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TRITIUM INCORPORATION FROM TRITIUM WATER AS AN INDEX OF METABOLISM

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This thesis describes the application of tritium incorporation from tritium water (THO) in two separate problems :

PART A deals with the problem of why seeds do not germinate at temperatures near $0^{\circ}C$.

PART B is a preliminary investigation of the metabolism of dry seeds, fungus spores and pollen.

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PART A

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PART A

THE METABOLISM OF IMBIBED SEEDS AT SUB-GERMINATION TEMPERATURES.

INTRODUCTION

THE PROBLEM

In part A of this thesis, the aim has been to answer the question: "Why do seeds not germinate at low temperatures?"

More fully, the question could be framed "What metabolic aberration(s) prevents seeds from germinating under conditions which are suitable for germination except that the temperature is too low?"

Chemists are all familiar with the rule of thumb that reaction rates halve for every 10° drop in temperature. Seed germination is an extreme exception to that rule. Seeds will germinate in a few days at 20° but at 0° or 5° most species fail to germinate even after many weeks.

Two plausible types of explanation might be hazarded:-

(a) one or more specific reactions, essential for germination, fail to begin. (This could be owing to exceptionally high energy of activation, or to inactivation of enzymes by low temperatures, a not unknown phenomenon.[3])

(b) all essential reactions begin, but "get out of step", i.e. run at relative rates which are unsuitable.

Of these, (b) may seem more likely to many chemists. It is very easy to imagine that some reactions would be slowed more than others over a given temperature range (about 20°), as postulated in (b). It seems less likely that one or more would be stopped.

Importance of the Problem

75% of the food eaten directly by man, on a dry weight basis, is seeds.[4] Aside from that and other plant products eaten directly, we depend of course on plants as the indirect source of meat and fish. Any work which might extend further north and south the cultivation of plants is therefore potentially important in our hunger-stricken world. It was conceivable that this thesis might point to means of germinating seeds in parts of the world where the cold had hitherto prevented germination.

The main reason for the work was, however, the intrinsic biochemical interest of the problem stated above. It was considered worthwhile to examine whether (a) or (b) or some other explanation applied to the phenomenon of seeds' not germinating at low temperatures.

What Is a Seed?

It would be inappropriate to give here a detailed discussion on the botany of seeds. On the other hand, some chemists may not know what a seed is, i.e. what is its place in the life history of a plant.

The seed, which is really an embryonic plant, is the diagnostic mark of the phylum Spermatophyta, which may roughly be said to consist of the coniferous plants (gymnosperms) and the flowering, fruit-bearing plants (angiosperms). The embryonic plant is produced by fusion of the male and female gametes, both of which are haploid (i.e. their nuclei contain only n chromosomes, n being a

constant for a given species). The zygote produced by this fusion is diploid (chromosome number = 2n) and divides and differentiates to give the seed, which when shed by the parent plant contains, in most kinds of seed-plant, the rudiments of a root and stem, as well as one or more special tissues containing stored food.

Oil, starch and protein serve as the main foods stored in different species. The chemical contents of seeds will not be reviewed here, because that subject has been reviewed elsewhere (e.g. refs. 1, 16) and it provides very little help in the problem under consideration here. A seed, like an adult plant, contains very many compounds: so many that a catalogue of them will not suggest confident predictions as to which reactions will occur in early germination.

Terminology in the subject of Germination

The most useful definition of the word "germination" is:[8] "that group of processes which causes the sudden transformation of the dry seed into the young seedling".

Evenari, leader of an active group in germination research, defines the word in this rather similar way:[9] "Germination is those processes, starting with imbibition and ending with protrusion of the root, which take place inside the seed and prepare the embryo for normal growth." Evenari's group has worked mainly on lettuce seeds, from which radicles protrude 15-18 hr. after the seed is wetted, at 20-25°.[9] He divides their germination into five phases:-

I Imbibition 0-3 hr. As the name implies, in this phase the seed imbibes water (and usually swells).

II Activation 3-13 hr. Apart from its place between phases I and III, this phase is poorly defined. It is described as "setting the trigger" for growth. This vague wording would not, one feels, satisfy many biochemists as a definition. The term 'activation' has been used in connection with germination of fungus spores,[10] referring only to the initial stages of germination, which break dormancy.

III Mitosis 13-15 hr. This phase is the start of rapid cell division, a process which of course extends into later phases.

IV Protrusion 15-18 hr.

V Growth 18 hr. onwards. (Reference to Evenari's definition of germination shows that, on that definition, phase V is not part of germination at all.)

