylacrylic acid, inhibits growth

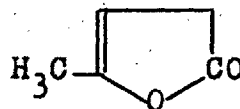


β -propionylacrylic acid.

of E. coli, Staphylococcus aureus and Pseudomonas pyocyanea at concentrations of from 1 in 2000 to 1 in 4000; the activity of the ethyl ester was much greater. The angelicalactones (α and β) are inhibitory towards E. coli at a concentration

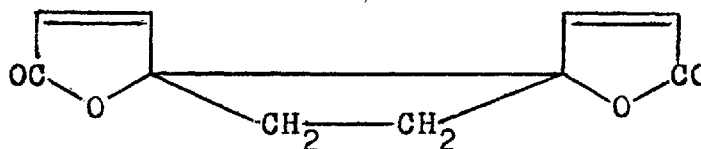


α -angelicalactone

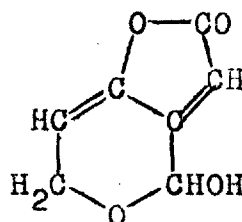


β -angelicalactone

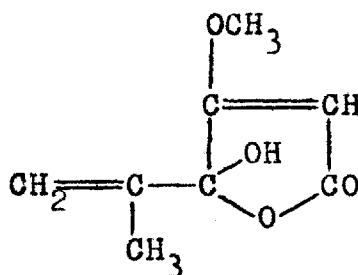
of 1 in 1000 (Baer et al.), while hexenolactone itself was shown by Kuhn et al. (1943) to inhibit growth of E. coli but not of certain Lactobacilli - a further example of a distinct differential capacity for inhibition. Similar observations on bacteria and yeasts were made by Brodersen and Kjaer (1946) and on filamentous fungi by Buston and Roy (1949). In attempts similar to those made by Hauschka et al. (1945) to overcome the inhibition caused by hexenolactone, Roy studied the effect of a number of vitamins and amino-acids, but only β -alanine, in a limited range of concentrations, was effective in counteracting the lactone (for *Fusarium* and *Phycomyces* species). The metabolic function of β -alanine has often been attributed to the fact that it is incorporated into pantothenic acid, itself an integral part of the co-enzyme A molecule. However, neither pantothenic acid nor pantoyl lactone could replace β -alanine as an antagonist to hexenolactone. May (1952) showed that, apart from suppression of growth, hexenolactone has some inhibitory effect on respiration and fermentation of Saccharomyces cerevisiae. Cavallito and Haskell (1945) and Geiger and Conn (1945) have discussed the mechanism of inhibition of bacterial growth by simple lactones such as anemonin, and more complex types such as patulin and penicillic acid,



Anemonin



Patulin



Penicillic Acid

and again have focussed their attention on the interaction between the lactone and SH groups, but point out that this simple explanation is not adequate to account for all

observed facts.

Further objections to the simple lactone - SH theory were put forward by May (1952). Since at least one of the enzymes involved in the glycolysis pathway is SH-dependent, it might be expected to show a sensitivity to hexenolactone; in fact, neither oxygen uptake nor CO₂ output by actively-fermenting cell-free yeast extracts was affected in the least by hexenolactone in concentrations as high as 1.5×10^{-3} M. Similar results were obtained with other micro-organisms; although inhibition of various dehydrogenases of intact cells could be demonstrated, in no instance was any effect observed on a cell-free preparation. May extended her studies to examine the effect of hexenolactone on other enzymes, some of which were recognised to be of the SH-dependent class, but only one enzyme was found to be affected, namely urease. (Slight inhibition of xanthine oxidase was occasionally observed, but later work failed to confirm this effect). May was therefore led to conclude that the inhibitory effect of hexenolactone was not due to any simple action on intracellular enzymes, at any rate those concerned with glycolysis and respiration, and she sought to explain the effect in terms of an alteration in the nature of the

cell-surface which would affect the passage of materials through the cell-wall. In an attempt to support this view she studied the effect of the lactone on the agglutination of cells of E. coli, and on the fragility and swelling of erythrocytes, but her experiments did not provide really convincing evidence in support of her views.

3) Inhibition of the germination of seeds and of the growth of plant tissues:

As early as 1910 Cameron had found that germination of seeds was delayed or suppressed when the seeds were treated with a dilute (1 in 10000) solution of coumarin. The same effect was studied by Köckemann (1934) who gave the name "blastocholine" to any substance which possessed this property, and such substances were found to be widely distributed in ripe fruits and seeds. Various attempts were made to isolate the substance responsible for the effect (Evenari, 1940; Cox, 1945; Konis, 1940; Akkermann and Veldstra, 1947) but without great success. However, it has been found that many unsaturated lactones possess blastocholine activity, and it appears that the activity is not greatly dependent on the specific structure of the whole molecule. Kuhn et al. (1943) found that both natural "parasorbic acid" from the mountain ash and the synthetic

DL-hexenolactone completely suppressed germination of cress seeds (Lepidium sativum) at dilution of 1 in 1000, and checked growth of roots of seedlings at 1 in 10000.

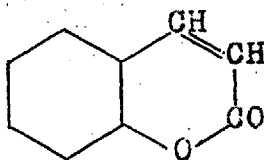
Roy (1948) found that no protection against the action of hexenolactone was given by (among other compounds) β -alanine, which was known (Hauschka, 1945) to protect Dugesia tigrina.

The inhibitory activity of certain unsaturated lactones on plants is not confined to the blastocholine effect on seeds. Apart from the effect of hexenolactone on seedling roots mentioned above, Buston and Roy (1949) found that the growth of excised tomato roots in a synthetic medium was completely suppressed by hexenolactone, (1 in 10000) and that neither thiamin nor nicotinic acid (both of which are required by tomato root cultures) gave any ~~no~~ protection.

A considerable amount of work has been done on the interaction between plant growth-inhibitors including unsaturated lactones and plant growth-substances (auxins). Much of the early work based on the supposition that the "natural auxins" ('a' and 'b' of Kögl) contained an unsaturated five-membered ring, but in view of the doubtful status of these "auxins" there is little point in speculations along these lines. Linser (1940) showed that

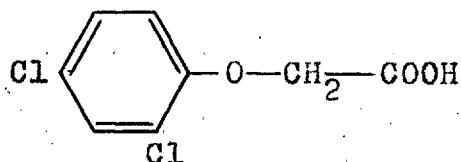
an extract of leaves of Syringa was antagonistic to heteroauxin (β -indolylacetic acid) both in the usual Avena test and as regards root development in Cochlearia amoracia. Juel (1946) found that tomato extracts could counterbalance the effect of "auxin" in maize extract, but also pointed out that since the tomato extract resembles that of maize in retarding growth of roots of pea the two extracts are not antagonistic in all their effects. Larsen (1947) has also shown antagonism between the auxins and certain lactones, including hexenolactone, in the Avena test, while Roy (1948) showed that this antagonism was partly overcome by β -alanine. This effect of the amino-acid was only apparent within a limited range of concentrations, just as was the effect of α -alanine, β -alanine, or cysteine noted by Hauschka for Dugesia tigrina. In the Avena test however, α -alanine was without effect.

Audus and Quastel (1947) made a fairly detailed study of the action of coumarin and showed that for a



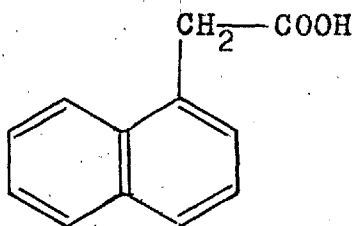
Coumarin.

variety of plant species root-growth was more sensitive than seed-germination, and also that the action of coumarin was a differential one, carrot being much more sensitive than cress to coumarin although affected to an equal extent by 2:4-dichlorophenoxyacetic acid. Protoanemonin was found



2:4-dichlorophenoxyacetic Acid.

to be considerably more active than coumarin, Veldstra and Havinga (1943) had also noted an antagonism between coumarin and α -naphthaleneacetic acid, a growth promoter



α -naphthaleneacetic Acid.

of the auxin type; and Veldstra has favoured the idea that the growth-promoters and growth-inhibitors exert their influence by affecting the permeability of various cell membranes.

Further investigations into the action of hexenolactone and closely related compounds on plant metabolism were carried out by Richards (1953) and Mehta (1954), by methods rather similar to those used by Thimann and his colleagues in studying the effect of iodoacetate, arsenite and fluoride on the plant tissues (Christiansen and Thimann, 1950).

Using the techniques devised by Thimann, Richards studied the carbohydrate balance in sections of stem of pea (Pisum sativum) and found that the uptake of water, under controlled conditions, was decreased to the extent of 61% by 4.5×10^{-3} M lactone, and that this decreased water-uptake was accompanied by a loss of materials from the stems, so that the dry weight of the stem sections showed a decrease of some 1.64% as compared with 0.83% for stems treated with water alone. Respiration, as measured by oxygen uptake of the stem tissues, was inhibited by some 15% during the first four hours of treatment with lactone, and to a much greater extent during the 16 - 20 hour period. All these observations are in agreement with those of Christiansen and Thimann on the effect of iodoacetic acid. These authors also reported that iodoacetate, alone of the growth inhibitors tested, caused a marked loss of easily diffusible cell-contents into the surrounding medium, and

stated that this loss could be stopped immediately by transferring the stems from the iodoacetate solution to an auxin solution. Richards observed that a similar exudation of simple substances from the stems was produced by hexenolactone, but while Christiansen found that fructose was the only sugar present in the exudate, Richards detected only glucose. Christiansen (1950,I), in discussing the results of his experiments on pea stem sections, concluded that many of the effects observed could be explained on the assumption that membrane permeability is in some way modified both by auxin and by iodoacetic acid, the two being antagonistic owing to a similarity of structure. He did not suggest that any specific enzymes, SH-dependent or other, were concerned.

It is evident from the above summary that hexenolactone is a substance capable of influencing a wide range of activities in many different animal tissues, plants and micro-organisms, but it is not easy to formulate in biochemical terms an explanation for its inhibitory activity. The simplest explanation, and one to some extent supported by some experimental findings, is that due to Hauschka outlined above - that the lactone acts by direct combination with the SH-groups of some essential enzymes, but that amino-groups (of enzymes) may also be involved. This simple explanation however does not account for all

the phenomena observed. It seems unlikely that the differential effect on connective and epithelial tissues could be thus explained, for SH enzymes concerned with growth are hardly likely to be restricted to connective tissue; nor to embryonic tissues at a particular stage of growth. Nor is it clear why the reversal of the action of the lactone by cysteine or β -alanine is never complete (in fact never exceeds about 70%) even in the presence of a considerable excess of these amino-acids. But the greatest objection is that test on isolated enzymes, even those known to be SH-dependent, have, with the exception of those on urease, given negative results; in none of the systems examined has a cell-free preparation been found sensitive to the lactone. A rather detailed study of urease inhibition was made by Spector (1956), who compared the effect of hexenolactone on the one hand and iodoacetate, mercuric salts and p-chloromercuribenzoate on the other. In no case was any indication obtained that these well-known SH-reactive compounds were concerned with the same active centres of the enzyme as was hexenolactone. The inhibition produced by mercuric chloride was completely reversible by cysteine; that produced by the lactone was not. Titration of the five SH groups of the urease molecule by p-chloromercuribenzoate was unaffected by the presence of

hexenolactone, nor was the amperometric titration of silver-nitrate-urease. Iodoacetamide which "spares" p-chloromercuribenzoate by combining with the "surface SH" groups of urease, had no such sparing effect when hexenolactone was the inhibitor. All these facts led Spector to conclude that although hexenolactone may react with simple SH compounds such as cysteine in aqueous solution, its inhibitory effect on urease is not to be explained in such simple terms, and this view was strengthened by the observation that it has no effect on papain, which is known to be SH-dependent. On the other hand a careful study of the electrophoretic behavior and of the electrometric titration curves of urease before and after treatment with the lactone led Spector to suggest that treatment resulted in a change in the "number, distribution or ionic strength" of the polar groups present in the enzyme-protein, probably by a suppression of certain acidic groups, but this suggestion was not further studied.

Scope of the present work.

From the later months of 1955 work on hexenolactone remained in abeyance until the present writer commenced these researches in January 1959. A review of the situation at that time suggested that the following points should be considered:

1) The possibility of direct investigations into permeability changes in membranes that might be produced by hexenolactone.

2) The need for a further investigation of the fermentation-respiration system in cells (yeast and other) (see on the next page).

3) The desirability of studying changes in the nitrogenous metabolism produced by hexenolactone in plant tissues, thus completing the comparison (begun by Richards) of the effect of iodoacetate and of the lactone on the major constituents of pea stems.

4) The possibility of studying changes in the distribution of various polar groups in proteins, as suggested by Spector.

1) Membrane permeability.

Direct investigations into permeability of cell-walls and membranes are without doubt most satisfactorily undertaken with the aid of isotopically labelled compounds such as ^{14}C glucose and ^{32}P phosphates. However no facilities were available for using such techniques and other methods had to be sought. The most promising methods seemed to be, for sugars, that of Burger, Helmová and Kleinzeller (1959) and for phosphate that of Goodman and

Rothstein (1957). These methods are described in detail in the "Experimental" section.

2) Cellular respiration.

May's investigation (1952) into the respiration and fermentation of glucose by Saccharomyces cerevisiae was some what superficial, and her methods are open to criticism. The sample of hexenolactone which she used was synthesised from sorbic acid by the method of Joly and Amiard (1947), and undoubtedly contained a certain amount of unchanged sorbic acid (see synthesis of lactones). At the time it was considered that this acid possessed none of the inhibitory activity shown by the lactones, since Hauschka (1944) had found it to have no effect on Dugesia tigrina. However, a direct test during the present work showed that sorbic acid markedly inhibits the respiration of yeast, and a search of the more recent literature disclosed the fact that its fungistatic activity has recently been revealed (Salunkhe, 1956; Shinkawa, 1957). Nor is it clear that May distinguished sufficiently between the carbon dioxide arising from anaerobic fermentation and that from aerobic respiration; her experiments with cell-free yeast preparations showed quite clearly that glycolysis was unaffected by hexenolactone but did not specifically cover the aerobic production of carbon dioxide. With intact yeast

cells she found that oxygen uptake was inhibited to the extent of between 20% and 40% during a four-hour period, but to obtain this degree of inhibition required an exceptionally high concentration (ca. 2×10^{-2} M.) of lactone. In view of the association of aerobic respiration with the mitochondria of cells, it seemed of interest to study the effect of lactone on these particles. It has been suggested (Chappell and Greville; 1958, 1959, 1960) that there may be a direct relation between the physical state of the mitochondria, as evidenced by their capacity for swelling, and their activity in sustaining the electron-transport system of cellular oxidation. Thus it was hoped that if hexenolactone did affect aerobic respiration some evidence might be forthcoming not only as to an inhibition of one or more stages in the tricarboxylic acid cycle but also as to some physical change in the mitochondria - and such a physical change might be the result of a modification of the mitochondria proteins, as suggested by Spector as an explanation of the urease-hexenolactone interaction. It has in fact been suggested by Chari-Bitron and Avi-Dor (1959) that some inhibitors of respiration act by causing "primary damage to the mitochondrial structure" rather than by affecting a specific enzyme. The experiments now described were therefore undertaken with these considerations in mind.

3) Nitrogen distribution in plant tissues.

The investigations into changes in distribution of nitrogenous substances present in pea stem tissue caused by exposure to lactone were planned to complete a survey of the gross effect on the major components of the tissue; the analytical methods used are all well-known and call for no special comment here.

4) Polar groups in enzyme-proteins.

Spector's study of the action of hexenolactone on urease - the only enzyme definitely known to be affected by the lactone - was largely concerned with an attempt to discover whether enzyme-SH groups were directly involved in the inhibition, and all evidence seemed to be against this view. Spector inclined towards the idea that the inhibition was caused by some alteration in the distribution of polar groups in the protein structure, and in support of this suggestion drew attention to an apparent change in the electrometric titration curve of urease following exposure to the lactone; indications were also obtained of a change in the electrophoretic behaviour of the treated enzyme. No detailed or extensive study of these preliminary observations was attempted. Recently certain refinements in the methods of electrometric titration have been introduced in this

laboratory by Dr. Russell, and it seems worth while to apply these methods in the present instance.

EXPERIMENTAL

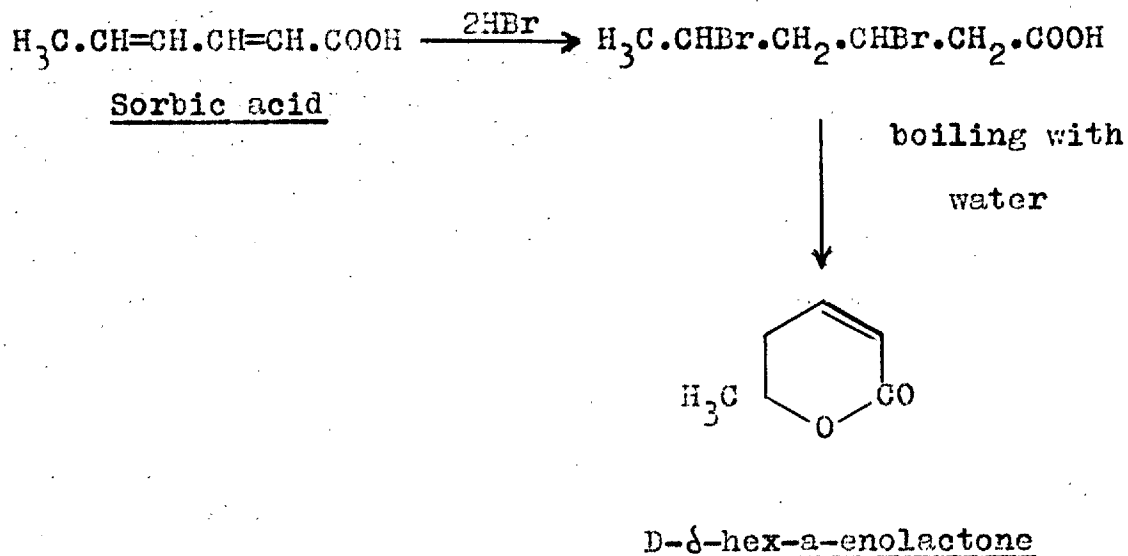
SYNTHESES OF LACTONES

Three methods of synthesis were investigated but only that described by Haynes and Jones (1946) gave a satisfactory product in sufficient yield. The method described by Medawar, Robinson and Robinson (1943) was not attempted.

1) The method of Kuhn and Jerchel (1943).

Sorbic acid (Light & Co.) was finely powdered in a ball-mill and 8.7 g. of the acid was added to 400 ml. of hydrobromic acid, previously saturated with HBr gas. The mixture was agitated thoroughly and stirred continuously throughout the reaction. Dry hydrogen bromide gas was passed through the reaction mixture at intervals to keep the hydrobromic acid fully saturated. At the end of six weeks crystals of 2:5-dibromohexoic acid were collected. The acid was recrystallised from carbon disulphide.

30 g. of 2:5-dibromohexoic acid and 350 ml. of water were heated under reflux for 2-3 hours. The solution was cooled and then extracted with ether. The ether extracts were dried over anhydrous sodium sulphate and the ether was distilled off. The residue left behind was distilled under reduced pressure (12 mm.) and the fraction distilling at 110° was collected.



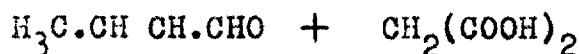
The objections to this method are firstly that the preparation of the dibromo-acid is an extremely tedious process. Sorbic acid is not appreciably soluble in concentrated hydrobromic acid, and although the reaction can be assisted by powdering the acid very finely, bromination still needs to be continued for six weeks or so. Further, the second stage of the process (removal of bromine) gives a poor yield of the lactone, and some amount of sorbic acid is re-formed. After two attempts this method was abandoned.

2) The method of Joly and Amiard (1947).

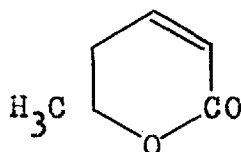
30 g. of crotonaldehyde, 45 g. of malonic acid and 45 g. of pyridine were refluxed together on an electric mantle. As the reaction commences, carbon dioxide is

evolved and this progressively diminishes towards the end of the reaction (12 - 15 hours). The reaction mixture was cooled in an ice-bath and 1:1 sulphuric acid added slowly while shaking till the solution was acid to congo red. A heavy, white precipitate of sorbic acid in the form of needles separated. The acid was recrystallised from a boiling mixture of 2 volumes of water and 1 volume of alcohol. The pure acid melted at 135° .