PREVIOUS WORK

On the question posed above, very little work has been reported. In oats, barley and rye, the higher the sucrose content of the grain the greater the resulting seedling's vigour and the lower the minimum temperature for germination.[5] Similarly, cold hardiness of three varieties of winter wheat was positively correlated with 4-aminobutyric acid content of the grain.[6] Addition of 4-aminobutyric acid to the germination medium greatly increased the germination % age of wheat at low temperatures, but did not

lower the minimum temperature below which no grains would germinate.[6] Swollen corn seeds kept at 0° for 12 days conducted proteolysis,[7] as judged by the decrease during that period of the ratio protein N/non-protein N from 3.5 to 1.2 in the germ, and from 2.6 to 1.4 in the endosperm. The concentration of amino N nearly doubled during the 12 days at 0°. Cucumber and spinach seeds formed more riboflavin during germination slowed by low temperature than they did in normal germination at room temperature.

Stumpf[153] soaked peas in water at 2° for 12 hr. and then homogenised them in acetone at 0° for 10 min. The resulting powder contained water-soluble enzymes capable of converting fructose diphosphate to acetaldehyde.

On the subject of the chemistry of seed germination at normal temperatures, the writer has read, in abstract and/or original, hundreds of papers. It is proposed to mention here only those which seem relevant to the problem dealt with in part A of this thesis. For one or more of the reasons which will be given immediately, a very high proportion of the literature on seed germination is irrelevant to this problem.

Firstly, the writer accepts the definitions of germination quoted above, and therefore rejected, as irrelevant to the present purpose, those papers in which the first measurement reported on the seeds was after protrusion. An example typical of many was Tang's[11] paper, entitled "Temperature characteristics for the pro-

duction of carbon dioxide by germinating seeds of Lupinus albus and Zea mays."[11] Despite this promising title, the work began by germinating the seeds until the radicles and hypocotyls were showing. The measurements then made on production of carbon dioxide, whatever their value otherwise, were clearly not on germinating seeds (but on young seedlings). Dozens of papers, some by well-known biochemists, purport to be on germinating seeds but, like Tang's, are irrelevant for the present purpose.

Again, many workers have analysed seeds at intervals during germination, avoiding the criticism just made, but have used the time-scale 1, 2, 4,... days. Now the results of the writer, presented later in this thesis, show that mustard seeds wet at 0° are conducting fewer reactions after several months than they are after 3-4 hr. at room temperature. Therefore experiments in which the first measurement is one day after wetting the seeds give results for a much more advanced stage of germination than this thesis deals with. It is, admittedly, possible that a result after one or two days of germination might give information on the much earlier stages. This is very unlikely, however. Furthermore, there is absolutely no way of telling whether a result gained after 24 or 48 hr. of germination would have been gained after 3-4 hr. Many scores of papers on germination are thus of very uncertain relevance to this thesis.

The literature on seed germination will be reviewed here, classified according to the experimental approach. Under each kind

of experiment will be discussed (a) those papers of likely relevance to the present problem (b) a few of the papers which, for one or other of the above reasons, are most probably irrelevant.

1. Nutrition studies on germinating embryos

It is the embryo (often a small fraction of a seed's bulk) which grows into an adult plant. Therefore several experiments have studied the nutritional requirements of excised embryos. This method of studying germination has not proved to be well suited to discovering the reactions which are important early in germination. Conclusions are of the form "Substance X is/is not (strike out whichever does not apply) required for growth of excised embryos of species Y". It can be seen, therefore, that information on the early reactions of germination is unlikely to accrue from this method, because, since growth or non-growth is what is observed, the early stages of germination are not being directly studied; and existence or absence of a requirement for substance X will not usually tell us much about intermediary metabolism. A further drawback is the slight possibility of seriously perturbing the embryo by excising it.

It is hardly surprising, then, that this method has provided no papers of apparent relevance to the present problem:

1(a) No papers

1(b) Mature rice embryos were excised and grown on agar to which had been added a particular sugar. The embryos grew well on sucrose, D-glucose, D-fructose, and maltose; but D-mannose

and D-galactose were little or no use.[12] Very immature barley embryos matured normally if supplied with, as well as sugar and minerals, a vitamin-free hydrolysate of casein.[53] This was interpreted as showing that amino-acids are important in early germination.

Although strictly it may belong in a category of its own, we mention here the fact that seeds with low vitamin E content germinate poorly.[14]

2. Measurements of concentrations of metabolites at successive stages of germination.

This has been one of the most widely applied methods. It suffers from these disadvantages:-

(i) it has usually proved too insensitive to detect the very early reactions in germination.

(ii) A negative result is inconclusive. This has not been realised by some workers. For instance, Evenari[9] found that lettuce seeds have a high fat content but this is "untouched" in germination, as are the 14 free amino-acids present in these seeds. The conclusion that the whole amino-acid metabolism was dormant until after protrusion was based on the experimental result that the concentrations of the free amino-acids were constant until after protrusion. It seems to have been overlooked by Evenari that constant concentration of a metabolite does not prove that it is not being metabolised; the alternative and often more likely explanation is that the metabolite is being formed and used

up at the same rate. An amino-acid might well be supplied by proteolysis and converted to, say, the corresponding α-oxo-acid, the two reactions proceeding at the same rate. On the other hand, if the concentration (on a dry weight basis) of a metabolite changes during germination, the unambiguous conclusion is reached that this metabolite has undergone chemical reaction.

2(a)

The Evenari group[9] measured the concentrations of fat, free amino-acids and sucrose in lettuce seeds germinating at 26°. They concluded that sucrose was the sole respiratory energy source. The ambiguity of the results on fat and amino-acids has been discussed in the last paragraph.

The concentration of 4-aminobutyric acid in Phaseolus radiatus seeds rose in the first six hours of germination.[15]

2(b)

In their well-known book, Crocker and Barton[16] collate many experiments on changes of chemical composition of germinating seeds, but the time scales are always far too coarse for the present purposes, e.g. the first measurement is after one or two days. Dozens more of this kind of experiment have been reported in the literature since that book. Many are reviewed in a more recent book on germination of seeds.[152] A few examples will be given here to show the sort of results gained after a day or two of germination.

Riboflavin concentration increased greatly in each of several species; nicotinic acid increased in some; biotin rose in

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some but fell in others; and aneurin showed no significant change. Embryos from barley which had been soaked overnight showed (very faint) absorption bands attributable to cytochromes a, b and c (ref. 157).

Much malic acid is synthesized during the sprouting of cereals and legumes.[169]

After 24 hr. on wet sand, barley grains no longer contain raffinose or sucrose, both of which had been present in the dry grain. Sucrose, but not raffinose, reappears after soaking for longer periods.[170]

3. Measurements of enzyme activities at successive stages of germination.

This method suffers from the drawbacks inherent in all enzymatic studies on cell-free extracts. These disadvantages are briefly discussed elsewhere in this introduction, in the explaination of the advantages of the method used in this thesis.

The other drawback is that this method has not proved sensitive enough to detect the very early reactions in germination.

3(a)

A thorough study of glycolytic enzymes in an aqueous extract of powdered peas showed that the Embden-Meyerhof pathway was the sole pathway of glucose phosphate breakdown.[154]

Lettuce seeds were extracted with phosphate buffer at 2°.[171] The supernatant after centrifugation was found, by conventional methods involving incubations of 30 min. or more, to contain

NADP-dependent glucose 6-phosphate dehydrogenase, and NAD-dependent alcohol dehydrogenase.

During the first 12 hr. of germination of seeds,[172] peptidase activity remained at a very low figure in the hull and endosperm, but increased greatly in the embryo and scutellum.

The first 24 hr. of germination of immature soybeans saw[173] an increase in the activity of a higher fatty acid dehydrogenase, acting on 9 common fatty acids and using either of NAD or NADP.

3(b)

This is a large category. 24 hr. or longer after wetting, various seeds have been found to contain very many different enzymes. Just a few will be mentioned here.

CoA-containing mitochondrial preparations from soybeans in an advanced stage of germination condensed oxaloacetate and acetate to give citrate.[174] Also, malate or oxaloacetate gave citrate from pyruvate. When oxidising a member of the Krebs cycle in the presence of one of the three amino-acids alanine, aspartic acid and glutamic acid, the mitochondria produced the other two of that trio. 4-aminobutyric acid was formed from glutamic acid.

Transamination has been observed after 24 hr. or longer in several species.[175-7] In wheat germinated 24 hr.,[178] dehydrogenases of citrate and malate were found to be very active in the germ; and of succinate and glutamate, in the endosperm.

Strong evidence for the glyoxylate cycle has resulted from several workers[179-182] but in all cases the seeds used had protruded rootlets i.e. germination was really complete.

4. Action of inhibitors

Inhibitors have been exceedingly useful in classical work on enzymes in cell-free extracts. Formulation of many metabolic pathways has rested largely on the detection of intermediate compounds when they "pile up" owing to the specific inhibition of enzymes.

For the study of the early reactions in seed germination, however, inhibitors have been of little use. Firstly, there is an ambiguity about negative results. Spedding[1] found all the normal early reactions in mustard seeds germinating in 10^{-5} M azide. One possible explanation is that a substance so foreign as azide would not be even appreciably absorbed into the cells of a whole organism. (This ambiguity does not apply, of course, in the interpretation of experiments with inhibitors in cell-free extracts.) Secondly, there is the serious drawback, already mentioned under method 1, that when growth or non-growth is the observed result, deductions about the early reactions of germination will probably not be possible.

4(b)

The embryos from wheat which had been soaked for 24 hr. yielded[183] cytochrome oxidase, which was inhibited by cyanide, ni-

trate and carbon monoxide, all of which also inhibit the respiration of whole embryos.