The acid mother liquor and the washings from the sorbic acid precipitation were then extracted four times with ether. The ether extracts were neutralised by washing with a solution of sodium carbonate, followed by sodium bisulphite and finally water. The ethereal solution was then dried over calcium chloride and the ether removed by distillation. The residue was fractionally distilled under reduced pressure. Hexenolactone is a colourless, oily liquid with a sweetish smell, b.p. 105° - 108° at 12 mm.



pyridine



D-δ-hex-a-enolactone

The authors of the method claimed that it was possible to obtain a 15% yield of the lactone by adjusting the temperature of the crotonaldehyde/malonic acid reaction, 70 - 72° being the optimum. In repeated tests the yield now obtained never exceeded 5%. It may be noted that Joly and Amiard do not mention any physical or chemical tests (other than a rather indefinite statement on boiling point) by which they checked the identity or purity of their product; they refer only to a test for biological activity. The chief objection to the method (which otherwise would be quick and simple) is that it is impossible satisfactorily to remove the last traces of sorbic acid from the product; even the appropriate fraction of the distillate yielded appreciable amounts of crystalline sorbic acid on standing for a few days at low temperature. Eisner, Elvidge and Linstead (1953) also rejected the method on the grounds that "purification of the product is difficult".

3) The method of Haynes and Jones (1946).

Freshly distilled propylene oxide (725 g.) was added during one hour to a solution of sodium acetylide in liquid ammonia, prepared from 230 g. of sodium in one litre of liquid ammonia. The acetylene flow of 100 ml. per minute was maintained for a further two hours and then stopped. The reaction mixture was then stirred for 24 hours after

which the reaction was interrupted by the gradual addition of ammonium chloride (600 g.) during one hour. After the addition of two litres of ether the mixture was stirred for another half an hour to complete the decomposition of the sodio-derivative. The ammonia was allowed to evaporate thoroughly with ether. The ethereal solution was dried over anhydrous sodium sulphate and evaporated. The residue was then distilled in nitrogen, yield 305 g., b.p. $74.6^{\circ}/100$ mm., n_D^{16} 1.4406.

The carboxylation of pent-1-yn-4-ol.

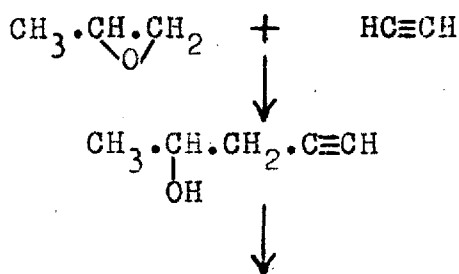
Ethyl magnesium bromide was prepared from one gram mol. of magnesium in ethereal solution and the excess ether was evaporated during the addition of sodium dried benzene (200 ml.). A solution of pent-1-yn-4-ol (half gram mol.) in benzene (100 ml.) was added to the ice-cold solution during $1\frac{1}{2}$ hours and the mixture refluxed for two hours. The Grignard complex solution was poured into a large excess of solid carbon dioxide (ca. 200 g.) in a steel autoclave of about one litre capacity which was then sealed and shaken at room temperature for 24 hours. The resulting complex was hydrolysed with ice and a 20% excess over the calculated quantity of 15% sulphuric acid at 0° C.

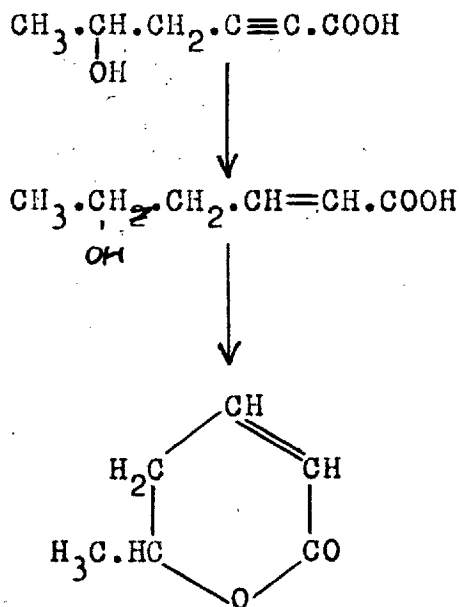
The aqueous solution was then saturated with salt

and extracted with ether. The ethereal solution was washed thoroughly with saturated bicarbonate solution. After extraction of any non-acid material with ether, the bicarbonate washings were acidified with 15% sulphuric acid, saturated with salt and extracted with ether. After drying over anhydrous sodium sulphate, the ethereal solution was evaporated giving the acetylenic hydroxy acid, usually as an oil, which solidifies on leaving at 0° C.

Reduction and lactonisation.

A solution of pent-1-yn-4-ol-carboxylic acid (42 g.) in methanol (110 ml.) was shaken with hydrogen in the presence of a palladium-barium sulphate catalyst (1.5 g. 5% palladium; Schmidt, 1919 and Shriner and Adams, 1924). The hydrogenation was stopped after the absorption of 6.6 litres of hydrogen. The catalyst was filtered off and the solvent evaporated from the filtrate after the addition of 50 ml. of benzene. Distillation of the residue gave the lactone of pent-1-^{car}~~yn~~-4-ol-1-carboxylic acid (18.5 g.), b.p. 110°C/15 mm., n_D^{19} 1.4730.





D-δ-hex-a-enolactone

This is a straightforward reaction provided precautions are taken at two stages. Firstly, in making the Grignard complex, all the apparatus and reagents to be used must be absolutely dry since the presence of moisture interferes with the formation of the complex. Secondly, it is essential that adequate pressure should be maintained in the steel autoclave during the carboxylation process. This is achieved by putting sufficient excess of solid carbon dioxide to allow for any loss during locking down the autoclave.

Physiological activity of the synthetic lactone.

As a check on the physiological activity of the synthetic lactone, the inhibitory effect on seed germination has been employed (cf. Joly and Amiard, 1947), Table I, which shows the results of such a test on a sample of hexenolactone prepared by the method of Haynes and Jones (1946), is typical; a parallel test on angelicalactone (L. Light & Co.) is included for comparison

25 clean seeds of mustard (Carter's Ltd.) were washed thoroughly with distilled water, and then allowed to remain for 2 hours in contact with the lactone solution to be tested; in a control experiment, the seeds were left for 2 hours in distilled water. The seeds were then transferred to filter-paper pads wetted with sufficient of the same solution and contained in petri dishes and allowed to germinate at room temperature. A visible breaking of the seed-coat by the radicle was taken as the criterion of germination. The results indicate that a suitable concentration of the lactone would probably be 5×10^{-3} M.

Table I

Effect of Hexenolactone and Angelicalactone
on the germination of mustard seeds.

25 seeds were taken for each experiment and the figures represent the number and percentage of germinated seeds.

Solution	Days				
		1st.	2nd.	3rd.	4th.
Control		18	19	21	24
	%	72	76	84	96
Hexenolactone		nil	nil	nil	nil
$1 \times 10^{-2} M$	%	0	0	0	0
Hexenolactone		1	1	1	1
$5 \times 10^{-3} M$	%	4	4	4	4
Hexenolactone		6	18	20	25
1×10^{-3}	%	24	72	80	100
Hexenolactone		15	17	18	22
$5 \times 10^{-4} M$	%	60	68	72	88
Angelicalactone		1	3	4	5
$1 \times 10^{-2} M.$	%	4	12	16	20
Angelicalactone		7	13	17	19
$5 \times 10^{-3} M$	%	28	52	68	76
Angelicalactone		14	18	19	22
$1 \times 10^{-3} M$	%	56	72	76	88
Angelicalactone		15	20	23	24
$5 \times 10^{-4} M$	%	60	80	92	96

MEMBRANE PERMEABILITY

Uptake of sugars and phosphate ions by yeast cells.

As stated in the Introduction, it seemed possible that a study of the passage of solutes into a tissue from a surrounding medium might yield information concerning permeability changes taking place in the tissue membranes, and for this purpose the passage of sugars and of phosphate ions into yeast cells was examined. It is true that some other metabolite or simple ion might be more directly concerned in a metabolic process affected by an inhibitor whose action results from some change in membrane structure, but these particular metabolites are easily estimated, and with reasonable accuracy, by methods not involving the use of isotopic tracer techniques. Yeast cells have been found (e.g., Burger et al., 1959) suitable for such studies.

Three strains of yeast have been used in the present work, namely Saccharomyces carlsbergensis No. 74 and No. 355, and S. cerevisiae No. 366 - all from the Nutfield collection, and kindly supplied by Dr. W.P.K. Findlay. The first-mentioned was chosen because it was found to possess a type of cell-wall exceptionally readily removable by the β -glucosidase of Helix pomatia (Eddy and Williamson, 1957, 1959), and it therefore seemed possible that this strain might more readily show ^{small} differences in permeability

induced experimentally.

The yeasts were maintained on malt agar slopes, and to prepare experimental material fresh cultures were made by inoculating from the slopes into portions (100 ml.) of 3% malt extract (Oxo Ltd.) and incubating at 30° for 18 - 24 hours in a shaker. At the end of this period the cells were removed at the centrifuge and washed three times with ice-cold 0.15M. sodium chloride, and finally re-suspended in the same medium. A measured aliquot of this suspension was taken, the cells were washed three times at the centrifuge with cold water and finally dried to constant weight at 98°, in order to obtain an estimate of the dry weight of cells per ml. of suspension. The average yield of yeast cells under standard conditions was 0.250 g. per 100 ml. of culture medium, and the suspension in sodium chloride was adjusted to contain 25 mg. dry weight cells per ml.

In studying the influx of sugar into the cells, the following procedure was adopted. 8 ml. portions of the stock suspension were diluted to 50 ml. with 0.1 M. phosphate buffer, pH 5.5 (final) (Gomori, 1955), so that the working suspension in the buffer contained 4mg. dry wt. cells/ml.

~~Since~~ It has been held by some workers (e.g. Rothstein, 1956) that the entry of a sugar into yeast cell

is not a simple diffusion, but is an energy-requiring process involving a phosphorylating system. It seemed possible, therefore, that some differences might exist between aerobic and anaerobic influx and experiments were made accordingly. For the former, four 100 ml. conical flasks, one containing 5 ml. of the 0.1 M. phosphate buffer as a control and three containing 5 ml. of a solution (5×10^{-3} M.) of the inhibitor, were taken for the experiments. The inhibitors studied were hexenolactone, angelicalactone and iodoacetic acid. In addition the flasks contained 10 ml. of the standard yeast suspension (40 mg. dry wt. of cells) and 5 ml. of a 20% (w/v) solution of the sugar to be tested. Burger et al. investigated the influx of a number of sugars, but as typical of those not metabolised, or only slowly assimilated, by yeast they used galactose and arabinose; the latter they considered to be incapable of fermentation by yeast under any circumstances. The same sugars were therefore used in the present work.

The flasks were incubated at 30° in a shaker, and 2 ml. sample were withdrawn at intervals of 10, 20, 30 and 60 minutes from the moment of addition of yeast. The samples were centrifuged at 2° , and the sedimented cells were quickly washed with ice-cold 0.15 M sodium chloride solution; it has been shown (Burger et al., 1959) that no efflux of sugar from

the cells takes place under this treatment during the first six minutes at least. After washing, the cells were suspended in 2 ml. water and disintegrated by being placed in a bath of boiling water for 20 minutes. This procedure results in the total liberation of the sugar content of the cells. The cellular debris was removed at the centrifuge, and the supernatant, made up to an appropriate volume, was used for the estimation of sugars.

For the anaerobic experiments, a stream of oxygen-free nitrogen was passed through the flasks before the addition of the yeast suspension and throughout the period of the experiment, but otherwise the procedure was similar.

Burger et al. have also shown that if two sugars are present in the medium surrounding the cells, there may be a competition between them at some stage in the process of influx and that neither is taken up to the normal extent; thus in the presence of glucose the uptake of galactose is notably diminished. Since glucose is apparently metabolised within the cells at a rate at least equal to the rate of influx, only galactose can be detected intracellularly, and the competition is manifested only by a lower maximum intake of this sugar. If however glucose metabolism is inhibited e.g. by iodoacetic acid, this sugar also accumulates within

the cells, and it is possible to follow uptake both of glucose and galactose and thus to gain further insight into the absorption process. Experiments in which both glucose and a nonmetabolisable sugar were present in the medium were therefore undertaken.

Analytical Methods:

For the estimation of sugar in the cell-extract it was sufficient, when only one sugar was being absorbed, to employ a direct method of determination, and the colorimetric method of Somogyi (1952) based on that of Nelson (1944) was used. A 1 ml. portion of the extract prepared as described above was diluted to 2 ml., and 2 ml. of the Somogyi's copper reagent was added; the mixture was heated in a boiling water bath for 10 minutes. After cooling, 2 ml. of Nelson's chromogenic reagent were added, and the cuprous oxide was brought into solution by mild agitation. The volume was made up to 25 ml. and the optical density of the solution was read in a Hilger photoelectric colorimeter using a red filter. Standard solutions of the same sugar were used in constructing a reference curve for determining concentrations.

Where two sugars were used in combination, attempts were made to distinguish between them and estimate them individually by chromatographic methods based on that employed

by Green and Stone (1952). The method is as follows:

A large sheet of Whatman No. 1 paper is creased at 4 cms. intervals to give an accordion pleat effect. The two edges of the paper were clipped together so as to form a pleated cylinder. The advantage of this method is that a relatively large sheet of paper can be placed in a comparatively small cylinder. The folds in the paper were made in the machine direction of the paper so that the solvent ascends in this direction.

The solvent employed was the one suggested by Albon and Gross (1950) and consisted of :

n-Butanol	5 parts,
Pyridine	3 parts,
Benzene	1 part,
Water	3 parts.

The mixture was shaken in a separating funnel and the top layer drawn off and used. About 1 cm. layer of the solvent was put in the bottom of the cylinder. The samples were applied to the paper by means of a capillary pipette, usually in increments of 0.01 ml. with intermediate drying in order to obtain spots with a diameter of about 1 cm. The pleated paper with the spots along the bottom edge was placed in the solvent, which was allowed to ascend the paper at room

temperature. The chromatograms were run overnight and the paper was then taken out and dried. The running was sufficient to give a good resolution of the sugars.

Albon and Gross suggest the use of three different solutions one after another for the development of the chromatograms. The dried paper is first dipped into an acetone solution of silver nitrate and after drying it is dipped into an alcoholic solution of sodium hydroxide. Finally, the paper is immersed into 6 N ammonium hydroxide solution until the excess silver nitrate is dissolved and then washed in running water for one hour and dried on glass plates.

In the modified method of Burger et al., which was preferred to that of Albon and Gross, the paper is first dipped into a solution prepared by adding 2.7 ml. of a saturated solution of silver nitrate to a litre of dry, redistilled acetone. After drying the paper is immersed into 1.3% w/v ammonia solution for clearing the background of the chromatograms. This solution is prepared fresh on each occasion. For a final clearing of the background, the paper is dipped into 20% sodium thiosulphate solution.

Difficulty was experienced with the method since it was always found that the spots on the chromatogram were

too dull for any accurate assessment to be made, and it seemed therefore preferable to use the Somogyi's method in all cases; this naturally gave figures only for "total reducing sugars", and estimates of galactose (e.g.) alone had to be made by indirect methods.

The results of these experiments are summarised in Tables II - Va.

Phosphate uptake by yeast cells.

Phosphate uptake by yeast cells has been studied by Goodman and Rothstein (1957) by a method depending upon direct estimation of inorganic phosphate remaining in a non-nitrogenous medium in which yeast cells have been suspended for various periods of time. (These authors have checked their method by the use of labelled ^{32}P also.) Following their technique, a quantity of commercial bakers' yeast was allowed to remain in suspension in distilled water for 24 hours, during which time it was aerated.

The cells were then removed at the centrifuge and re-suspended in "tris" buffer (0.02M, pH 4.5 - 5.0 with the addition of 0.02M. KCl). The suspension was adjusted to contain 100 mg. dry cells per ml. of medium, Goodman and

Table II

The influence of Hexenolactone, Angelicalactone and Iodoacetic Acid on the influx of arabinose into yeast cells under aerobic conditions.

Conditions as detailed in the description above.

Yeast strain: Saccharomyces carlsbergensis No. 74.

Figures represent $\mu\text{g.}$ of sugar uptake per mg. dry yeast cells.

Inhibitor	Time of uptake (minutes)				
	10	20	30	60	90
None	22.00	37.00	46.00	59.00	63.00
Hexenolactone $5 \times 10^{-3} \text{M}$	20.00	36.00	44.00	59.00	62.00
Angelicalactone $5 \times 10^{-3} \text{M}$	22.00	37.00	45.00	58.00	62.00
Iodoacetic Acid $5 \times 10^{-3} \text{M}$	14.00	25.00	34.00	44.00	48.00

All results represent mean of not less than four determinations.

Table II

The influence of Hexenolactone, Angelicalactone and Iodoacetic Acid on the influx of galactose into yeast cells under aerobic conditions.

Conditions as detailed in the description above.

Yeast strains: (a) Saccharomyces carlsbergensis No. 74,
(b) Saccharomyces carlsbergensis No. 355,
(c) Saccharomyces cerevisiae No. 366.

Figures represent μ g. of sugar uptake per mg. dry yeast cells.

Inhibitor	Yeast	Time of uptake (minutes)			
		10	20	30	60
None	a	10.50	20.00	29.50	42.00
	b	5.00	7.00	10.00	15.00
	c	4.75	6.50	10.50	14.75
Hexenolactone $5 \times 10^{-3} M$	a	9.50	19.00	28.00	40.00
	b	5.00	6.50	9.50	14.00
	c	4.50	5.50	9.50	14.50
Angelicalactone $5 \times 10^{-3} M$	a	10.00	19.50	28.50	41.00
	b	5.00	7.00	10.00	14.00
	c	4.50	6.00	9.75	14.75
Iodoacetic Acid $5 \times 10^{-3} M$	a	3.50	6.50	10.00	17.00
	b	3.50	5.00	7.00	10.50
	c	2.50	3.25	4.00	5.25

All results represent mean of not less than four determinations.

Table III

The influence of Hexenolactone, Angelicalactone and Iodoacetic Acid on the influx of galactose into yeast cells under anaerobic conditions.

Conditions as detailed in the description above.

Yeast strains: (a) Saccharomyces carlsbergensis No. 74,

(b) Saccharomyces carlsbergensis No. 355,

(c) Saccharomyces cerevisiae No. 366.

Figures represent $\mu\text{g.}$ of sugar uptake per mg. dry yeast cells.

Inhibitor	Yeast	Time of uptake (minutes)			
		10	20	30	60
None	a	17.00	29.00	42.00	56.00
	b	4.25	6.25	9.00	13.00
	c	4.25	7.00	9.75	15.75
Hexenolactone $5 \times 10^{-3} \text{M}$	a	16.00	27.00	40.00	53.00
	b	4.00	5.50	8.50	12.00
	c	3.50	6.50	9.50	15.00
Angelicalactone $5 \times 10^{-3} \text{M}$	a	17.00	28.50	41.00	54.00
	b	4.00	6.00	9.00	12.50
	c	3.75	6.75	9.75	15.25
Iodoacetic Acid $5 \times 10^{-3} \text{M}$	a	10.00	17.00	22.00	32.00
	b	3.00	4.00	7.00	10.00
	c	2.50	4.00	5.50	7.00

All results represent mean of not less than four determinations.

Table IV.

The influence of Hexenolactone, Angelicalactone and Iodoacetic Acid on the influx of glucose into yeast cells under aerobic conditions.

Conditions as detailed in the description above.

Yeast strains: (a) Saccharomyces carlsbergensis No. 74,
(b) Saccharomyces carlsbergensis No. 355.

Figures represent $\mu\text{g.}$ of sugar uptake per mg. dry yeast cells.

Inhibitor	Yeast	Time of uptake (minutes)			
		10	20	30	60
None	a	4.50	5.00	5.50	5.00
	b	2.00	2.00	1.50	1.50
Hexenolactone $5 \times 10^{-3} \text{M}$	a	5.00	7.00	4.00	1.50
	b	2.00	2.50	1.50	1.00
Angelicalactone $5 \times 10^{-3} \text{M}$	a	4.50	6.00	3.50	2.00
	b	2.00	2.00	1.00	1.00
Iodoacetic Acid $5 \times 10^{-3} \text{M}$	a	7.50	14.50	19.00	25.00
	b	3.00	6.50	8.50	10.00

All results represent mean of not less than four determinations.

Table IVa.

The influence of Hexenolactone, Angelicalactone and Iodoacetic Acid on the influx of glucose into yeast cells under anaerobic conditions.

Conditions as detailed in the description above.

Yeast strains: (a) Saccharomyces carlsbergensis No. 74,

(b) Saccharomyces carlsbergensis No. 355.

Figures represent $\mu\text{g.}$ of sugar uptake per mg. dry yeast cells.

Inhibitor	Yeast	Time of uptake (minutes)			
		10	20	30	60
None	a	3.50	4.00	5.00	5.00
	b	2.50	2.50	3.00	3.00
Hexenolactone $5 \times 10^{-3} \text{M}$	a	5.00	5.50	6.50	7.00
	b	3.00	3.50	3.50	4.00
Angelicalactone $5 \times 10^{-3} \text{M}$	a	4.00	5.50	6.00	6.00
	b	2.50	2.50	3.00	3.50
Iodoacetic Acid $5 \times 10^{-3} \text{M}$	a	22.00	28.00	33.50	38.50
	b	3.50	7.00	9.00	11.00

All results represent mean of not less than four determinations.

Table V.

The influence of Hexenolactone, Angelicalactone and Iodoacetic Acid on the influx of galactose in the presence of 1% glucose into yeast cells under aerobic conditions.

Conditions as detailed in the description above.

Yeast strains: (a) Saccharomyces carlsbergensis No. 74,

(b) Saccharomyces carlsbergensis No. 355.

Figures represent $\mu\text{g.}$ of sugar uptake per mg. dry yeast cells.

Inhibitor	Yeast	Time of uptake (minutes)			
		10	20	30	60
Control	a	1.50	4.50	3.50	2.00
	b	1.00	2.00	2.50	3.50
Hexenolactone $5 \times 10^{-3} \text{M}$	a	1.50	4.50	3.00	1.50
	b	1.50	3.00	3.50	4.00
Angelicalactone $5 \times 10^{-3} \text{M}$	a	1.00	4.50	3.50	2.50
	b	1.00	2.00	2.00	3.50
Iodoacetic Acid $5 \times 10^{-3} \text{M}$	a	1.00	2.00	2.50	3.00
	b	3.00	4.50	6.00	9.00

All results represent mean of not less than four determinations.

Table Va.

The influence of Hexenolactone, Angelicalactone and Iodoacetic Acid on the influx of galactose in the presence of 1% glucose into yeast cells under anaerobic conditions.

Conditions as detailed in the description above.

Yeast strains: (a) Saccharomyces carlsbergensis No. 74,

(b) Saccharomyces carlsbergensis No. 355.

Figures represent μ g. of sugar uptake per mg. dry yeast cells.

Inhibitor	Yeast	Time of uptake (minutes)			
		10	20	30	60
Control	a	3.50	7.50	4.00	2.25
	b	2.00	2.00	2.50	3.50
Hexenolactone $5 \times 10^{-3} M$	a	3.50	7.00	3.50	2.00
	b	2.00	2.00	2.00	3.00
Angelicalactone $5 \times 10^{-3} M$	a	3.50	7.50	4.00	2.50
	b	2.00	2.00	2.50	3.50
Iodoacetic Acid $5 \times 10^{-3} M$	a	5.50	7.00	9.00	10.50
	b	2.00	3.50	6.00	8.00

All results represent mean of not less than four determinations.

Rothstein confirmed the observation of Hevesy et al. (1937) that in the absence of an exogenous substrate only a negligible uptake of phosphate occurred with "starved" yeast cells, and further that among seven likely substrates glucose was most effective in promoting influx of phosphate. Glucose was therefore included in the medium at a concentration of 0.3M. After bubbling a stream of air through the suspension for 30 minutes at room temperature to establish a uniform rate of metabolism, orthophosphoric acid was added to give an initial concentration of 0.02M., and taking the moment of the addition of phosphate as zero, samples were withdrawn for analysis at intervals up to 120 minutes. Each sample was rapidly centrifuged, the supernatant liquid decanted off, and inorganic phosphorus estimated by the method of Fiske and Subbarow (1929) modified by the addition of 15% of alcohol (v/v) before colour development, as recommended by Goodman and Rothstein. When the effect of an inhibitor was to be examined, it was added in suitable concentration immediately before the addition of the inorganic phosphate.

Table VI summarises the observations made.

Table VI.

The effect of Hexenolactone on the Phosphate uptake by
Bakers' yeast under aerobic conditions.

Conditions as detailed in the description above.

Figures represent $\mu\text{g.}$ of Phosphate uptake per 100 mg.
(fresh weight) of yeast cells.

Time (minutes)	Control	Hexenolactone $5 \times 10^{-3} \text{M}$
15	28.63	24.39
30	52.07	45.48
45	67.94	60.02
60	78.99	68.75
90	90.84	77.81

* 3 replicates

CELLULAR RESPIRATION

The effect of hexenolactone on cellular respiration.

The experiments of May (1952) on the influence of hexenolactone on oxygen uptake and carbon dioxide output by intact yeast cells indicated that a moderate inhibition (up to 20% with 2.5×10^{-2} M. lactone) could be observed. However, as already noted, this effect was not found with cell-free extracts of yeast (or of certain bacteria). These observations have been confirmed, using the three strains of yeast now being studied. In addition, carbon dioxide output under anaerobic conditions was investigated, for which purpose the Warburg flasks were connected to a supply of oxygen-free nitrogen throughout the test. The gas was allowed to pass through the flasks for 15 minutes, after which connection was made to the manometers, the N_2 supply cut off, and the usual readings for CO_2 output were made. However, as shown in Table VII under these conditions no inhibition results from the presence of lactone.

May did not consider the aerobic phases of glucose metabolism, although under her experimental conditions these would have contributed to the gas exchange of the intact cells. Aerobic respiration involving the citric acid cycle is considered to be located mainly if not entirely in the mitochondria, but the techniques used by May in preparing

Table VII.

The influence of Hexenolactone on the production of CO₂
by yeast cells under anaerobic conditions.

Conditions as detailed in the description above.

Yeast strains: (a) Saccharomyces carlsbergensis No. 74,

(b) Saccharomyces carlsbergensis No. 355.

Figures represent ul. of CO₂ production per 3 mg. dry
yeast cells.

Time (minutes)	a		b	
	Control	Hexenolactone 5x10 ⁻³ M	Control	Hexenolactone 5x10 ⁻³ M
5	5.82	5.22	4.33	4.10
10	35.55	32.90	13.51	12.92
15	66.11	63.22	25.22	24.53
25	122.92	122.19	45.60	45.07
35	191.31	189.97	70.96	73.12
45	248.19	250.27	114.96	119.87
55	308.79	310.76	166.99	172.65
65	-	-	229.22	238.13
75	-	-	286.00	300.54

extracts were such that the survival of any mitochondria would have been most unlikely, and the carbon dioxide produced by these extracts would thus have arisen by glycolysis alone. Thus these extracts would not have been suitable for studying mitochondrial activity, nor indeed did May attempt any such study. This aspect of the problem has now been investigated.

Yeast cells are not the most convenient material from which to prepare mitochondrial suspension, which are in fact most commonly obtained from animal tissues such as liver etc. For a few experiments rat liver was used but difficulty in obtaining supplies of this tissue led to the use of plant material for routine tests. Not many workers have used plant material for this purpose, largely because of the sensitivity of the plant mitochondria and the extreme care which must be taken if they are to be isolated with their vital properties unimpaired. Laties (1953a) has made a detailed study of the effects of the physical environment on the oxidative and phosphorylative capacities of mitochondria from immature inflorescences of Brassica oleracea and has drawn attention to the need for preserving the of tonicity of the surrounding medium within rather narrow limits. The malic, α -ketoglutaric and succinic oxidase systems are all damaged in hypotonic suspensions; this damage, which

is rapid and irreversible, is associated with a swelling which Latices considers may be a prerequisite to such injury. When mitochondria are suspended in distilled water in the absence of other protoplast components the malic and ketoglutaric systems suffer the most damage, while the succinoxidase system is relatively unaffected. The injury is lessened by the presence of dilute solutions of neutral salts, or avoided by the use of slightly hypertonic solutions of mannitol or sucrose containing small amounts of phosphate. James et al. (1957) while working on the respiration of chlorophyllous cells from young leaves of Vicia faba found that the most suitable preparative and experimental medium was one containing 0.3M. sucrose, 0.67M. phosphate buffer pH 7.3, 1.8×10^{-3} M. $MgSO_4$ and 2×10^{-3} M. versene. Cauliflower inflorescence was found to be a very satisfactory source of active mitochondria.

Cauliflower heads, as ~~freshly~~ as possible, were obtained commercially; these were washed with tap water, rinsed with distilled water and blotted as dry as possible. They were then cooled in a refrigerator to bring them to 2°. The topmost 2 - 3 mm. of the flower was cut off with a chilled knife, and the remainder of the head discarded. The buds were homogenised with an approximately equal quantity of pre-cooled medium prepared as above described. After

macerating to a smooth paste the homogenate was strained through a single layer of muslin. The strained liquid was centrifuged at $2400 \times g$ at 0° for 10 minutes to throw down cell debris etc., after which the supernatant was recentrifuged at $10,000 \times g$ (MSE angle head centrifuge) at 0° for 20 minutes. The mitochondrial pellets thus thrown down were re-suspended and recentrifuged at $10,000 \times g$ for 20 minutes, and finally suspended in the sucrose-phosphate medium. The following tests were performed on this suspension: Nitrogen content - this was estimated by the usual micro-kjeldahl method. Absorption spectrum - the suspension was examined in the Beck micro-spectrometer; on the addition of a few crystals of sodium dithionite two distinct bands were visible, namely at 603 and 563-552 m μ . Staining test - staining for 5 minutes with Janus Green B (1 in 8000) gave a green-black staining of active mitochondria. Only preparations which gave a satisfactory response to the absorption and staining tests were used in the manometric experiments, or in the later experiments on mitochondrial swelling.

Oxygen uptake by cauliflower mitochondria.

A freshly prepared suspension, containing 1 mg. N per ml. was used to study the oxygen uptake of mitochondria,

the respiration substrate being one or other of the members of the citric acid cycle, namely citrate, cis-aconitate, α -ketoglutarate, succinate, fumarate or malate, 0.5 ml. portions of the suspension were taken in the Warburg flasks, and to each was added 1.4 ml. of the usual medium. 0.4 ml. of the substrate (usually 0.02M.) was placed in the side arm of the flask, and the centre cup contained 0.2 ml. of 10% KOH. In order to retain the correct tonicity, the substrate was dissolved in the standard suspension-medium, as was also the inhibitor (hexenolactone or other when used). The solution of the inhibitor was added to the mitochondrial suspension; all components were kept in a tray of ice until immediately before use. The manometer bath was kept at 30°, and for equilibration 10 minutes period was allowed. After this time the taps were closed and the substrate tipped into the reaction chamber; manometric measurements were taken over a suitable period of time. The observations are summarised in Tables VIII - XIV .

The step in the citric acid cycle which has received the most detailed study is perhaps that involving the oxidation of succinic acid. The enzyme concerned with this stage seem to be unusually resistant to changes in the physical environment, such as occur during the preparation of tissue extracts, and also to chemical inhibitors. However

Table VIII.

Respiration of mitochondria: The effect of Hexenolactone
on the uptake of oxygen by mitochondria.

Each flask contains 3.0 ml. of liquid including 0.2 ml.
of 10% Potassium Hydroxide in the centre cup.

Substrate: 0.02 M Sodium Citrate.

Figures represent μ l. of O_2 uptake by cauliflower
mitochondria per mg. of total N.

Time (minutes)	Control	Hexenolactone $1 \times 10^{-3} M$	Hexenolactone $5 \times 10^{-4} M$
5	6.80	4.00	2.50
10	14.30	9.20	6.90
15	20.70	15.20	12.20
20	28.50	21.30	18.00
30	40.90	33.40	29.20
40	49.54	39.70	36.80
50	54.80	45.20	43.00
60	61.30	50.40	47.70
70	65.00	54.70	51.50
80	68.70	58.50	55.00
90	72.40	61.20	58.30

Effect of Hexenolactone on the uptake
of oxygen by mitochondria.
Substrate: Sodium Citrate.

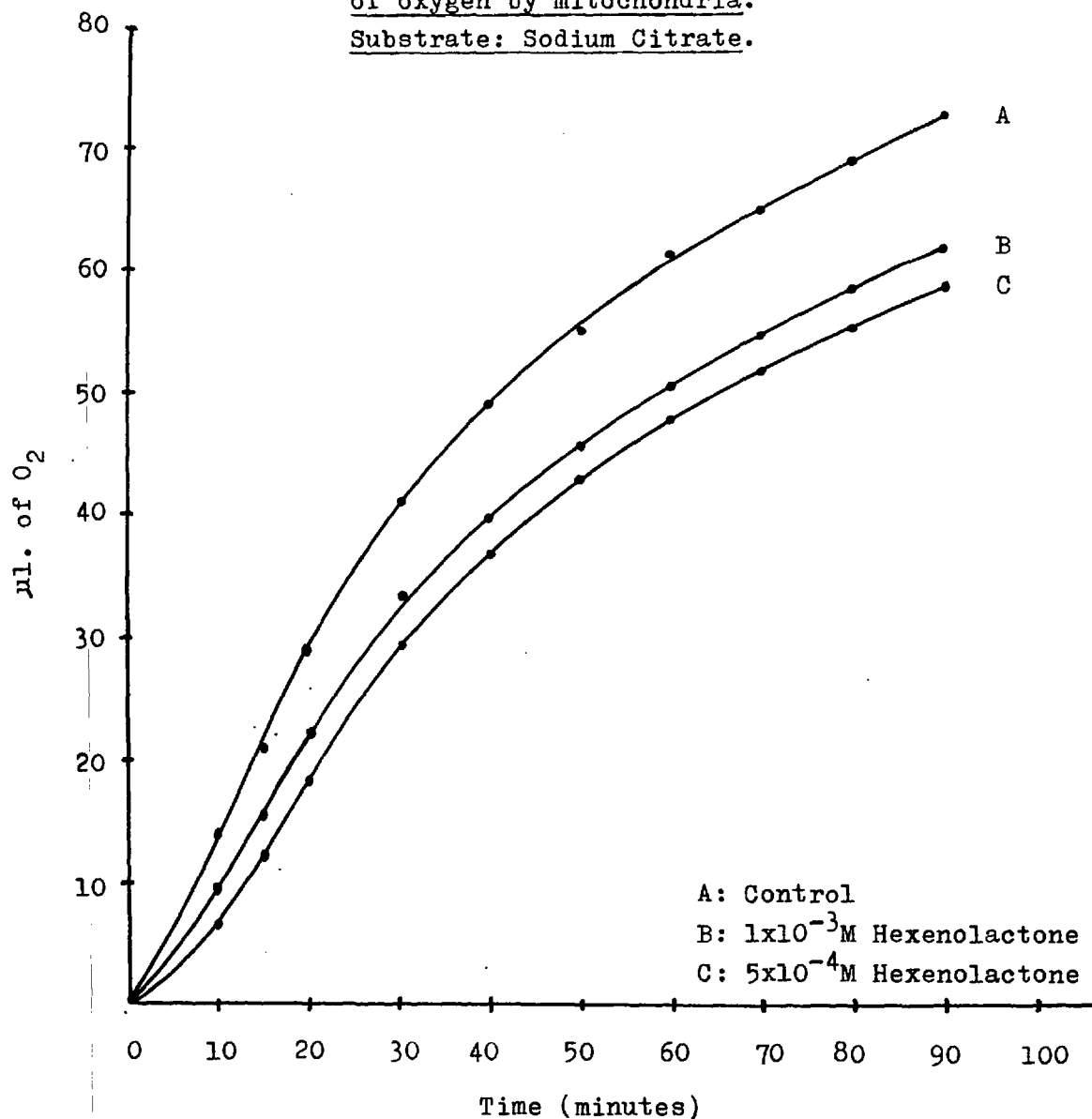


Table IX.

Respiration of mitochondria: The effect of Hexenolactone
on the uptake of oxygen by mitochondria.

Each flask contains 3.0 ml. of liquid including 0.2 ml.
of 10% Potassium Hydroxide in the centre cup.

Substrate: 0.02 M cis-aconitic acid.

Figures represent μ l. of O_2 uptake by cauliflower
mitochondria per mg. of total N.

Time (minutes)	Control	Hexenolactone $1 \times 10^{-3} M$	Hexenolactone $5 \times 10^{-4} M$
5	4.50	3.50	4.25
10	10.25	8.00	9.80
15	18.56	14.00	17.50
20	28.85	22.22	26.80
25	36.93	29.50	35.00
30	43.00	35.50	42.00
40	53.50	45.73	51.00
50	61.50	53.10	59.05
60	69.00	59.02	66.10
70	75.23	64.45	71.59
80	80.57	68.67	76.50
90	85.17	72.50	80.50

Effect of Hexenolactone on the uptake
of oxygen by mitochondria.

Substrate: cis-Aconitic Acid.

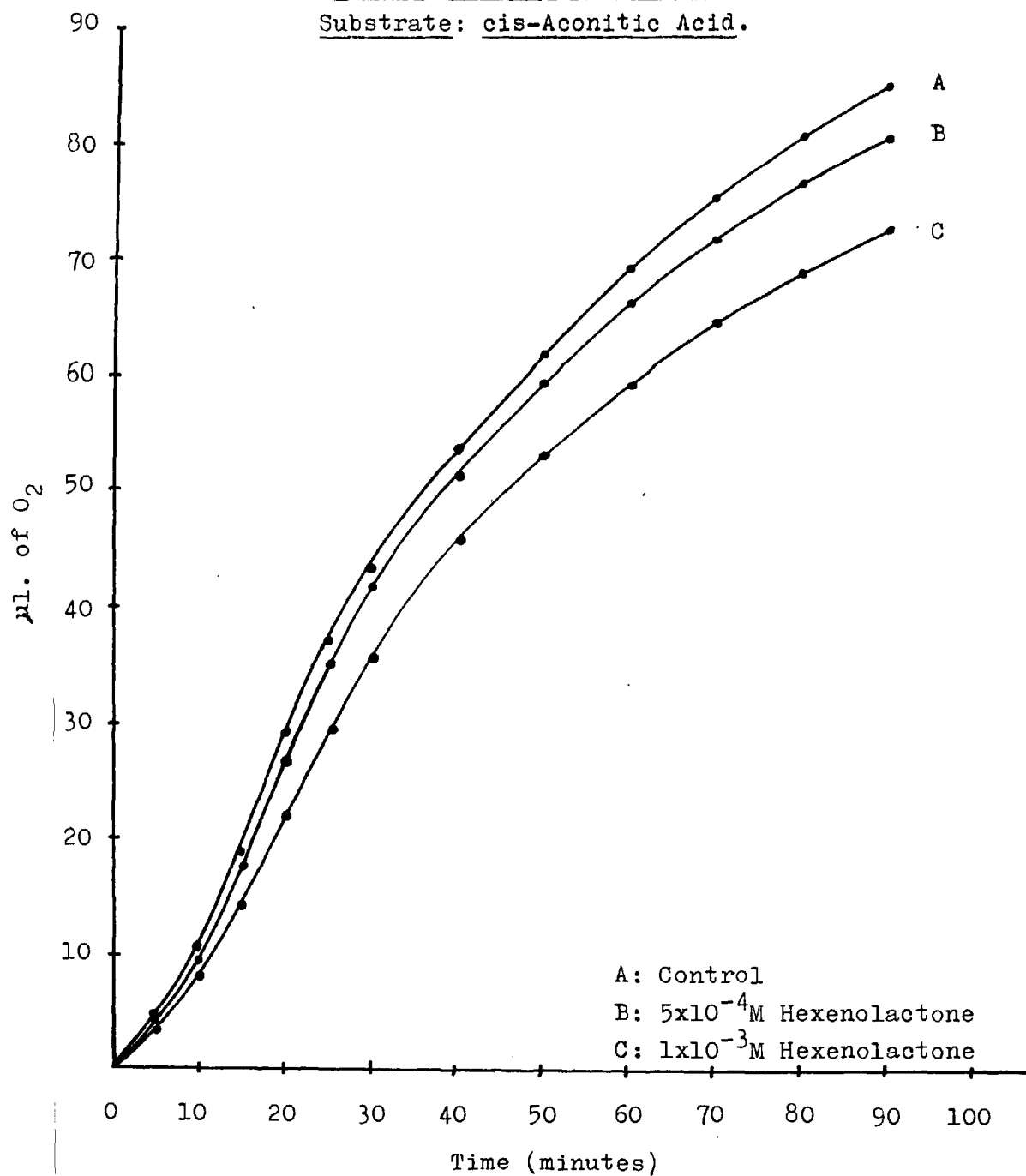


Table K.