Carbon dioxide inhibits germination of seeds of pine,[184] Brassica alba,[185] and 10 other species.[186] When the partial pressure of oxygen is as in air, high partial pressures (20-30%) of CO_2 are needed to inhibit germination.[186]

Cress and mustard seeds moistened with dilute solutions of salts of lead, copper, zinc, beryllium or thallium germinate slowly or not at all.[187] Seeds germinate 0% in soil containing 10 ppm 2, 4-dichlorophenoxyacetate.[188]

Lettuce seeds can germinate, though only slowly, in an atmosphere 80% N_2, 15% CO_2, 5% O_2 (ref. 189). 13 common species can germinate in partial pressures of O_2 below 5% (ref. 190). In one of these species, [illegible] germination rate increased to near normal on addition of ATP.[190] Some seeds even germinate under pure nitrogen or argon![191]

5. Measurements of Respiratory Quotient (R. Q.)

This method has the virtue of studying the whole, unperturbed organism. A value of 1.0 for the R.Q. of a tissue is usually taken to mean that complete oxidation of carbohydrate is the sole cause of oxygen uptake and carbon dioxide output. However, the value 1.0 for the R.Q. of a seed could conceivably arise as a "weighted average" of other types of metabolism, some of which would, if proceeding alone, give a value below 1.0, but others of which would

give a value above 1.0. If these proceeded at appropriate rates they could "balance out" to a net value R.Q. = 1.0.

The main drawback of R.Q. for the present purposes is that even if one could conclude from it that, say, glucose was being oxidised to carbon dioxide, one could not tell by what pathway the oxidation was being effected.

5(a)

R.Q. values have been measured within a few hours of wetting seeds.[195-7] The conclusions have been limited by the considerations mentioned immediately above.

5(b)

A few values exist of R.Q. for seeds in very advanced stages of germination (e.g. ref. 198).

6. Use of Isotopes

Experiments with isotopes have great potential for providing the sensitivity in early germination which most other methods lack. In view of that potential, it is surprising how few relevant results are in the literature. One prospective difficulty is introducing the appropriate labelled substance into the cells of the whole seed.

6(a)

Possibly the most important paper in the literature on the early reactions of seed germination (overlooked by Spedding[1] in his review[112] of the subject) is by Haber and Tolbert. They intro-

duced isotopes into lettuce seeds under conditions which lead to protrusion of the radicles 15-18 hr. after the seeds were moistened. The aim was to study metabolic reactions in the first three of Evenari's five stages (see the start of this introduction). Imbibition, Evenari's stage I, was complete in 4 hr., or if the seeds were punctured, 15-30 min. From $H^{14}CO_3^-$, ^{14}C was incorporated into soluble compounds within an hour (unpunctured seeds). After 90 min. compounds labelled were alanine, glycine, glutamic acid, glutamine, aspartic acid, asparagine, serine; malic, citric, succinic, fumaric and glyceric acids. By the end of imbibition, 1/3 of the total fixed ^{14}C was in malic acid. From $H_2{}^{32}PO_4^-$, whole seeds incorporated ^{32}P into only ethanol 1-phosphate, up to protrusion; and that result was considered by the authors to be probably an artifact created in extraction. From protrusion onward, many phosphates became labelled, as in adult plants. Punctured seeds, however, labelled phospholipids and phosphorylcholine with ^{32}P within 3 hr. of moistening. Before protrusion, intact seeds incorporated no ^{35}S from carrier-free labelled sulphate; but punctured seeds incorporated ^{35}S within 3 hr. into many compounds, e.g. cysteine and methionine. The difference between whole and punctured seeds was thought to be due to poor permeability of the seed coats to phosphate and sulphate. If that explanation is correct, which seems highly likely, it constitutes an example of the difficulty mentioned in the previous paragraph.

No such difficulty can arise in the use of this thesis' method to study germination, if the assumption is granted that there is no serious discrimination against the absorption of $^{3}HO^{1}H$ molecules during imbibition of THO. The highly important results of Spedding[1] on germinating mustard seeds lead to conclusions in accord with those of Haber and Tolbert, namely, transaminations and reactions of the Krebs cycle seem important very early in germination. In summary, Spedding found that metabolism of the amino-acids 4-aminobutyric acid, aspartic acid, alanine and glutamic acid occurred within 10 min. of wetting mustard seeds with THO at room temperature. Within 30 min. of wetting, the seeds were also metabolising citric, malic and succinic acids. Soon these were joined by fructose and what were tentatively reported as phosphates[1] but have since (Spedding, pers. comm.) proved to be amino-acids. Lipids were being metabolised within 3 hr.

Other unidentified labelled compounds appeared later in the germination process.

6(b)

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In the first 72 hr. of germination, Phaseolus radiatus seeds metabolised exogenous, uniformly-labelled glutamate -^{14}C mainly to $^{14}CO_2$. Probably this was achieved by passing glutamate into the Krebs cycle as α-oxoglutarate. Similar experiments with labelled glucose gave labelled aspartate and glutamate.

Radioactive wheat seeds, obtained by injecting acetate -^{14}C into the stems of the parent plants, were germinated and the fate

of the ^{14}C was observed.[194] Unfortunately, the time scale 1, 2, 3,... days was used, so McConnell can hardly be said to have studied "the onset of germination", as he claimed. Much of the ^{14}C appeared as $^{14}CO_2$. Several results "indicated that glutamic acid was reutilized, after re-entry into the Krebs cycle, for the biosynthesis of seedling protein." McConnell's method is one answer to the difficulty of getting isotopes into seeds.

PRINCIPLES OF THE METHOD USED IN THIS PROJECT

Tritium water (THO) is introduced into a living organism. It is confidently expected to penetrate into all cells. After a suitable period of time, the organism is killed and analysed to find which compounds, if any, have become labelled with tritium.

Three kinds of label can result:

1. Instantly exchangeable ("labile") labels. Hydrogen atoms of -OH, -SH, $-NH_2$ and $-CO_2H$ groups are ionizable and equilibrate with the hydrogen of intracellular THO within a small fraction of a second, to give -OT, -ST, $-NT_2$ and $-CO_2T$ respectively.

2. Slowly exchangeable ("semi-labile") labels. Tritium bonded to a carbon atom adjacent to an enolisable carbonyl group is slowly exchangeable with the hydrogen of water.

$$R'\text{-}\underset{\underset{T}{|}}{CH}\text{-}\underset{\underset{O}{\|}}{C}\text{-}R \quad \overset{\text{(slow)}}{\rightleftharpoons} \quad R'\text{-}CH = \underset{\underset{OT}{|}}{C}\text{-}R \quad \overset{\text{(fast)}}{\rightleftharpoons} \quad R'\text{-}CH = \underset{\underset{OH}{|}}{C}\text{-}R$$

3. Non-exchangeable ("stable") labels. These are C-T labels not of type 2.

The bracketed nomenclature seems inferior in the opinion of some, because the term "stable tritium atom" is objected to.

Labels of types 1 and 2 are removed in the extraction and subsequent treatment of the killed organism. More precisely, one

should say that labels of types 1 and 2 are reduced to specific activities far below the detectable minimum. This is achieved by exchange with relatively huge excesses of instantly exchangeable hydrogen in the form of non-tritiated solvents, e.g. ordinary water, ethanol etc. (see "Methods", below, for experimental details).

Now the crux of the method is that non-exchangeable incorporation of tritium from THO can occur solely as a result of metabolic activity. Any compounds still tritiated after removal of labels of types 1 and 2 must have become labelled in metabolic reactions.

In many biochemical reactions hydrogen from water becomes non-exchangeably attached to carbon atoms of metabolites. Thus tritium incorporation can be used as an index of metabolism.

The writer has surveyed the biochemical literature[20,52,81] and listed most known biochemical reactions in these categories:

A. Reactions in which non-exchangeable incorporation of tritium from THO is expected to occur.

B. Reactions which are not expected to entail non-exchangeable incorporation of tritium.

C. Reactions of which the mechanisms are not well enough known for a confident prediction to be made as to whether tritium would be non-exchangeably incorporated. In most of these cases, conducting the reaction in THO would probably be a valuable means of gaining information on the mechanism.

The lists of reactions appear below. Meantime, suffice it to say that non-exchangeable labelling of metabolites is expected in the Embden-Meyerhof sequence, the Krebs cycle, the hexose monophosphate shunt, the fatty acid spiral, mevalonic acid biosynthesis, pyrimidine biosynthesis, transamination, and many other important metabolic pathways.

Advantages of this Method

1. Metabolism is studied in vivo

Biochemists have become so used to inferring biochemical pathways from enzymological studies on cell-free extracts that many of them would now regard almost suspiciously any studies of intermediary metabolism not performed on purified enzyme preparations.