Respiration of mitochondria: The effect of Hexenolactone
on the uptake of oxygen by mitochondria.

Each flask contains 3.0 ml. of liquid including 0.2 ml.
of 10% Potassium Hydroxide in the centre cup.

Substrate: 0.02 M α -ketoglutaric acid.

Figures represent μ l. of O_2 uptake by cauliflower
mitochondria per mg. of total N.

Time (minutes)	Control	Hexenolactone $1 \times 10^{-3} M$	Hexenolactone $5 \times 10^{-4} M$
5	1.50	0.50	1.00
10	5.00	2.00	3.50
15	9.50	3.40	6.50
20	14.20	6.80	10.80
30	28.80	16.50	23.00
40	44.10	31.00	38.00
50	51.50	38.80	46.00
60	56.40	42.30	48.60
70	59.50	44.50	51.25
80	62.20	47.00	54.00
90	65.60	50.00	57.00

Effect of Hexenolactone on the uptake
of oxygen by mitochondria.

Substrate: α -keto-glutaric acid.

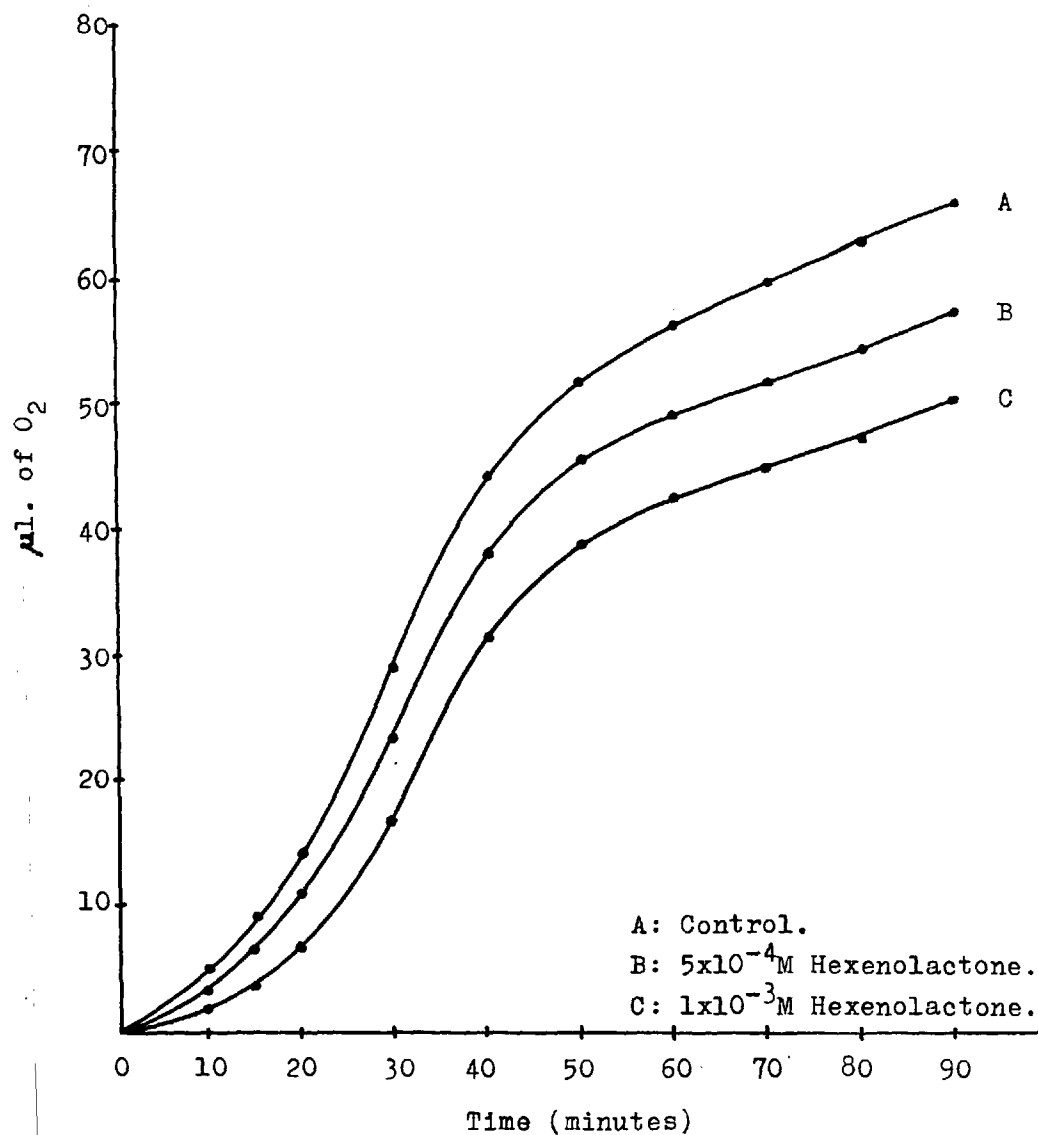


Table XI.

Respiration of mitochondria: The effect of Hexenolactone
on the uptake of oxygen by mitochondria.

Each flask contains 3.0 ml. of liquid including 0.2 ml.
of 10% Potassium Hydroxide in the centre cup.

Substrate: 0.025 M Sodium Succinate.

Figures represent μ l. of O_2 uptake by cauliflower
mitochondria per mg. of total N.

Time (minutes)	Control	Hexenolactone $1 \times 10^{-3} M$	Hexenolactone $5 \times 10^{-4} M$
5	25.0	22.0	23.0
10	52.5	47.5	50.0
15	83.0	73.5	76.5
20	115.0	101.0	105.0
25	145.7	128.0	133.0
30	178.2	157.0	162.5
40	241.6	215.0	221.3
50	300.6	265.0	278.0
60	348.5	305.0	324.9
70	397.2	347.5	375.0
80	435.2	384.7	411.4
90	476.9	423.2	452.6

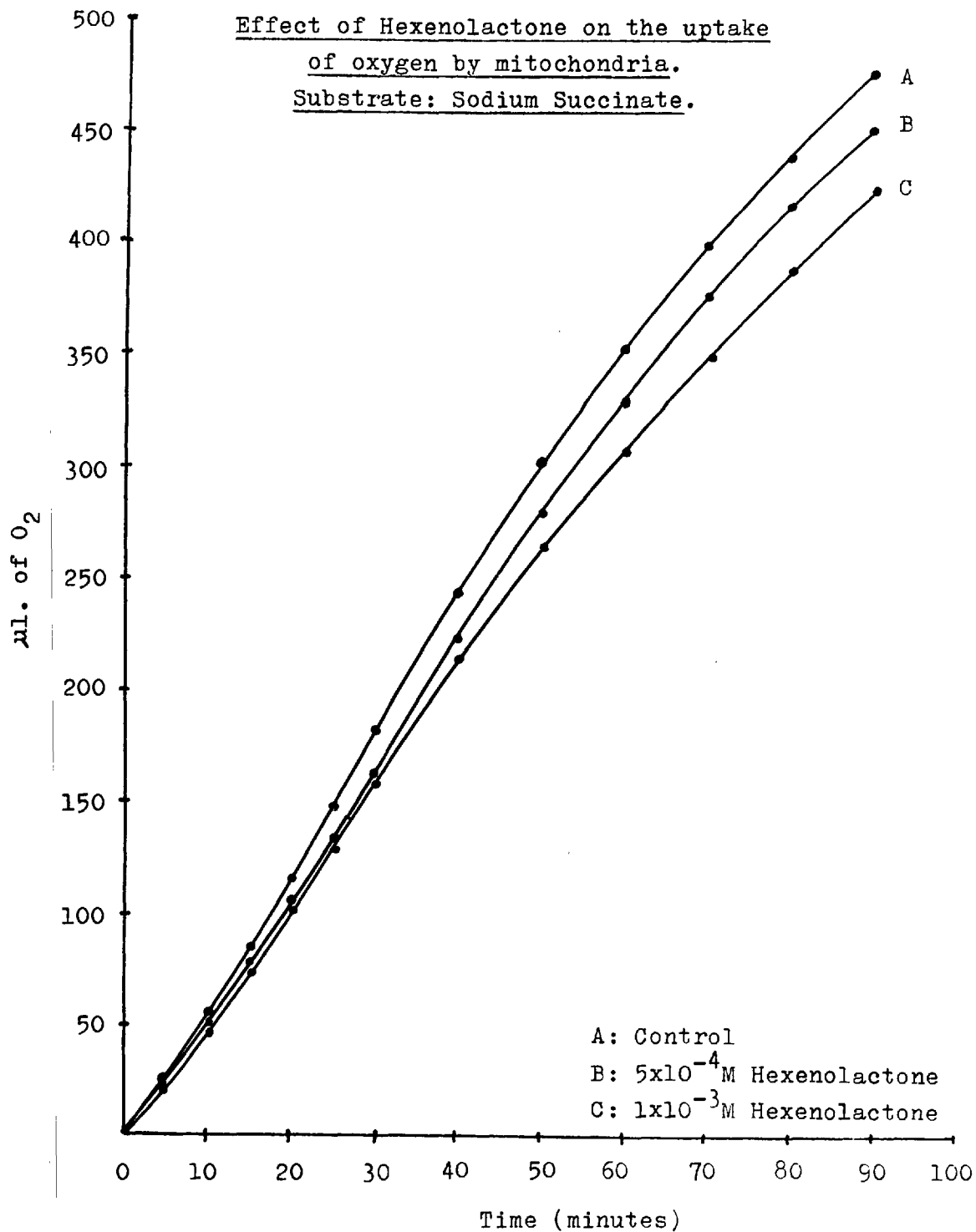


Table XII.

Respiration of mitochondria: The effect of Hexenolactone
on the uptake of oxygen by mitochondria.

Each flask contains 3.0 ml. of liquid including 0.2 ml.
of 10% Potassium Hydroxide in the centre cup.

Substrate: 0.02 M Sodium Fumarate.

Figures represent μ l. of O_2 uptake by cauliflower
mitochondria per mg. of total N.

Time (minutes)	Control	Hexenolactone $1 \times 10^{-3} M$	Hexenolactone $5 \times 10^{-4} M$
5	2.50	1.00	1.50
10	6.00	3.20	4.50
15	10.50	5.50	7.50
20	16.00	9.00	11.50
25	21.00	12.80	15.50
30	26.50	16.70	19.50
40	35.00	23.50	27.00
50	41.70	30.00	34.00
60	46.50	34.20	39.10
70	50.90	39.00	43.00
80	55.90	42.90	47.20
90	60.00	46.80	50.50

Effect of Hexenolactone on the uptake
of oxygen by mitochondria.
Substrate: Sodium Fumarate.

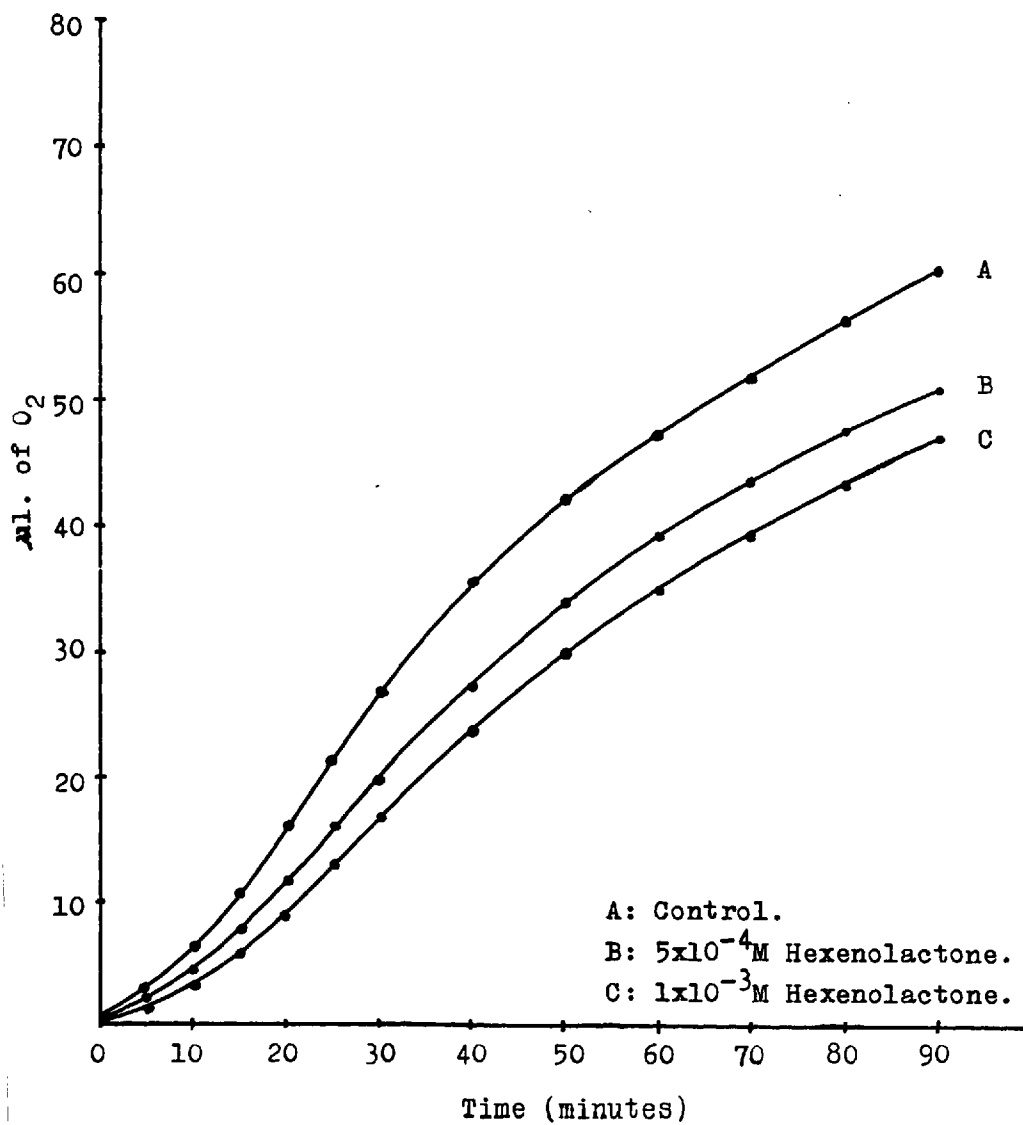


Table XIII.

Respiration of mitochondria: The effect of Hexenolactone
on the uptake of oxygen by mitochondria.

Each flask contains 3.0 ml. of liquid including 0.2 ml.
of 10% Potassium Hydroxide in the centre cup.

Substrate: 0.02 M Sodium Hydrogen Malate.

Figures represent μ l. of O_2 uptake by cauliflower
mitochondria per mg. of total N.

Time (minutes)	Control	Hexenolactone $1 \times 10^{-3} M$	Hexenolactone $5 \times 10^{-4} M$
5	3.00	1.50	2.00
10	7.20	4.50	5.50
15	14.00	10.00	11.20
20	22.20	16.50	19.20
25	30.90	23.50	27.40
30	38.70	30.00	33.60
40	51.30	40.00	43.50
50	61.90	48.00	52.50
60	69.50	54.40	59.00
70	76.00	58.50	63.30
80	82.20	63.00	68.40
90	88.30	67.10	72.20

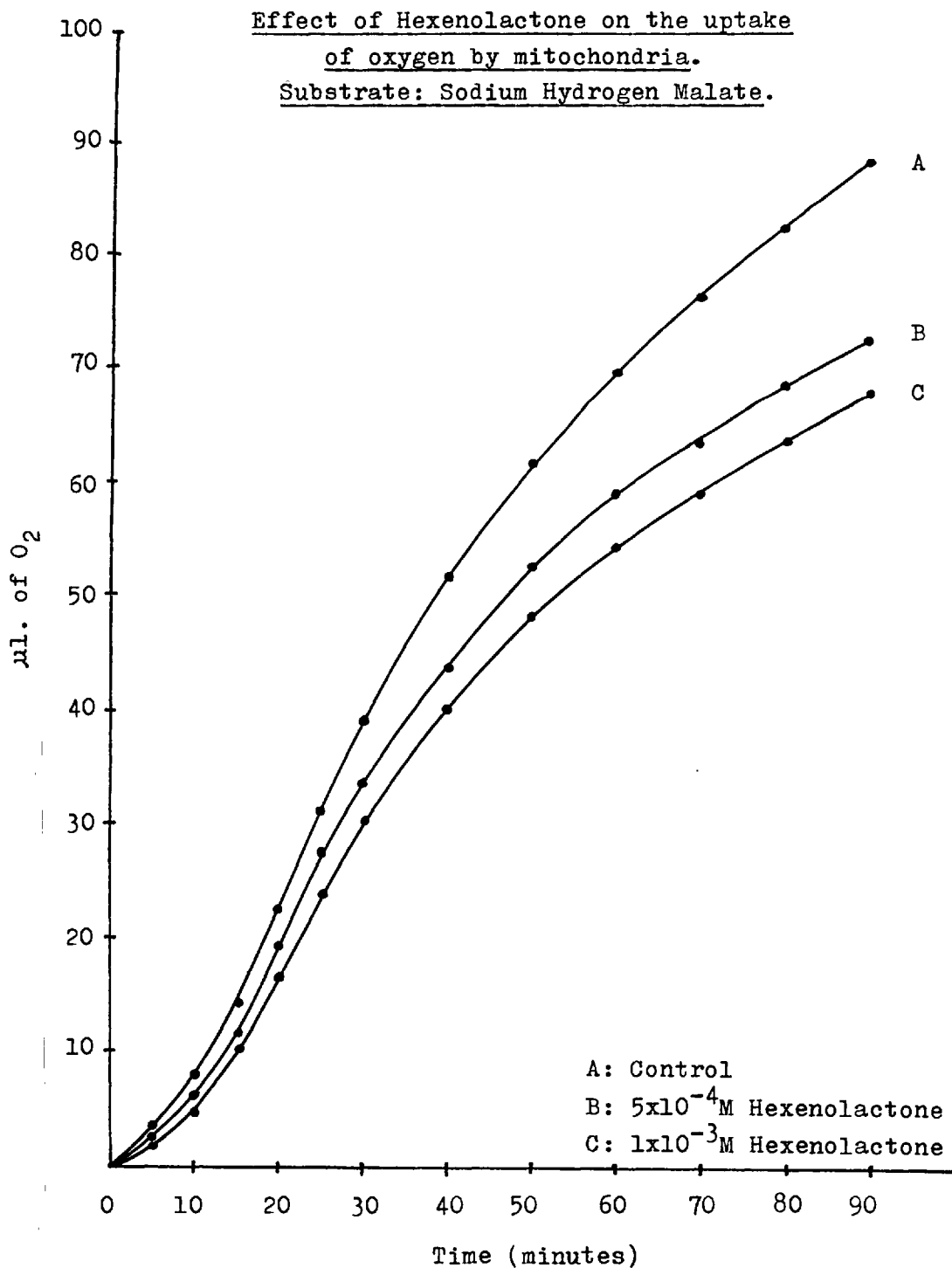


Table XIV.

Respiration of mitochondria: The effect of Hexenolactone
on the uptake of oxygen by mitochondria.

Substrate	Molarity	O ₂ uptake (ul./hr./mg.N)		
		Control	Hexenolactone	
			$1 \times 10^{-3} M$	$5 \times 10^{-4} M$
Sodium Citrate	0.02	61.30	50.40	47.70
cis-aconitic Acid	0.02	69.00	59.02	66.10
α -ketoglutaric Acid	0.02	56.40	42.30	48.67
Sodium Succinate	0.025	348.50	305.00	324.90
Sodium Hydrogen Malate	0.02	69.50	54.40	59.00
Sodium Fumarate	0.02	46.50	34.20	39.10

Table XV.

Respiration of mitochondria: The effect of Malonate on
the uptake of oxygen by mitochondria.

Each flask contains 3.0 ml. of liquid including 0.2 ml.
of 10% Potassium Hydroxide in the centre cup.

Substrate: 0.005 M Sodium Succinate.

Figures represent μ l. of O_2 uptake by cauliflower
mitochondria per μ g. of total N.

Time (minutes)	Control	Malonate 0.0025M	Malonate 0.005M	Malonate 0.01M
5	25.0	7.5	5.0	2.5
10	56.0	17.0	8.0	5.0
15	97.5	30.0	14.0	8.0
20	144.0	45.0	22.0	10.0
25	192.0	60.0	32.5	15.0
30	241.0	72.0	43.0	25.0
35	284.0	85.0	55.0	35.0
40	325.0	100.5	63.5	40.0
45	366.0	112.5	75.0	45.0
50	400.0	125.0	82.0	50.0
60	469.5	146.0	100.0	65.0
70	525.0	176.0	120.0	78.0
80	580.0	200.0	135.0	90.0
90	630.0	225.0	148.0	100.0

Table XVI.

Respiration of mitochondria: The effect of Malonate on
the uptake of oxygen by mitochondria.

Each flask contains 3.0 ml. of liquid including 0.2 ml.
of 10% Potassium Hydroxide in the centre cup.

Substrate: 0.025M Sodium Succinate.

Figures represent μ l. of O_2 uptake by cauliflower
mitochondria per mg. of total N.

Time (minutes)	Control	Malonate 0.00025M	Malonate 0.0005M	Malonate 0.001M
5	25.0	20.0	15.0	10.0
10	56.0	44.0	36.5	27.5
15	97.5	70.0	60.0	48.0
20	144.0	98.0	85.0	67.5
25	192.0	127.0	112.5	90.0
30	241.5	160.0	142.0	115.0
35	284.0	190.0	170.0	134.0
40	325.0	221.5	197.0	155.0
45	366.0	255.0	225.0	177.0
50	400.0	290.0	260.0	196.0
60	469.5	342.0	301.5	226.5
70	525.0	396.0	350.0	273.0
80	580.0	440.0	392.5	310.0
90	630.0	485.0	435.0	345.0

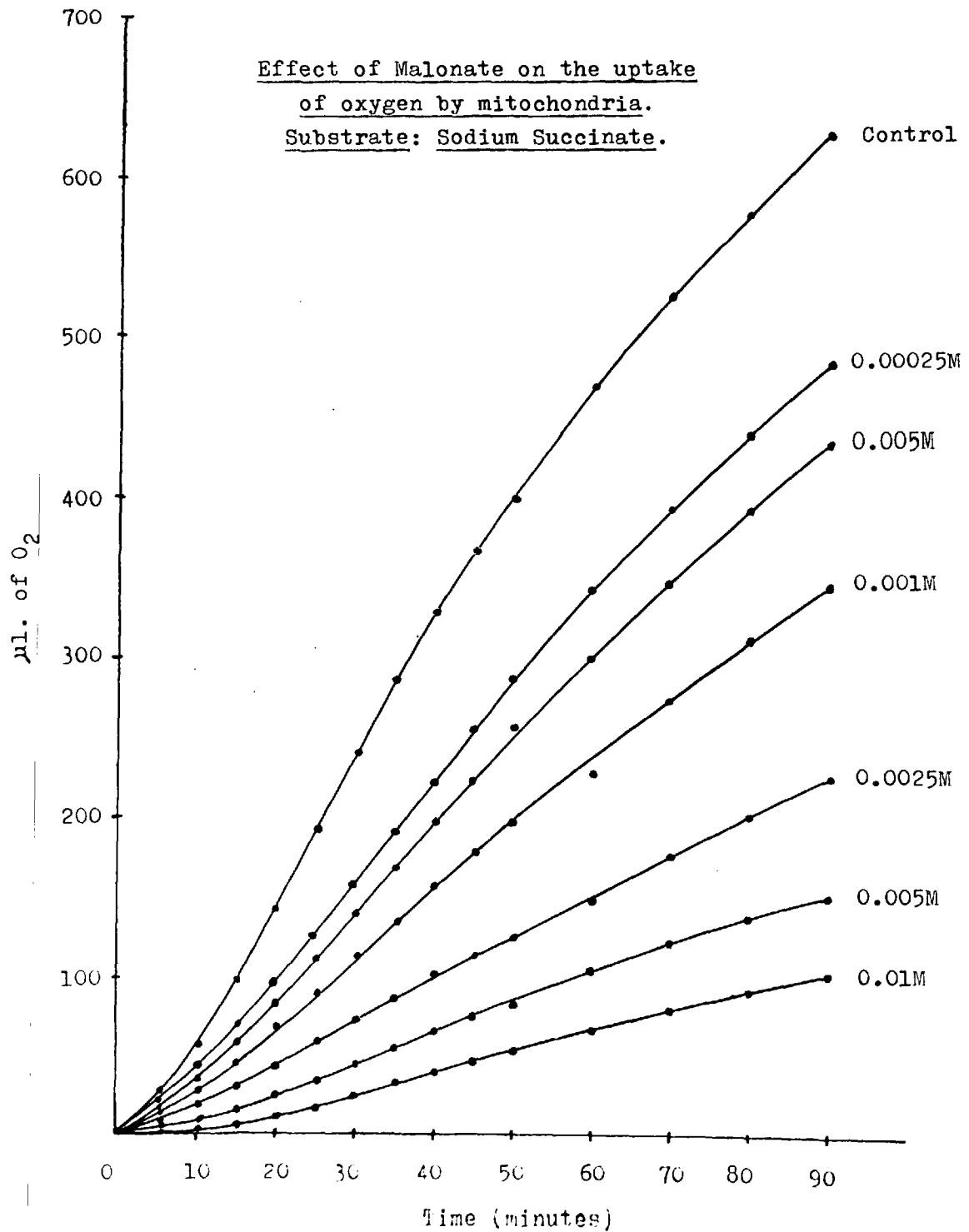


Table XVII.

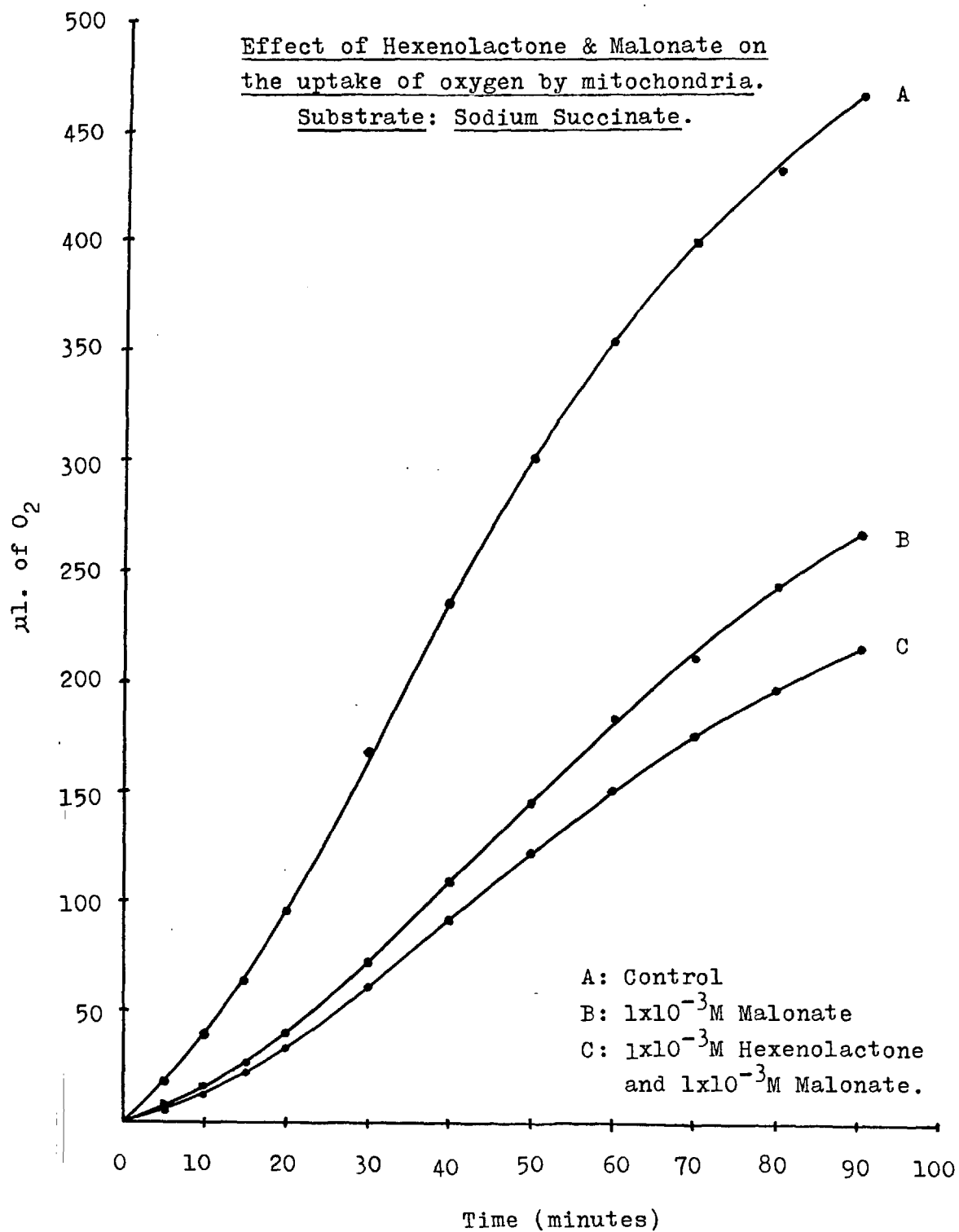
Respiration of mitochondria: The effect of Hexenolactone
and Malonate on the uptake of oxygen by mitochondria.

Each flask contains 3.0 ml. of liquid including 0.2 ml.
of 10% Potassium Hydroxide in the centre cup.

Substrate: 0.025 M Sodium Succinate.

Figures represent μ l. of O_2 uptake by cauliflower
mitochondria per mg. of total N.

Time (minutes)	Control	Hexenolactone $1 \times 10^{-3} M$	Malonate $1 \times 10^{-3} M$
		Malonate $1 \times 10^{-3} M$	
5	19.40	6.00	7.00
10	40.00	12.50	15.00
15	65.20	22.00	26.40
20	95.80	33.50	41.20
30	170.00	59.50	75.90
40	235.30	93.10	110.80
50	302.50	121.80	144.00
60	352.70	153.90	185.60
70	400.10	178.30	210.30
80	429.40	200.70	245.10
90	469.50	220.10	268.10



it has long been known that a competitive inhibition at substrate level exists between succinate and malonate, and it therefore seemed of interest to test whether this particular inhibition was further affected (enhanced) by the presence of hexenolactone together with malonate, since it might be that different active centres of the enzyme would be concerned. The results of such an experiment are shown in Tables XV - XVII.

Mitochondrial swelling.

A considerable amount of work has been recorded concerning changes in the physical condition of isolated mitochondria resulting from contact with certain substances, which, it has been suggested, influence the structure of the mitochondrial membrane in some manner. This sensitivity to the composition of the surrounding medium was apparently first noted by Harris (1943), who observed a marked swelling of the particles in the presence of certain inorganic ions. Later investigations showed that this swelling was not a simple osmotic phenomenon, although the osmolarity of the medium played some part in governing the degree of swelling but that chemical activity such as respiration was necessarily involved. A complete explanation of the

phenomenon is still not forthcoming, however, but since various inhibitors such as amytal and antimycin have been shown to influence the course of swelling it was thought worth while to carry out some tests with hexenolactone.

Cleland (1952) used a simple optical-density technique for following the course of the swelling; his method has been commonly adopted and was finally used here. Cauliflower mitochondria, prepared as previously described, were used with the difference that the suspension was made in 0.3M. sucrose in 0.2M. Tris buffer pH 7.4 (Chappell and Greville, 1959); N 0.3 mg./ ml.

In some early experiments 1 ml. portions of this suspension were placed in small test-tubes (60 mm. x 7 mm.), and at a noted time portions of 0.04M. phosphate (pH 7.4) were added, to give a final concentrations of phosphate ranging from 0.001M. to 0.02M., a control was free from added phosphate. The tubes were held either at room temperature or in an incubator at 30°. According to Hunter et al. (1956), whose experimental conditions were similar to those described here, the degree of swelling can be estimated by observing visually the decrease in turbidity of the suspension, but although observations were made over a period of 120 minutes no visible change could be detected. Hunter et al. do not state the duration of their

of their tests, but Chappell and Greville (1959) indicate that 30 minutes should be ample for maximum swelling to occur. It was noted however that in all the tubes to which phosphate had been added the mitochondria had settled out overnight, the compactness of the sediment varying with the amount of phosphate present; there was no precipitate in the control (phosphate-free) suspension. It seemed evident therefore that a phosphate-induced swelling had indeed taken place, but that visual observation was not sufficiently sensitive to detect the changes taking place during the first two hours of exposure. Since Hunter et al. used rat-liver mitochondria, the experiment was repeated with this material, kindly supplied through the Department of Zoology of this College but no difference in behaviour could be found, and no explanation is offered as to why the observations of Hunter et al. could not be reproduced.

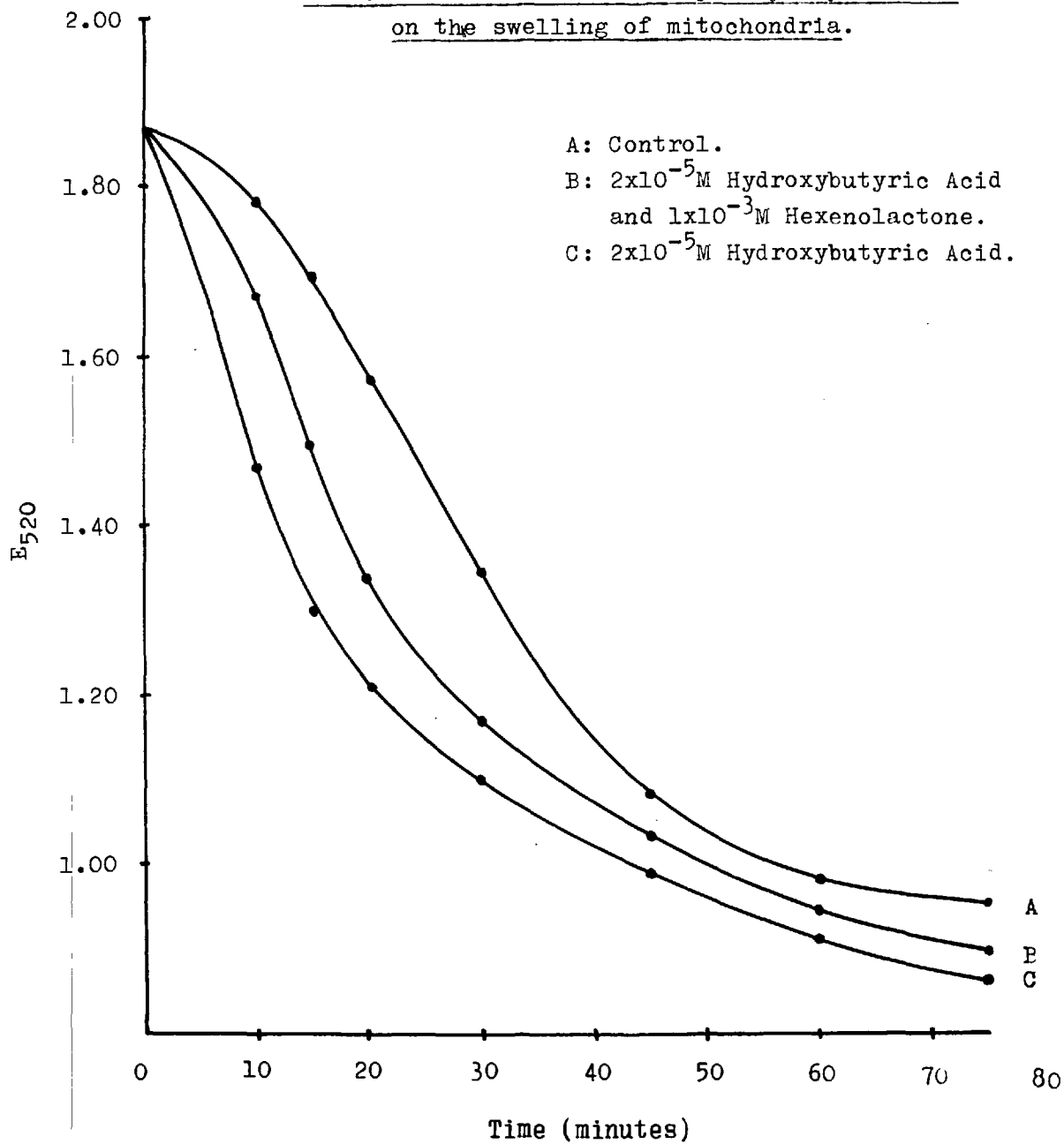
The technique of Chappell and Greville (1959) in which estimations of turbidity are made by means of an electric spectrophotometer seemed to offer a more satisfactory method and was therefore adopted in further experiments. The suspensions to be examined were held in the optical cell of a Hilger "Uvispek" spectrophotometer throughout the whole course of an experiment, as this found a more satisfactory procedure than transferring portions of