However, it should be remembered that the goal in studying intermediary metabolism is, by definition, the discovery of the reaction pathways in the living cell. It does not seem too extreme, therefore, to say that studies on cell-free extracts are in a sense makeshift, indirect ways of inferring what reactions occur in living cells. Such indirect approaches are open to dangers. For instance, consider the astonishingly high catalase activity of many cell-free extracts. Does it not seem incredible

that living cells decompose hydrogen peroxide at anything like corresponding rates? Some plant extracts show polyphenol oxidase activity[99] far beyond credible figures for the intact cells. Liver, kidney, moulds and bacteria have yielded active D-amino-acid oxidases[17]; yet it seems very doubtful whether oxidation of D-amino-acids occurs naturally in liver or kidney, mainly because these organs probably never contain any D-amino acids! It seems relevant to quote some remarks on spores[18]:

"In our studies with fungus spores, we have found the intact, viable spores to possess fairly high enzymatic activity - the enzymes we have worked with being invertase, an atypical ascorbic acid oxidase, and a sulfhydryl oxidase. The activity of these enzymes is much higher than could possibly be necessary for metabolic requirements of the cell. Furthermore, we can completely inactivate these enzymes without impairing the metabolic activity of the cell. Thus, it is quite possible that there are enzymatically active proteins in spores and possibly vegetative cells too, that have no particular catalytic function essential to the metabolism of the cell. The enzymes I have mentioned are apparently localized on the external surface of the plasma membrane of the spore. Their primary function may thus be as structural proteins in the membrane".

Further, it may be pointed out that the very making of a cell-free extract destroys to a large extent the spatial

organisation, which is important for the metabolism *in vivo*. Chemicals are mixed which had been kept separate in the living cell.

The writer does not desire, let alone undertake, to demolish the whole concept of using cell-free extracts in metabolic studies. It is, however, insisted that whenever possible the living cell (or even better, the whole living organism) should be studied. Historically, the use of other less direct and less conclusive approaches arose largely through necessity. The advent of isotopic tracers provided a most welcome means for studying metabolism *in vivo*. Isotopes have been used to discover "new" pathways, such as the path of carbon in photosynthesis[19], and to reinforce previous knowledge (e.g. on the existence *in vivo* of the Krebs cycle[93]).

2. *It is applicable to some biochemical problems on which other methods have not made (and probably could not make) much progress.*

For instance, if one is asking "What reactions occur in spores or seeds within 10 minutes of wetting them?" it is of little use to begin by extracting these propagules with aqueous solutions and then test the extracts for enzymic activities. However, these problems have been attacked with marked success[1,2] by the method used in this thesis.

Similarly, questions on the metabolism (if any) of resting seeds, spores and pollen seem hard to tackle because one is precluded from wetting these propagules (for they would then be no longer resting but germinating). Part B of this thesis describes an encouraging preliminary study of a new method in this field.

3. The experiments are essentially cheap and simple

A great deal of information on the metabolic pathways in a hitherto unexamined species should be available for a relatively low outlay of money and man-hours.

4. Being a radiochemical method it is fairly sensitive

At the start of part B is presented, with comments, a set of assumptions on which to calculate the sensitivity of the method. The conclusion is that only 10^{-9} mole of a metabolite would have to react before it became labelled to an extent detectable by the method (described under "Methods", in part A). This figure of 10^{-9} mole has an uncertainty of several orders either way, depending on the validity of the assumptions. The reader is referred to the start of part B for details on this.

Forerunners of the Method used in this thesis

Quite soon after deuterium was discovered, Schoenheimer[57] fed D_2O to mice and discovered that hydrogen from body water is incorporated into fats. Also he concluded that steroid biosynthesis began from small molecules.

Over a period of 13 days, mice injected with a total of 1.2 millicuries of THO bound organically 0.5-1% of the tritium. The biological half-life was as brief as one day[58].

Phaseolus vulgaris (red kidney bean) plants of which the nutrient solution contained THO (100 μcuries/ml.) showed a plateau in decrease of tritium activity in the nutrient solution, after c. 12 hr. Bound tritium took c. 60 hr. to reach equilibrium[59].

THO vapour was supplied to soybean leaves and shown to be in relatively rapid equilibrium with the non-exchangeable hydrogen fixed on photosynthesised sugars[60].

Liver slices incorporated[61] tritium into fatty acids, mainly at odd-numbered carbon atoms, from a solution of $KHCO_3$ and glucose in THO.

The first applications of tritium incorporation as a means of surveying most of an organism's metabolism at once were by Spedding[1] and Edwards[2]. On the germination of seeds and fungus spores they made many discoveries, particularly important in that measurements were able to be made very early in germination - within a few minutes

of wetting the dry seeds or spores. Few previous methods had afforded results so early in the germination process. Neither worker, however, made a systematic survey of known metabolic reactions and pathways for the purpose of predicting which metabolites would, would not, and might become labelled. Their surveys were not extensive, and contained several mistakes. For instance, Spedding predicted that oxidative deamination of amino-acids would not give rise to labelled metabolites; and Edwards concluded that pyruvate would become labelled in the glycolytic sequence (whereas it most likely would not be found to be labelled by the methods used, owing to keto-enol tautomerism, which will exchange out the hydrogens of the methyl group). It is too much to hope that the survey presented here is quite free from errors or omissions. However, it does represent the first exhaustive survey of this kind, and can be fairly claimed to be a considerable advance.