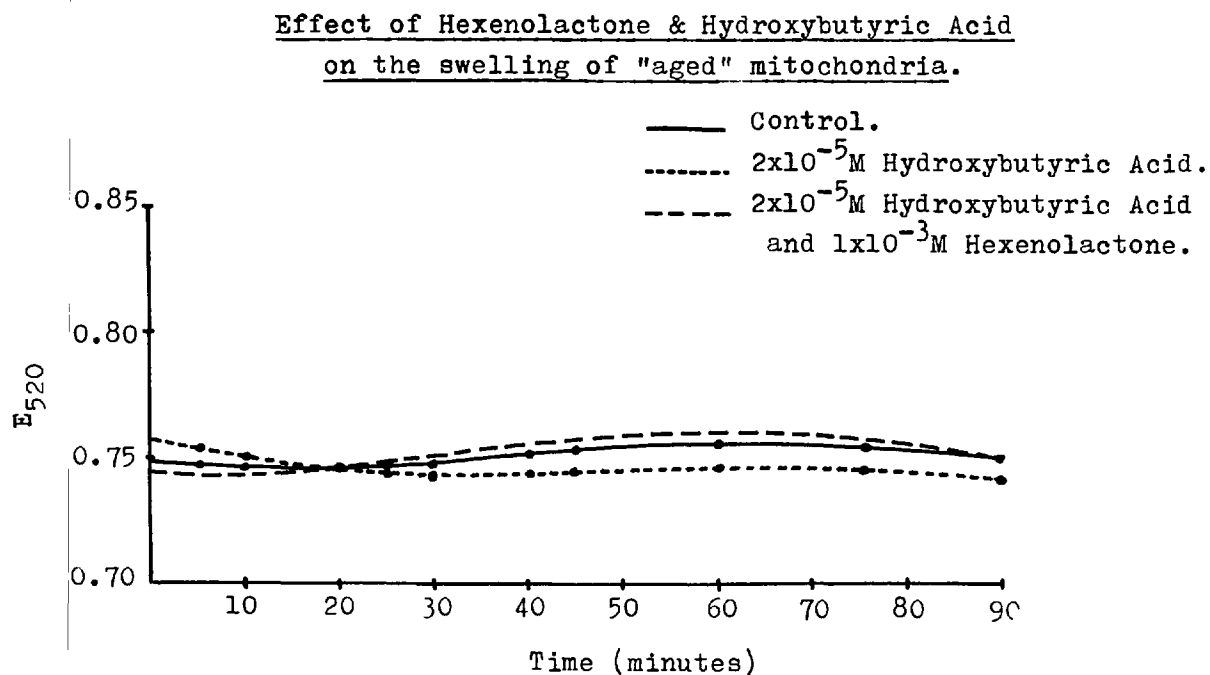
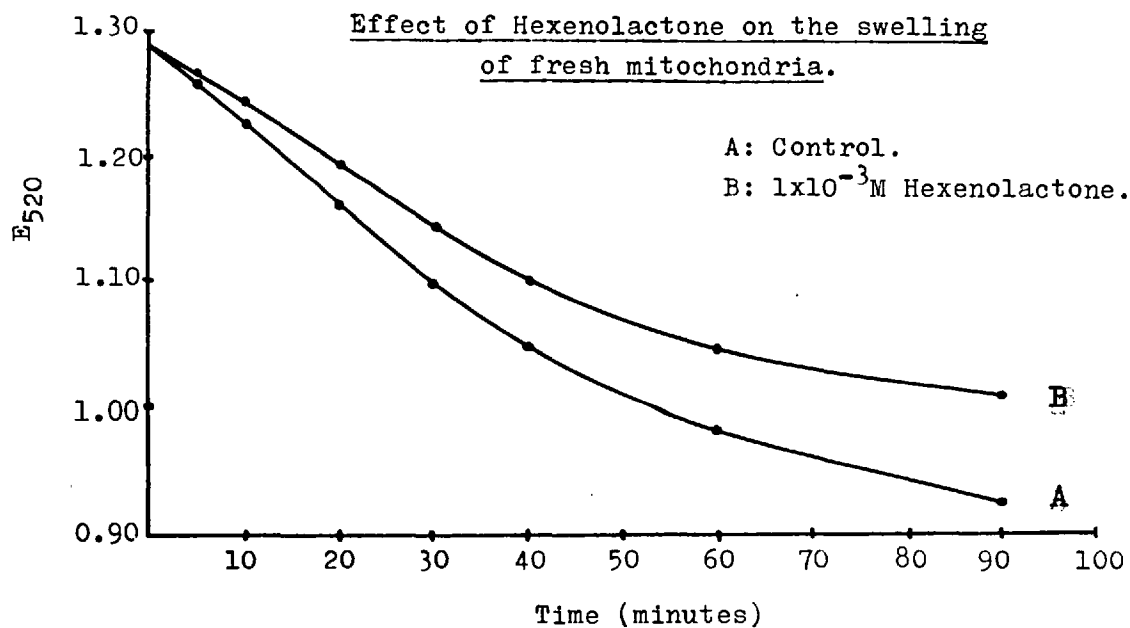
a suspension from another vessel. Each cell contained 1 ml. of the suspension (0.3 mg./ml.); to the control cell was added 1 ml. of the tris buffer, and to the other cell a suitable amount of hexenolactone dissolved in 1 ml. of buffer. To each was added 0.5 ml. of phosphate to give a final concentration 0.02M., and the optical density of each cell measured as quickly as possible, using a 520 m μ filter (light path 1 cm.). Readings were taken at suitable intervals for a period of 90 minutes in order to follow the change in translucency resulting from phosphate-induced swelling during that period. In other experiments, the mitochondria were held in contact with the hexenolactone (1×10^{-3} M.) before the addition of phosphate, to see whether changes in the physico-chemical nature of the mitochondria involved a time factor. The results of these tests are shown on page no. 82.

In these experiments care was taken to use freshly prepared suspensions of mitochondria, since these are known to undergo very definite changes during even a few hours at temperatures as low as 0°. It appears (Chappell and Greville, 1959) that the particles themselves contain certain amounts of materials which serve as substrates for respiration, and that exhaustion of these, which takes place fairly rapidly, leads to diminished activity of the mitochondria. However, to a great extent the enzymes of the mitochondria are still

capable of functioning, and the provision of a suitable respiratory substrate will restore much of the original activity including the capacity to respond to phosphate ions. A particularly suitable substrate has been found in β -hydroxybutyric acid (Na salt) at a concentration of 2 mM. The addition of this substrate may cause even freshly prepared mitochondria to show an increased sensitivity to phosphate, as shown on page no. 82; possibly even the unavoidable delay involved in preparation of the suspensions results in a certain wastage of the endogenous metabolites contained in the mitochondria in vivo. Chappell and Greville performed some experiments in which mitochondria were allowed to "age" by being maintained for 24 hours at 0°, after which their activity was restored by the addition of β -hydroxybutyrate and their sensitivity to phosphate studied. Similar experiments in which cauliflower mitochondria were allowed to "age" showed however that these particles did not respond to the restorative treatment, and had apparently irreversibly lost their capacity to swell - a further example perhaps of the greater sensitivity of plant preparations compared with animal. (see page no. 83).

Effect of Hexenolactone & Hydroxybutyric Acid
on the swelling of mitochondria.





NITROGEN DISTRIBUTION
IN PLANT TISSUES

Investigations on the metabolism of stem tissues.

I) Preparation of materials.

For this investigation sections of the stems of seedlings of Pisum sativum (Meteor) were used, as by previous workers in this and other laboratories. Certain difficulties were encountered at the beginning in obtaining uniformly growing and healthy seedlings. These difficulties were also experienced by earlier workers (Richards, 1953 and Mehta 1954). For sterilizing the pea-seedlings, Richards used 0.2% Mercuric Chloride solution whereas Mehta used 1.0% Chloramine T solution. However, both noticed a certain amount of fungal contamination even after sterilizing the seeds. Taking these facts into consideration the methods of sterilization were changed. Seeds were sterilized by exposing them to Ultra-violet light for half an hour from each side, and then soaked in sterilized water for 15 to 18 hours. It was noticed, however, that this exposure of seeds to Ultra-violet light often caused inhibition of the growth, and as a result of many attempts the writer concluded that it is impossible to get seedlings free from fungal contamination. Finally, the following procedure was adopted. Pea-seeds were soaked in freshly boiled and cooled water and then kept in an incubator for

15 - 18 hours. At the end of this period they were washed thoroughly, first with tap-water and then with sterilized water. The seeds were then placed over filter papers (previously soaked in sterile water) in petri dishes and kept in an incubator and allowed to germinate for 48 hours, during which period the radicles grow to a length of 2 to 3 cms. The seedlings were then placed on the specially perforated lid of a plastic box containing sterilized water in such a way that their radicles would pass through the small perforations and become immersed in the water. The seedlings were then grown in the incubator maintained at 25° in a dark room. Through all the stages of their growth - from the beginning to the end of the experiments - the seedlings were kept in darkness or in a minimum red light when inspection or manipulation was necessary.

For experimental purposes only unaffected and healthy seedlings were chosen. Seedlings were harvested after 12 - 14 days when those stems having a well-developed third internode, but which had not developed a fourth internode longer than 1 - 2 mm. were selected for experiments. Sections exactly 20 mm. long were cut from the most apical part of the third internode. Sections thus prepared formed the test material for all experiments. Stem-sections were divided into random samples of 10 to 15 sections according to different

experiments. These sections were washed with distilled water and blotted dry quickly. They were transferred to black weighing bottles and weighed.

II) The influence of hexenolactone and other compounds on the growth and dry weight of pea-stem sections.

Christiansen and Thimann (1950,I) have shown that a close parallel exists between the elongation of pea-stem sections and the increase in fresh weight when sections were allowed to grow for 24 hours floating on the surface of the test solutions. The following test solutions were taken - Water; Auxin - 1 mg./litre (β -indolyl acetic acid from Light & Co. was used as a source of auxin.); Hexenolactone, $1 \times 10^{-3}M.$; Angelicalactone, $1 \times 10^{-3}M.$; and Iodoacetic acid, $6 \times 10^{-4}M.$ Hexenolactone, angelicalactone and iodoacetic acid were made in solution containing 1 mg./l. auxin. All the test solutions were adjusted to pH 6.5 with H_2SO_4 or KOH. Sections of the pea-stems prepared as above were transferred into 10 ml. of each test solutions separately and then incubated for 24 hours. This treatment was carried in small open crystallizing dishes (5 cm. diameter) so that stem sections floated on the surface of the solutions; this has been shown essential since the presence of oxygen is necessary for the growth to take place. At the end of

this growth period the sections were blotted dry and weighed again. From the difference in weight percentage increase in fresh weight was calculated. The results are shown in Table XVIII.

For estimation of changes in dry weight, samples of the sections grown for 24 hour periods in different test solutions as mentioned earlier were immediately dried to constant weight for 48 - 60 hours at 95°. The dry weight results are expressed in Table XIX.

It is evident from these results that any increase in fresh weight was accompanied by a loss in dry weight and that the decrease in water uptake was accompanied by a proportionally greater loss of cell-wall materials.

III) Influence of Hexenolactone and other compounds on a nitrogen distribution.

(a) Total nitrogen : Total nitrogen was determined in samples of 10 sections each. At the end of 24 hour growth period the sections were blotted dry and then transferred to 30 ml. Kjeldahl flasks and the procedure according to Pregl et al. (1945) was followed.

(b) Determination of non-protein nitrogen : - After 24 hour growth period the samples of sections (in

batches of 15) were macerated with cold water and treated with equal volume of 5% trichloroacetic acid in order to precipitate all the proteins. The mixture was filtered and the residue was washed thoroughly; the combined filtrate and washings were evaporated to a suitable volume and then incinerated with concentrated H_2SO_4 in usual manner for determination of nitrogen.

(c) Total protein : - This was estimated indirectly by subtracting the values of non-protein nitrogen from the values of total nitrogen and the difference so obtained was multiplied by the factor 6.25.

(d) Cell-wall protein : - Samples of 20 sections each were grown for 24 hours in different test solutions and then macerated with sufficient quantity of cold water; after filtering, the pulp was washed three times with cold water. The water extract was discarded and the pulp was re-extracted with hot 2% sulphuric acid. The sulphuric acid extract was used for determining nitrogen content and cell-wall protein was calculated accordingly by multiplying by the factor 6.25.

(e) The plasma-protein : - This was obtained by subtracting the values of cell-wall protein from that of total protein.

(f) Total Amides : - After 24 hours samples of 20 sections each, grown in different test solutions, were ground with sufficient cold water to a fine paste. This was extracted three or four times with cold water and then treated with 5% trichloroacetic acid to precipitate all protein, which was then removed by filtration. The filtrate was hydrolysed with 5% H_2SO_4 under reflux for 1 hour. After cooling, the solution was diluted to 50 ml. 10 ml. of this solution were used for each nitrogen estimation by micro-distillation into boric acid solution.

(g) Free Amino-acid : - This was calculated by subtracting the values of nitrogen of the total amides from that of non-protein nitrogen. The presence of small amount of bases etc., as shown by Christiansen and Thimann (1950,III), is of minor importance; and therefore the above difference in nitrogen is considered to represent amino acid. The amino acid nitrogen was multiplied by 6.25 to give the quantity of free amino acid.

(h) Chromatographic analysis of amino acids.

Attempts were made to separate the free amino acids by using high-voltage paper electrophoresis in one direction followed by chromatographic separation at right angle as suggested by Dixon et al. (1958) and Efron (1959).

Whatman No. 1 paper was first wetted with formic acid-acetic acid buffer pH 2 (Gross, 1956) and the edges were placed between paper wicks fed from the small cell containing the electrodes immersed in the same buffer. A potential difference of 1.2 kv. was then applied to the electrodes, producing a current of approximately 9 mA. for 15 minutes. The amino-acids travel towards the cathode. The paper was dried in an oven. The dried paper was then used for chromatography in the right angle direction, using phenol as a solvent. After drying, the paper was sprayed with 0.2% ninhydrin.

This combined electrophoresis-chromatography method seems quite satisfactory, but the lack of suitable equipment, especially efficient cooling device forced the writer to abandon the method.

Two-way chromatographic separation of amino-acids.

Alternatively, the following two-way chromatography method was used. A sufficient amount of the extract was applied to one corner of the Whatman No. 1 paper (size 24" x 24") and the chromatogram was run in a descending direction, first in a solvent containing butanol, acetic acid and water in the proportion : 250 : 60 : 250 (Woiwod, 1949) in one direction and then in phenol in right angle

direction. The paper was dried and sprayed with 0.2% ninhydrin solution.

Table XVIII.

The effect of Hexenolactone and other compounds on the growth of pea-stem sections, expressed as a change in initial fresh weight.

Treatment	Per cent change in fresh weight.	Average change in % in fresh weight.	Number of experiments.
Water	+ 17.50	---	43
Auxin, 1.mg./l. (control)	+ 32.00	+ 32.00	37
Hexenolactone $1 \times 10^{-3} \text{M}$	+ 14.96	- 53.25	22
Angelicalactone $1 \times 10^{-3} \text{M}$	+ 26.55	- 17.00	20
Iodoacetic Acid $6 \times 10^{-4} \text{M}$	+ 19.92	- 37.75	20

The solutions of Hexenolactone, Angelicalactone and Iodoacetic Acid were made in a solution containing Auxin (1 mg. per litre).

Table XIX.

The effect of Hexenolactone and other compounds on the dry weight of pea-stem sections, expressed as a change in initial fresh weight.

Treatment	Dry wt. % of initial fresh wt.	Average % change in dry weight.	Number of experiments performed.
Initial	6.148	---	15
Water	5.614	-8.68	12
Auxin (1 mg/1.)	5.469	-11.04	10
Hexenolactone $1 \times 10^{-3} M$	5.663	-7.88	10
Angelicalactone $1 \times 10^{-3} M$	5.739	- 6.65	8
Iodoacetic Acid $6 \times 10^{-4} M$	5.652	- 8.10	8

The solutions of Hexenolactone, Angelicalactone and Iodoacetic Acid were made in a solution containing Auxin (1 mg. per litre).

Table XXa.

The influence of Hexenolactone and other compounds on
the nitrogen distribution in pea-stem sections.

Treatment	Total Nitrogen.	Non-protein Nitrogen.	Protein Nitrogen by difference.
Initial	6.998	4.845	2.153
Water	6.608	4.030	2.578
Auxin (1 mg. /l.)	6.795	3.974	2.821
Hexenolactone $1 \times 10^{-3} \text{M}$	5.910	3.283	2.627
Angelicalactone $1 \times 10^{-3} \text{M}$	6.719	4.006	2.713
Iodoacetic Acid $6 \times 10^{-4} \text{M}$	6.298	4.108	2.190

The solutions of Hexenolactone, Angelicalactone and
Iodoacetic Acid were made in a solution containing
Auxin (1 mg. per litre)

Table XXb.

The influence of Hexenolactone and other compounds on
the nitrogen distribution in pea-stem sections.

Treatment	Calculated protein.	Cell-wall nitrogen.	Cell-wall protein.	Plasma protein.
Initial	13.446	0.516	3.231	10.215
Water	16.112	0.627	3.917	12.195
Auxin (1 mg. /1.)	17.631	0.565	3.527	14.104
Hexenolactone $1 \times 10^{-3} \text{M}$	16.419	0.555	3.471	12.948
Angelicalactone $1 \times 10^{-3} \text{M}$	16.956	0.572	3.596	13.360
Iodoacetic Acid $6 \times 10^{-4} \text{M}$	13.687	0.544	3.399	10.288

The solutions of Hexenolactone, Angelicalactone and Iodoacetic Acid were made in a solution containing Auxin (1 mg. per litre).

Figures represent per cent of the initial dry weight.

Table XXc.

The influence of Hexenolactone and other compounds on
the nitrogen distribution in pea-stem sections.

Treatment	Amide Nitrogen.	Non-protein Nitrogen.	Amino-acid Nitrogen.
Initial	0.905	4.845	3.940
Water	0.886	4.030	3.144
Auxin (1 mg. /l.)	0.927	3.974	3.037
Hexenolactone $1 \times 10^{-3} \text{M}$	0.779	3.283	2.504
Angelicalactone $1 \times 10^{-3} \text{M}$	0.847	4.006	3.159
Iodoacetic Acid $6 \times 10^{-4} \text{M}$	0.749	4.108	3.359

The solutions of Hexenolactone, Angelicalactone and Iodoacetic Acid were made in a solution containing Auxin (1 mg. per litre).

Figures represent per cent of the initial dry weight.

Table XXI.

Summary of changes in nitrogen distribution in pea-stem sections produced by Hexenolactone and other compounds.

Treatment	A	B	C	D	E	F
Initial level	6.998	4.845	2.153	0.516	0.905	3.940
Loss in water	-0.390	-0.815	+0.425	+0.111	+0.019	-0.796
Loss in Auxin (1 mg. /l.)	-0.203	-0.871	+0.668	+0.049	-0.022	-0.903
Loss in Hexenolactone $1 \times 10^{-3} M$	-1.088	-1.562	+0.474	+0.039	-0.126	-1.436
Loss in Angelicalactone $1 \times 10^{-3} M$	-0.279	-0.839	+0.560	+0.056	-0.058	-0.781
Loss in Iodoacetic Acid $6 \times 10^{-4} M$	-0.500	-0.737	-0.037	+0.028	-0.156	-0.581

Figures represent per cent of the initial dry weight.

A : Total Nitrogen,

B : Non-protein Nitrogen,

C : Protein Nitrogen,

D : Cell-wall Nitrogen,

E : Amide Nitrogen,

F : Amino-acid Nitrogen.

Table XXII.

Chromatographic analysis of the amino-acids of sections
of pea-stems.

Amino-acid	Initial	Water	Auxin	HL	AL	IAA
L-alanine	+	+	+	+	+	+
Asparagine	+	+	+	+	+	+
Aspartic acid	+	+	+	+	+	+
Glutamine	+	+	-	-	-	-
Glutamic Acid	+	+	+	+	+	+
Glutathione	+	+	+	-	-	+
Glycine	+	+	+	+	+	+
Leucine	+	+	-	-	-	-
Serine	+	+	+	+	+	+
Threonine	+	+	-	-	-	-
Tyrosine	+	+	-	-	-	-
Valine	+	+	-	-	-	-

HL :- 1×10^{-3} M Hexenolactone,

AL :- 1×10^{-3} M Angelicalactone,

IAA:- 6×10^{-4} M Iodoacetic Acid:

POLAR GROUPS IN ENZYME PROTEINS.

Influence of hexenolactone on the polar groups
of enzyme proteins.