Apart from experiments using D_2O or THO for *in vivo* experiments, isotopic hydrogen has been useful in studying the mechanisms of enzymatic reactions *in vitro*. A complete review is not required here, but only a mention of some results relevant to this thesis. The mechanisms of several reactions in the citric acid cycle, glycolytic (Embden-Meyerhof) sequence, hexose monophosphate shunt, squalene biosynthesis via mevalonate, and some other pathways, have been illuminated by experiments with isotopic hydrogen.

These are described in the discussions of pathways, immediately after the lists of enzymatic reactions. A common type of application has been the determination of the proximal source of bound hydrogen. Water, $NAD(P)H_2$, and intramolecular hydrogen shifts have all in different cases proved to be the source of hydrogen newly bound to carbon. Some fascinating stereochemical aspects of enzymes (e.g. aldolase[43], fumarate hydratase[26], etc.) have been revealed. Measurements of isotope effects have not yet been much use; in those cases (e.g. refs. 49, 62) where measurements have been made, few deductions about mechanisms have been possible.

The Isotope Effect

Spedding[1] stated, without much supporting evidence, the opinion that isotope effects were very important in the interpretation of tritium incorporation from THO. That was in his introduction. He never again[1] mentioned isotope effects. Edwards[2] concluded from several papers that the isotope effect "may be considerable", but that it will not cause any drastic changes in mechanism. She expected the germination of spores in THO would be "the same" as in ordinary water.

The following literature review, while perhaps not exhaustive, is more extensive than those by Spedding and Edwards. It points to a conclusion which is contradictory to Spedding's but similar to Edwards', though rather more cautiously framed than hers.

The isotopes of hydrogen differ more widely in atomic weight than those of any other element. A key assumption in the use of THO in this thesis is that the path of tritium can be used to infer the path of protium. At worst, isotope effects could conceivably invalidate this assumption. The literature on hydrogen isotope effects *in vivo* gives little ground for fear, however. Most experiments have used deuterium; often as almost pure D_2O. In several such cases (see below) important isotope effects have occurred. However, in the THO added to organisms for this thesis, only about one in 300 of the hydrogen atoms is a tritium atom. (This was calculated from the specific activity, 5 C/ml. Thanks are due to R. J. Furkert for an independent calculation giving the same result, 0.3 atom % tritium). For comparison, the hydrogen in natural water is 0.02 atom % deuterium[63].

A basis for the theoretical explanation and prediction of deuterium isotope effects[64] is the difference in zero-point energy between a bond to deuterium and the corresponding bond to protium. However, theory is not yet adequate to predict values of isotope effects for enzymatic reactions in general. It is useful[65] to distinguish between the primary and secondary isotope effects. In the latter, the isotope is not involved at the reaction site. An example would be the decarboxylation, in ordinary water, of phenylalanine labelled with tritium at the *para* position of the

benzene ring. Secondary isotope effects are most unlikely to be of magnitudes appreciable for our present purpose. On the other hand, primary isotope effects could be, with the hydrogen isotopes especially, of considerable magnitude. For instance, a theoretical limit at 298°K predicted for the isotope effect in breaking O-H bonds, is[64] $k_H/k_D = 10.6$ (where k = specific rate). Tritium primary isotope effects could conceivably, on some theoretical predictions[65], be as serious as $k_H/k_T = 100$.

In the absence, however, of any adequate theory predicting tritium isotope effects in vivo, we must turn to experimental values. G. N. Lewis[66] discovered that tobacco seeds germinated only slowly in 50% D_2O, and not at all in 100% D_2O. Germination of wheat[67] was greatly delayed in 100% D_2O, but did at length occur. Seeds soaked in 25% or 40% D_2O for 25 hr. germinated but slowly. The effect was more marked with 40% D_2O, but in neither case was any lasting effect on structure or enzyme activity observed[68]. Two varieties of Pisum sativum seeds germinated in D_2O up to 40%, but above 50% D_2O not at all[69]. A possible slight inhibition of germination of Lupinus seeds in 1:2000 deuterium solution was alleged[70]. D_2O did not stimulate seed germination at low temperatures[69].

Nutrient solutions for two cultures, (a) and (b) of a mould, Aspergillus sp., were made up with (a) double-distilled water, (b) 0.47 mole % D_2O. At harvest after equal times, the felts of colony (a) weighed 16 times those of colony (b)[71]. However, 0.46%

D_2O had the same effect as distilled water (i.e. 0.02% D_2O) on the growth of Aspergillus niger, the percentage germination of Erysiphe graminis tritici (a powdery mildew) conidia, the growth of wheat roots and the respiration rate of wheat seedlings[103]. Male mice supplied with 30% D_2O become incapable of effecting fertile matings[104]. A possible explanation is based on alteration of properties of DNA. Similar conclusions have been pointed to by other work[105,106]. Possibly the hydrogen bonding in the DNA macromolecules is significantly different from normal when a high percentage of isotopic hydrogen is present. We may confidently expect no such aberrations when only 0.3% of the hydrogen atoms are tritium.