The electrometric titration curve of a protein is capable of furnishing information concerning the ionising groups present in the molecule, and from such data it is possible in favourable cases to detect changes in ionic condition brought about by external means. Since ionisation is undoubtedly concerned with the distribution of "active centres" in an enzyme protein, Spector was led to examine the electrometric titration curve of urease before and after treatment with hexenolactone, but the differences which she observed were too slight to permit of any notable deductions. Since Spector's investigations, Russell (private communication) has made a detailed study of the techniques of electrometric titration, and has evolved a procedure whereby the accuracy and sensitivity of the titration is appreciably increased. This is done by conducting the titration in such a way that the reagent (acid or alkali) is added in equal amounts (by volume) at brief regular intervals of time. Russell considers that his method more readily gives information concerning ionising centres on the protein surface and these centres might reasonably be regarded as primarily involved in the initial stages of enzymic activity. Russell's method was

therefore, used to construct titration curves of egg-albumin and of urease, as typical examples of a soluble non-enzymic protein and of a lactone-sensitive enzyme protein. The procedure was as follows:

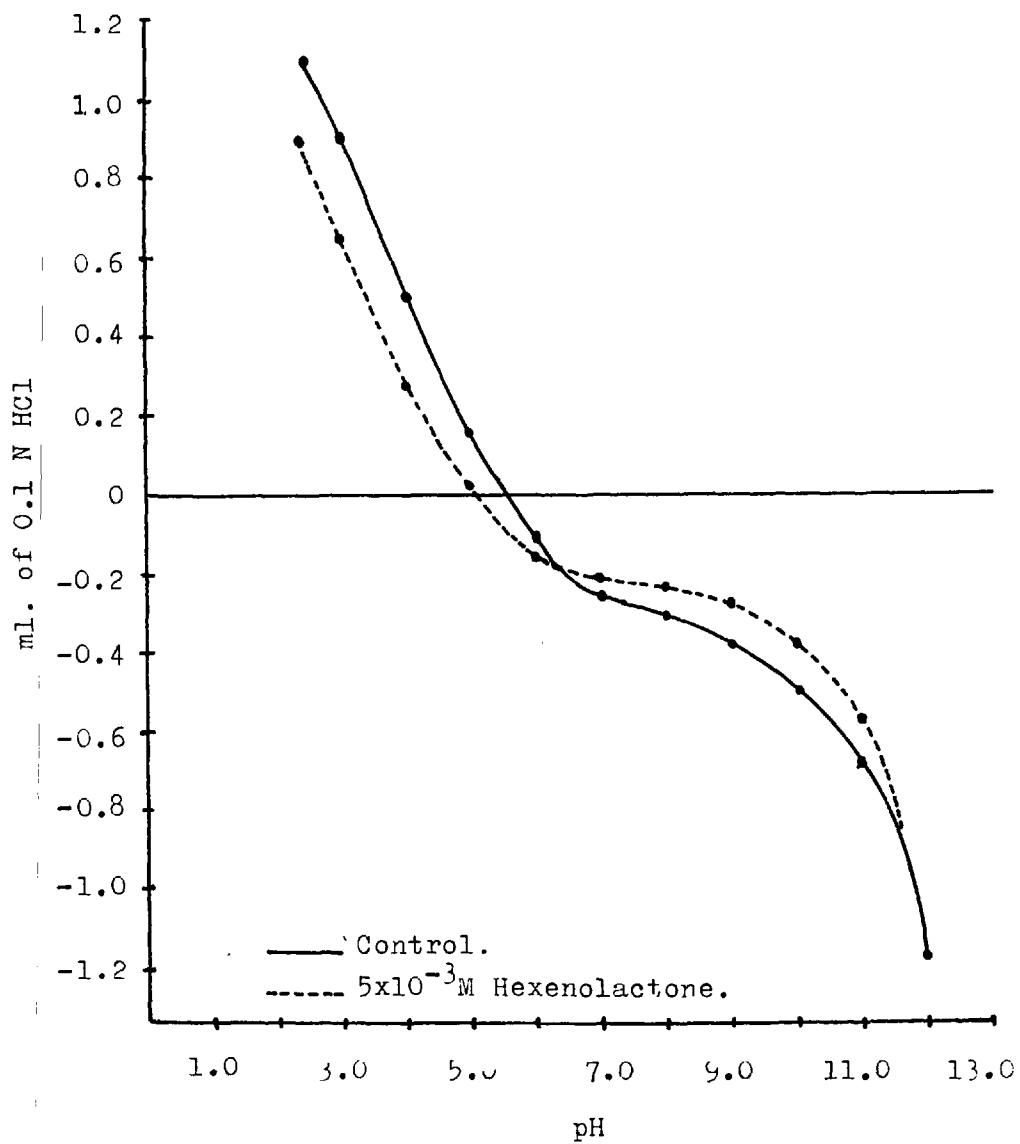
Egg-albumin: 5 ml. of distilled water was added to 5 ml. of a 2% egg-albumin (B.D.H. crystalline) solution. To this was added 1 ml. of 0.3N (approximately) NaOH and thoroughly mixed. After 5 minutes 0.1 ml. of 0.1N HCl was added from a burette, mixed well and the pH was noted immediately (PYE pH meter): Further 0.1 ml. portions of HCl were added by adjusting the time in such a way that ten seconds were allowed for adding HCl, ten seconds for mixing and ten seconds for taking the pH readings.

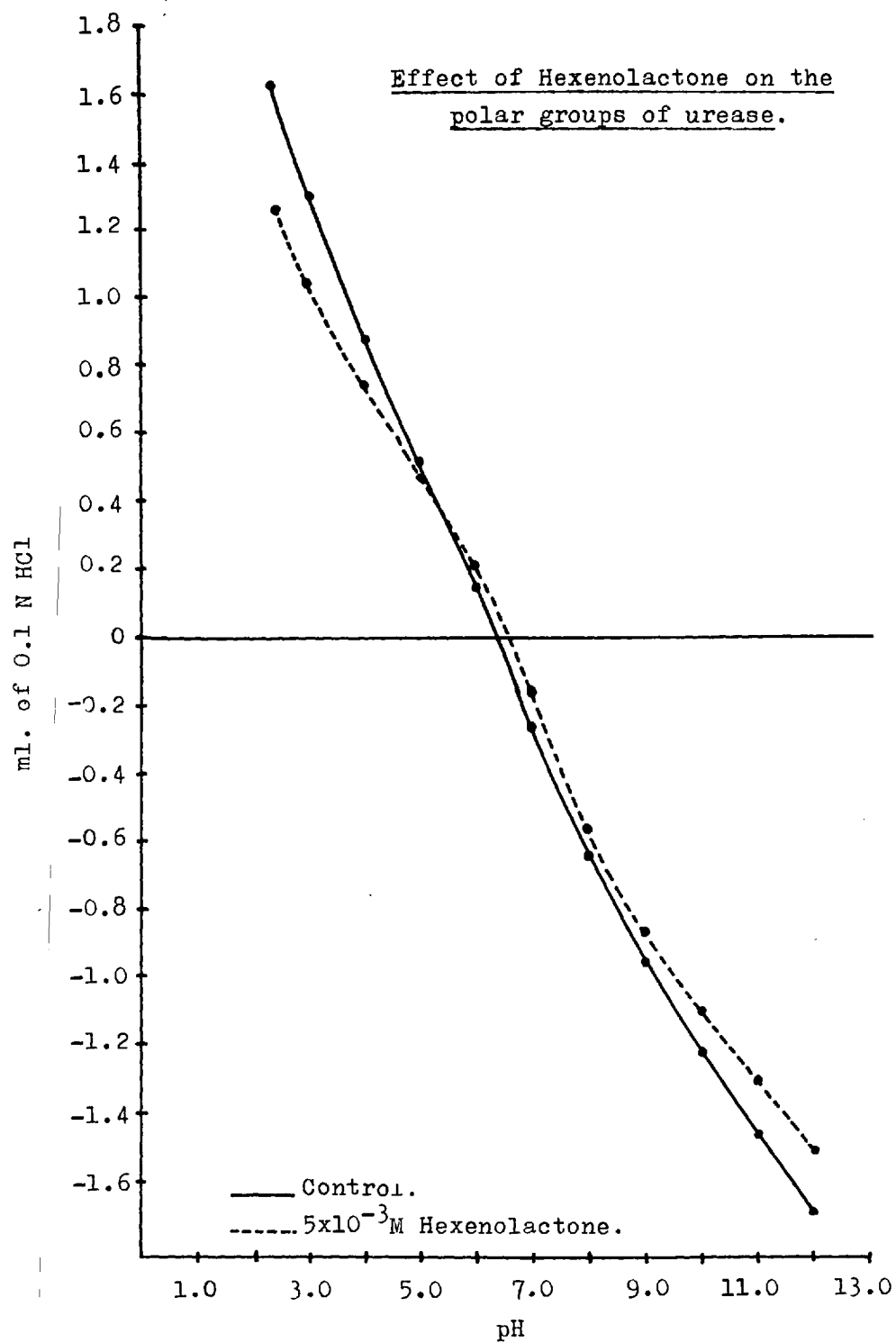
In another experiment 5 ml. of hexenolactone ($5 \times 10^{-3}M$ final concentration) were added to 5 ml. of a 2% solution of egg-albumin. After keeping in contact for two hours, 1 ml. of 0.3N NaOH was added. The solution was then titrated, after five minutes, against 0.1N HCl as above. The controls of water and hexenolactone were also treated in the same manner.

Urease: Crystalline urease obtained from Sigma Chemical Co., U.S.A. was used for these experiments. The experimental procedure was the same as described above.

The curves are shown on pages 102 and 103.

Effect of Hexenolactone on the
polar groups of egg albumin.





DISCUSSION

The first objective of the present work was to test whether any positive experimental evidence could be obtained to support May's theory that the inhibitory activity of hexenolactone was due to an effect on membrane permeability rather than to a direct action on any enzyme system. May attempted to investigate the permeability of erythrocytes treated with hexenolactone, but could not by chemical means detect any changes. She did however notice appreciable changes in cell-volume and in agglutination behaviour, and considered that the lactone had in some way altered the nature of the cell-surface - possibly by neutralisation or redistribution of the surface charge. A similar phenomenon was noted with cells of E. coli, which flocculated more readily after treatment with the lactone, and since the effect was greater than could be accounted for by any change of pH of the medium she considered that here again the surface had been modified in some way by the lactone with a consequent change in permeability. Except for a fruitless attempt to detect an increased efflux of cell materials from lactone-treated yeast, May did not investigate directly the passage of solute molecules through any cell-walls or membranes. This has now been done, and since the system chiefly studied by May, namely fermentation by yeast, involves primarily the uptake of glucose and of phosphate by the cells, attention was focussed mainly on these metabolites.

Since glucose is normally taken up into some metabolic pathway (glycolytic or other) at a rate equal to that at which it enters the cell, it is not possible to estimate directly the rate of uptake except in the presence of some metabolic inhibitor; however the influx of a sugar which is not metabolised e.g. arabinose or (for most strains of yeast), galactose can readily be followed, as was done by Burger et al. (1959), and the technique of these authors has been used.

The first requirement for the utilisation of a sugar or other metabolite is that it should enter the cell, i.e., pass through the surrounding cell-wall and/or membrane. The cell-wall of yeast is a complex structure; in fact its complexity is only now being fully appreciated. It appears that the outer, mechanical structure consists of one or more polysaccharides of the gluco-mannan type; but if there is any resemblance to many bacterial cell-walls one may expect also the presence of a polyol-phosphate-protein complex. Within the relatively thick polysaccharide layers lies a more delicate membrane, that of the protoplast. While it is possible that each structure plays a part in controlling the transfer of solutes, it would seem likely that the major role would fall upon the membrane of the protoplast and upon the protein components of the outer wall; certainly it is not

easy to imagine any sensitivity to external environment in a polysaccharide unit. It has been found possible to remove, by the action of the β -glucosidases of Helix, the outer cell-wall and leave the protoplast intact, and different strains of yeast have been found (Eddy and Williamson, 1959) to differ markedly in the ease with which this can be done. This suggests that important differences exist between the outer structure of the different strains, and for this reason more than one strain has been used in the present work. S. carlsbergensis no. 74 is reputed to be especially suitable for the preparation of protoplasts, and direct measurement of the influx of the non-fermentable sugar galactose does indeed indicate that its cells are more permeable than those of the other strains tested (nos. 355 and 366) - or else that the cells are able to accommodate a much higher permanent internal concentration of the sugar (Table IIIa). Strain no. 74 seems also similarly permeable to arabinose - possibly even more so, since the influx during 60 minutes is, on a molar basis, some 20% higher than for galactose. However it appears that hexenolactone (5×10^{-3} M.) exerts no appreciable influence upon the ability of the cells to accumulate galactose, or upon the rate of influx, since although the intracellular concentration of the sugar (and of arabinose in the case of no. 74) is almost always lower in

the lactone-treated cells; the decrease is too slight to be considered significant. With nos. 355 and 366, galactose uptake was not appreciably lessened under anaerobic conditions (atmosphere of nitrogen), either without or with hexenolactone, but the rather curious observation was made that anaerobic conditions seemed to favour influx of the sugar in the case of no.74, although here also hexenolactone was without effect. A possible, though unlikely, explanation, would be that galactose is removed by an oxygen-requiring mechanism, and that the higher level attained anaerobically more truly represents the amount of intake. Galactose influx is influenced by iodoacetate ($5 \times 10^{-3}M.$), and the degree of inhibition now observed is in agreement with the work of Burger et al.(1959).

Only a very small intracellular accumulation of glucose can be detected, since this sugar is metabolised as rapidly as it is absorbed; a "steady state" level is attained within a short time (less than 20 minutes) and is maintained for at least 60 minutes - probably much longer. The level was found to be appreciably higher for no.74 than for the other strains; with no. 355 anaerobic conditions seem to favour a slightly higher "steady state" level. As noted by Burger et al., and shown also by the figures quoted in Table IV, iodoacetate, by checking the metabolism of glucose, allows

this sugar to accumulate within the cells, the concentration/time ratio giving the usual form of curve. Hexenolactone however seems to cause a different effect. During the initial stages (up to 20 minutes) its effect resembles at least superficially that of iodoacetate, in that the glucose level begins to rise, although less rapidly than in the presence of iodoacetate. This difference may be due either to the fact that iodoacetate is a more efficient inhibitor of fermentation than hexenolactone, or that the lactone does in fact cause a slower entry of glucose. (The latter explanation would indicate a difference in the behaviour of the lactone towards galactose and glucose). After this initial period, a marked difference in behaviour between the two inhibitors is observed; whereas with iodoacetate the glucose level continues steadily to rise, with hexenolactone (and to a less extent with angelicalactone) the level falls sharply, to below the "steady state" level. Again there is more than one possible explanation. The entry of fresh sugar may be more strongly inhibited; or the permeability of the membrane may have been so changed as to permit an actual efflux of sugar from the cell; or the lactone now actually stimulates the metabolism of glucose. The first two possibilities are similar in that each would be connected with a permeability change, and one taking place more markedly as the time of contact between membrane and lactone lengthens. The effects

noted are more marked in the case of no.74, in keeping with the apparently greater susceptibility of this strain, but still follow the same trend in no. 355.

Under anaerobic conditions, certain differences in pattern can be seen. The glucose concentration in the "steady state" for no.74 is unaffected, but that for no.355 is perceptibly higher. Iodoacetate allows a considerably greater accumulation of glucose under anaerobic conditions, suggesting that an oxygen-requiring path of glucose metabolism, insensitive to iodoacetate, exists. Anaerobically, in the presence of hexenolactone, the glucose level for no.74 does not show the marked rise and fall so noticeable in aerobic conditions; instead the concentration continues to rise steadily during the 60-minute test period (cf. iodoacetate).

The presence of glucose (1%) together with galactose (5%) has been shown by Burger et al. to inhibit almost entirely the uptake of the latter sugar into yeast cells, the concentration attained in 90 minutes being only about 6% of that attained in the absence of glucose. A few experiments (Tables V and Va) largely confirmed this observation, although in the case of no. 355 the maximum level attained was appreciably higher, being some 20% of that reached without glucose. In the present experiments attempts to distinguish between glucose and galactose (quantitatively) in cell-

-extracts were unsuccessful, and only "total reducing sugar" was estimated (and recorded in the Tables). However, no appreciable effect on total sugar influx could be attributed to hexenolactone. All that can be said is that, as with glucose alone, iodoacetate allows a greater intracellular concentration of sugar to be built up anaerobically than aerobically, and that this **effect** is not found with hexenolactone.

From a study of the literature it seems that there is at present no clear picture of the mechanism by which sugars enter living cells of yeasts or bacteria. Entry may take place merely by physical means (diffusion), governed by the relative concentrations on either side of the boundary and with the cell-wall or membrane acting purely as a mechanical sieve. With a non-metabolised sugar this process should result in a more or less equal distribution of sugar molecules (assuming that these could pass the pores of the "sieve") on either side; and with a metabolisable sugar, the process would continue as the molecules within the cell were broken down. In either case it is not easy to envisage an influx against a concentration gradient, yet the figures of Burger et al. show that this may indeed take place. No such effect was found in the present experiments with galactose,

but it was noted with arabinose, and this suggests that some positive transport mechanism must be involved. The existence of such systems has been postulated by many workers. Thus Rothstein (1956) has argued that glucose enters the yeast cell under the influence of a phosphorylating system located within the cell-wall; Cohen and Monod (1957) suggest that certain permeases are concerned with the entry of oligosaccharides into cells of E. coli; and Stein and Danielli (1956) postulate a "facilitated diffusion" mechanism. The observation of Burger et al., of the inhibitory effect of glucose on galactose influx, suggests that if an enzyme system is involved it must be the same for both sugars, although the enzyme may have a much greater affinity for glucose than for galactose (Burger et al. suggest about 100 times greater). The present work (Table III) indicates that, whatever the mechanism of influx, that concerned with galactose in absence of glucose is insensitive to hexenolactone, while that concerned with glucose is sensitive. If therefore the lactone acts by altering the physico-chemical state of the surrounding membrane, the alteration is not sufficient to affect the galactose uptake system.

The idea that hexenolactone actually stimulates aerobic utilisation of glucose is at entire variance with all its recognised properties. The simplest explanation

therefore of the effect of the lactone on glucose metabolism under aerobic conditions would be that the sugar enters the cell normally at first, and since its catabolism is slightly inhibited by the lactone a somewhat increased level can be observed during the first 20 minutes. However, during this time the lactone is in some way modifying the membrane or cell-surface; by about 30 minutes its effect has become evident so that much less glucose now enters the cell, while that already present disappears; thus the balance of entry and utilisation is altered; breakdown exceeds entry, and the level falls below the "steady state" which persists in the absence of lactone. To summarise, the lactone acts by inhibiting to a small degree aerobic catabolism, and more markedly by inhibiting entry of fresh glucose through some action on the absorbing membrane. This latter effect would seem more readily explained if the uptake involves the participation of a membrane-bound enzyme (or if the membrane itself has enzymic properties - cf. muscle fibres); in this case alteration of the membrane structure would result in loss of enzyme activity and thus the loss of capacity to promote transport of metabolites. Finally in this connection there is the observation that under anaerobic conditions (Table IVa), there is slight but steady rise in the level of glucose in the presence of hexenolactone; this resembles the effect of iodoacetate but is much less marked.

It is unfortunate that in the present no distinction between intracellular galactose and glucose was possible, in the experiments in which both sugars supplied to the cells; in the absence of this information discussion of the figures in Table IV and IVa is unprofitable.

The experiments on phosphate uptake were less extensive, and no numbered strain of yeast was used. However the tests with commercial baker's yeast gave sufficient evidence that hexenolactone produced a small but consistent inhibition of phosphate uptake amounting to some 15% under the conditions used, ($5 \times 10^{-3}M$ lactone at 30° for 90 min.). This seems a somewhat small degree of inhibition, but it is not to be assumed that this alone represents the sum of the activity of the lactone - depletion of available phosphate within the cell may be but one contributory factor, although a significant one. The mechanism which causes the decreased uptake is not certain. A small ion such as phosphate might be expected to pass freely through a cell membrane without the intervention of any energy-yielding metabolic system but evidence is available to show that this simple view is not correct. It seems likely that the phosphate is incorporated into one or more of the glycolytic intermediates during its passage through the membrane; 2:3-diphosphoglycerate and ATP have been suggested. This has been demonstrated for various animal tissues e.g., erythrocytes (Gourley, 1952),

see-urchin eggs (Lindberg, 1950), and for micro-organisms (Kamen and Spiegelman, 1948; Wiame, 1949). Further, the process is affected by inhibitors, among which hexenolactone may now be included. Since the fundamental reactions involved in phosphate uptake seem to be based on surface-bound enzymes situated in the membranes, there is nothing to contradict the view that hexenolactone acts by modifying the nature or distribution of active centres on that surface.

As mentioned in the Introduction (p.19) no previous investigation has dealt with the effect of hexenolactone on mitochondrial systems, and for the reasons then stated this seemed a desirable line to follow. The stages in the mitochondrial-dependent tricarboxylic acid (TCA) cycle which were studied did not comprise the whole sequence, but were representative as including both simple oxidations (dehydrogenations) and oxidative decarboxylations. The results (Table XIV) show that the different stages vary in their sensitivity to the lactone, but that all are affected to some extent. The degree of inhibition varies from about 12% when succinate forms the substrate to over 27% in the case of fumarate, the lactone concentration being $1 \times 10^{-3}M$. It is of interest to note that the succinate system, which shows the greatest oxygen uptake, is least affected, while the relatively feeble α -ketoglutarate system (and still more so

the fumarate system) is most strongly inhibited. The succinate enzyme is well-known to be one of the "toughest" of the respiratory enzymes, possibly because it is more directly linked to the cytochrome system than the others of the TCA cycle. These results indicate that the oxidative systems based on mitochondrial activity are definitely sensitive to the action of hexenolactone.

It has long been known that malonate exerts a substrate-competitive inhibition of the succinate system, and Table XVII shows that the action of hexenolactone can be distinguished from that of malonate; it is in fact superimposed on the malonate inhibition, but not in a strictly additive sense. A point of interest is that the relative effect of the lactone in presence of malonate seems to increase with time; during the first five minutes of contact the additional inhibition amounts only to some 6% over the malonate level, but this rises steadily to about 10% in 30 minutes - again due possibly to a slow but continued action of the lactone on the mitochondrial surface or structure.

The experiments on the phosphate-induced swelling of mitochondria may be considered at this point, since they also indicate that hexenolactone affects the activity of the mitochondria. With fresh mitochondria, over a period

of 90 minutes, the swelling is quite appreciably reduced (lactone concentration $1 \times 10^{-3} M$) and the shape of the time/swelling curve resembles that of the cyanide-inhibition curves of Chappell and Greville (1960). The swelling is slightly increased in the presence of β -hydroxybutyrate (found by Chappell and Greville to be a very suitable oxidisable substrate for mitochondria) and in the presence of this substrates the lactone still suppresses the swelling. These results were only obtained when freshly prepared mitochondria were used; with mitochondria that had been stored overnight at 0° (called by Chappell and Greville "aged mitochondria") not even the addition of β -hydroxybutyrate permitted any detectable phosphate-induced swelling. Chappell and Greville have shown that mitochondrial swelling, however induced, is dependent upon the presence of an oxidisable substrate (either added, or, in the case of quite fresh mitochondria, endogenous) and upon the maintenance of activity in at least part of the electron-transport mechanism of mitochondria. The oxidisable substrate (which could be glucose, a member of the TCA cycle or β -hydroxybutyrate) would not be destroyed by the lactone, but the transport mechanism, being particle-bound, would be open to damage by any substance capable of disturbing the essential orientation of the particle-bound enzymes. Such damage would be manifested by an inhibition

both of oxidations and of induced swelling.

The picture of hexenolactone-induced inhibition which has been developed in this Discussion, and which is supported by the experiments herein described, seeks to show that the effect of the lactone is due to its capacity for disorganising those membranes through which metabolites must necessarily pass or that in those cases where the existence of a definite boundary membrane is in doubt (e.g. mitochondria) some similar disorganisation affects the orientation of the particle-bound enzymes. Recently this basis of inhibition has been suggested in the case of a number of other substances such as certain polyene antibiotics active against fungi and yeasts. Henis and Grossowicz (1959) have found that heptamycin completely and instantaneously inhibits the uptake of inorganic phosphate by Candida albicans, S. cerevisiae and other yeasts, although it is without action on the carbon dioxide output of cell-free extracts of yeast, or upon the esterification of inorganic phosphate by the extracts. These authors located the site of activity on the cell surface. In a later paper (1960) they extended their observations, and again noted the contrast between the inhibition of intact cells and insensitivity of cell-free extracts. They suggested that

the fungistatic activity of heptamycin was due to its preventing phosphate uptake. In this connection also, Marini, Arnow and Lampen (1961) have found evidence to indicate that another polyene antibiotic, nystatin, directly damages cell membranes causing irreversible changes. This view of the mode of action is based upon indirect observations, not upon any direct demonstration by physical or chemical means of a change in the nature of the cell-wall or membrane. However, some slight evidence that the lactone may cause some re-orientation of acidic or basic groups in a protein molecule may be found in the electrometric titration curves (pages 102 and 103) for egg albumin and urease. The change in iso-ionic point is in each case only small, but it may be significant. Egg albumin seems to be more affected than urease, but there is no reason to suppose that all proteins (or all enzymes, or all protein-containing membranes) are affected to the same degree. Indeed, the fact that the lactone shows differential inhibition argues against such an idea.

The property which first directed attention to hexenolactone is its capacity for differential inhibition of animal tissues, and in particular the fact that it has

little influence on the growth of the connective tissue.

If a purely chemical basis for inhibition is sought it would seem that one must consider a region of sensitivity elsewhere than in the metabolic pathways common to all types of tissue; for instance, glycolysis is a pathway common to nearly all types of cell and it is difficult to imagine that interference with it would have more far reaching results in one type of tissue than in another. It is therefore suggested that hexenolactone does not affect directly the metabolic pathways in the sense that any one step is inhibited in the way that, for instance, fluoride inhibits a single step in glycolysis. Instead, this work has sought to find an explanation for the physiological activity of hexenolactone in some influence which it has on cellular boundaries (cell-walls or membranes) or on some organs (if this word can be applied) which although perhaps not bounded by a definite membrane yet (as with mitochondria) depend ^{on} ~~for~~ their structure ^{for activity.}. Attention has perhaps been focussed upon the protein components of these systems since these are undoubtedly the seat of enzymic activity. It has been widely held that the passage of substances through a membrane, is an enzymic process and not merely one of physical diffusion. If hexenolactone does indeed act by modifying the protein part of a membrane, why does it not affect the protein of soluble enzymes such as those concerned in glycolysis? May it be because the

membrane proteins, be they enzymes or not, are in close association with non-protein material (e.g., polyol phosphate, polysaccharide, lipid) which maintains them in a state more sensitive to external influences ? It is possible to imagine a protein molecule held in a more or less "expanded" condition by a polyol phosphate, whereby its active groups would be as it were spread out on a surface, open to attack by physiologically active materials in the surrounding medium. If this view is valid, it might be rewarding to make a detailed comparison of the membranes present in epithelial and connective tissues.

As stated in the Introduction, the experiments on the growth of pea-stem sections were undertaken in order to complete the work of Richards and Mehta comparing the effects produced by hexenolactone with those of iodoacetate, arsenite and fluoride, as recorded by Thimann and his colleagues. It had been observed that the effects of the lactone resembled those of iodoacetate rather than of the other two inhibitors. Thus during the growth period in water or in auxin solution, soluble sugar in the tissue decreases, and a small proportion of the decrease is accounted for by fresh synthesis of cell-wall material; in the presence of any inhibitor the decrease is accentuated but the increase in polysaccharide is

relatively very slight. A difference between iodoacetate and lactone is that while the main sugar that appears in the surrounding medium is, with the former, fructose, with the latter it is only glucose. Another effect of the lactone, not seen with iodoacetate, is an enormous increase in respiration within a rather narrow range of concentrations of lactone; and further it seems from measurements of respiratory quotient that the normal transformation of fat to sugar is depressed by iodoacetate but not by hexenolactone.

As regards nitrogen metabolism, Christiansen and Thimann (1950) found that there was a very large decrease in the amount of free amino-acid in the stems during a 24-hour period in the absence of the inhibitors, and that this was accompanied by a rise in protein and in asparagine, in approximately equimolar amounts. Iodoacetate inhibited this change, but did not alter the general pattern thereof. Protein synthesis always ran closely parallel to growth, and Christiansen and Thimann observed that the conversion of amino-acid to equal amounts of protein and asparagine was an essential feature of growth ("critical for growth"). They also found that iodoacetate causes an appreciable exosmosis of nitrogenous material, including a high proportion of amino-acids, from the stems, and that this exosmosis was characteristic of iodoacetate, not being shown

by arsenite or fluoride. Variations in ammonia, glutamine and protein of the cell-wall were insignificant.

In the present work the most notable effect of hexenolactone was to cause a very large decrease in the non-protein nitrogen of pea stems (Table XXI). The loss amounted to about 33% of that initially present, compared with a loss of about 16% in a water control and a slightly greater amount in iodoacetate. Some of this loss was accounted for by protein synthesis but there was no indication of an equivalent production of asparagine as found by Christiansen and Thimann. The loss was in fact almost entirely due to exosmosis of amino acids. It is evident that hexenolactone and iodoacetate differ in some features of the type of effect they produce, and especially in the far greater exosmosis produced by the lactone. No evidence was obtained that protein synthesis is necessarily accompanied by an equivalent formation of asparagine as postulated by Christiansen and Thimann. It may be that the lactone causes so rapid and extensive a loss of amino acids that the asparagine forming mechanism cannot function to an appreciable degree. This fact seems also to be in agreement with the view already put forward that the effect of the lactone is in some way centred upon the membranes and boundary tissue of cells. It is clearly desirable that these structures should be more closely investigated.

S U M M A R Y .

1) Three methods for the synthesis of hexenolactone have been tested, and that of Haynes and Jones, based upon the condensation of propylene oxide and sodium acetylide is preferred.

2) The uptake of glucose, galactose and arabinose by yeast cells under aerobic and anaerobic conditions has been studied, using three strains of Saccharomyces, namely S. carlsbergensis nos. 74 and 355, S. cerevisiae no. 366. All three strains show the same type of response to hexenolactone, although no. 74 is most affected. Glucose uptake is inhibited, slightly during the first 20 minutes of contact, but more markedly on longer contact with the lactone. Uptake of galactose or arabinose is unaffected.

3) Uptake of inorganic phosphate by yeast cells is appreciably inhibited by hexenolactone.

4) Hexenolactone has been shown to reduce the respiration (oxygen uptake) of mitochondria freshly prepared from cauliflower. Six members of the tricarboxylic acid cycle were used as substrates for respiration, and all were affected; but the inhibition was most marked with the most weakly-respiring systems (α -ketoglutarate, fumarate) and least with the most vigorous (succinate).

5) Inhibition of the succinate system due to malonate was enhanced by hexenolactone, the degree of enhancement increasing with time of contact with the lactone.

6) The phosphate-induced swelling of fresh mitochondria in the presence of a respiration-substrate is inhibited by hexenolactone.

7) Hexenolactone has been shown to produce changes in the ionisation pattern of certain proteins, as shown by a shift in the iso-ionic point given by electrometric titration.

8) The nitrogen distribution of sections of pea-stem tissue exposed to hexenolactone has been studied. The most notable effect of the lactone is to cause a very considerable exosmosis of the amino-acids of the tissue.

9) Possible reasons for the observations made are discussed, and it is suggested that all are in agreement with the theory that the effect of hexenolactone is chiefly due to its influence on the permeability of tissue membranes. In this connection a comparison is made with certain fungistatic antibiotics.

APPENDIX

It was noticed that when hexenolactone, prepared by the method of Joly and Amiard (1947), was allowed to stand at low temperatures for a few days crystals of sorbic acid separated from the preparation. The lactone was more yellowish in colour than the pure sample. It was then thought that this yellow colouration might be due to the presence of sorbic acid, which, it was observed, was difficult to remove completely from the lactone preparation.

The inhibitory effect of this lactone on various tissues has been reported by Mehta and Richards (pea-stem) and by May (yeast). The fungistatic activity of sorbic acid has recently revealed by Salunkhe and Shinkawa. In order to see whether sorbic acid itself has any inhibitory effect on yeast, the following experiments were undertaken.

The effect of sorbic acid on the oxygen uptake by yeast.

The conventional Warburg Manometric Techniques were used to measure the oxygen uptake by yeast. Each manometer flask contained 0.2 ml. of 10% Potassium Hydroxide in the centre cup; 0.5 ml. of 0.5M glucose in the side arm; 1.0 ml. of yeast suspension containing yeast cells equivalent to 3 mg. of dry weight of cells, 0.8 ml. of 0.1M phosphate buffer and 0.5 ml. of $5 \times 10^{-3}M$ (final concentration)

sorbic acid solution were placed in the flask. The sorbic acid solution was replaced by an equal volume of the buffer solution in the control. All the solutions (except potassium hydroxide) were made in 0.1M phosphate buffer, pH 5.5.

All the flasks were then transferred to the Warburg bath maintained at 30° and equilibrated for 20 minutes while shaking at 115 strokes per minute. After equilibration all the taps were closed and the solution from the side arm was tipped into the flask and thoroughly mixed. Oxygen uptake was then measured at regular intervals of time. The results are summarised in Table A.

The effect of sorbic acid on the carbon dioxide production by yeast.

All the reactants used in the above experiment were employed here in the same quantities except that the potassium hydroxide solution in the centre cup was replaced by an equal amount of distilled water. The manometer readings were taken at regular time intervals and the volume of carbon dioxide was calculated. The results are summarised in Table B.

Table A.

Respiration of yeast: Effect of Sorbic Acid on the oxygen
uptake by yeast.

Each flask contains 3.0 ml. of liquid including 0.2 ml.
of 10% Potassium Hydroxide in the centre cup.

Substrate: 0.5 ml. of 0.05 M Glucose.

Figures represent μ l. of O_2 uptake by 3 mg. dry yeast cells.

Time (minutes)	Control	Sorbic Acid $5 \times 10^{-3} M$
5	3.5	2.0
10	8.5	4.5
15	15.0	9.5
20	23.0	16.5
25	34.0	25.5
30	47.0	33.0
35	61.5	41.5
45	90.0	57.5
55	115.5	74.0
65	141.0	89.0

Table B.

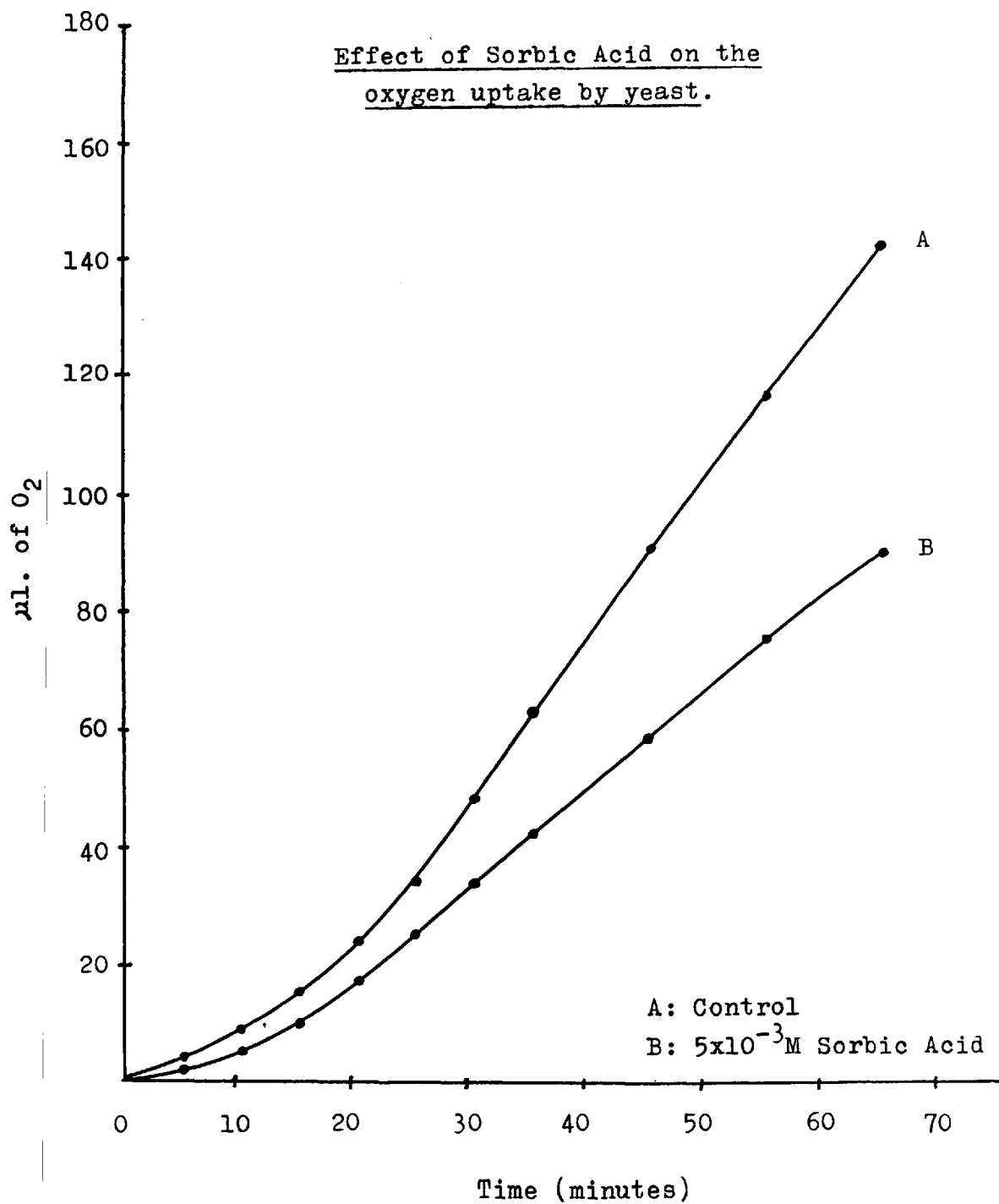
Fermentation by yeast: Effect of Sorbic Acid on the CO₂
production by yeast.

Each flask contains 3.0 ml. of liquid including 0.2 ml.
of distilled water in the centre cup.

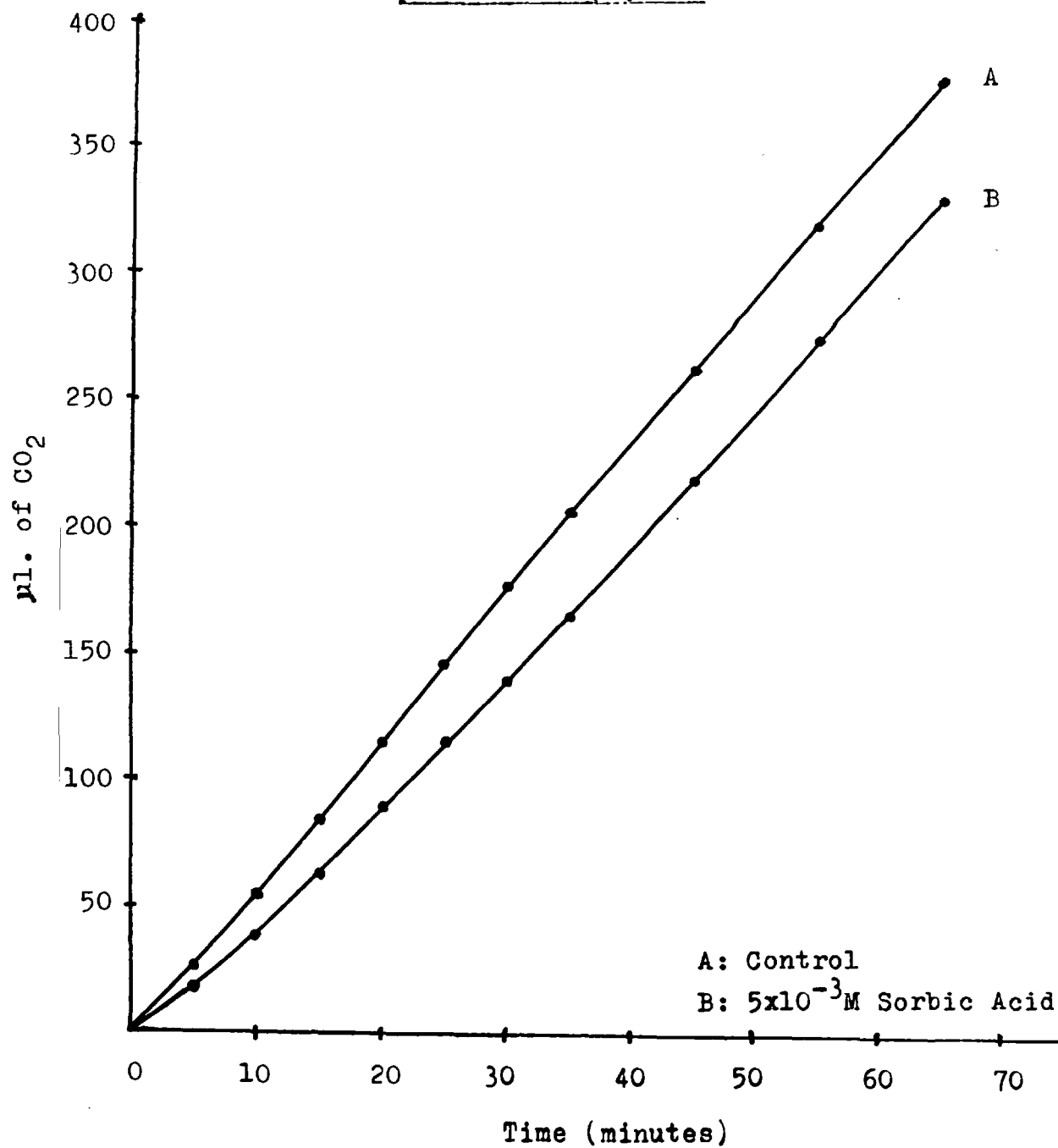
Substrate: 0.5 ml. of 0.05 M Glucose.

Figures represent μ l. of CO₂ produced per 3 mg. dry
yeast cells.

Time (minutes)	Control	Sorbic Acid $5 \times 10^{-3} M$
5	25.0	17.5
10	54.3	37.5
15	85.0	61.8
20	115.0	90.0
25	148.0	116.0
30	176.5	138.0
35	205.5	169.5
45	262.0	220.0
55	321.0	275.0
65	378.0	332.0



Effect of Sorbic Acid on CO₂
production by yeast.



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