Pollen of Narcissus papyraceus germinated well in 18% or 57% D_2O[72].

Algae growing rapidly in a medium containing 23.5 mole % D_2O and 1 mC/ml. THO, and no other hydrogen source than water, incorporated deuterium at half the rate for protium, and tritium at 90% the rate for deuterium[73]. This agreed well with the theoretical prediction of Eyring and Sherman[74] that the maximal deuterium isotope effect (k_D/k_H), at ordinary temperatures, would be $\frac{1}{2}$. More isotope effect occurred[73] in the biosynthesis of lipid than of protein, nucleic acid or starch.

The body water of nursing rats was labelled[63] with 2.5% D_2O and 1 μC/ml. THO. Similarly labelled drinking water was supplied

for two weeks. The mothers and pups were then killed and analysed, revealing that the ratio of atoms T/D in the fats of the mammary gland was 80% of the T/D ratio in the body water. However, other workers[75] found no significant isotope effect in the body water, liver, pelt or residual carcass of rats which had drunk water containing 98.5 mole % D_2O and 0.50 mC (i.e. 2×10^{-5} atom % of T) THO. The rats had been fed this water for five weeks but ordinary water after that for 60 days until killing. The T/D ratio remained constant in all tissues after incorporation.

Pseudomonas sp. (a unicellular marine animal) gives off hydrogen gas, containing only about $\frac{1}{5}$ the deuterium concentration (atom %) of the water it lives in[76]. Hydrogen-adapted Scenedesmus shows an isotope effect (k_H/k_T) of 0.6 in incorporating hydrogen from molecular hydrogen gas[62].

The maximum rate (initial rate with high substrate concentration) of the condensation reaction which feeds acetyl groups into the citric acid cycle is 1.4 for acetyl-CoA compared with 1.0 for acetyl-CoA deuterated in the acetyl group[82]. For the conversion of L-tyrosine to tyramine-1-d_1 in D_2O, k_H/k_D = 2.0 (ref. 102).

In the enzymatic reaction (4.2.99.2 in the List) O-phospho-homoserine + H_2O = threonine + phosphate, k_H/k_D = 6.7 and k_H/k_T = 40. These[49] are very high compared with the few other recorded values for hydrogen isotope effects in enzymatic reactions.

Succinate in which the hydrogen on carbons 2 and 3 was 77 atom % deuterium was oxidised at 40% of the normal rate by a succinic oxidase system[84].

Greater retention of C-T than of C-H bonds occurred[85] in the utilisation of the methyl group of methanol for the biosynthesis of labile methyl (choline, creatine).

The literature on hydrogen isotope effects in living organisms indicates, then, with a few possible exceptions, that THO in which only 0.3 atom % of the hydrogen is tritium will not cause gross metabolic aberrations through the kinetic isotope effect. We may expect, however, that specific rates for tritium will differ somewhat from those for the corresponding reactions of ordinary hydrogen. Branching ratios will almost certainly be abnormal; i.e. the molar ratio of products B and D when a compound A breaks down will be different in THO than in ordinary water. We reiterate here the much greater magnitude of primary isotope effects compared with secondary ones. Also we bear in mind that only qualitative (and not quantitative) conclusions will be sought.

The assumption then seems fairly well justified that the path of tritium <u>in vivo</u> will not be qualitatively different from the path of ordinary hydrogen.

Radiation Damage

The experiments for this thesis involved introducing THO of specific activity 5 C/ml. into living organisms. Some dilution is expected from inactive water and other exchangeable hydrogen already present in the organisms, but this dilution would probably not reduce the specific activity even one order of magnitude.

Black mustard seeds (Brassica nigra, extremely closely related to the species used for this thesis) are exceedingly resistant to radiation damage, compared with other seeds.[86] At room temperature, they germinated well despite heavy irradiation from ^{60}Co at 700 kr./hr. (r.=Roentgen), the dose beginning when the seeds had been imbibing water for six hr. The dose to prevent germination of half a large sample was 0.95×10^6 r., an exceptionally high figure for seeds. This unusual resistance to radiation damage might well be shown, in some degree at least, by the seeds used for this thesis.

Nevertheless the possibility of radiation damage had to be carefully looked into.

Most measurements of radiation damage have been with X-rays or gamma rays. The β-rays of tritium, mean energy 5.69 keV (i.e. 5.69×10^3 electron volts)[87] are among the weakest known. Their range is only 0.7 mg. cm^{-2}.[107] It was conceivable they might have an anomalous relative biological effectiveness (RBE); that is to say, a given dose of radiation from tritium, measured as production

of ion pairs in air, might do less damage to an organism than the same dose from a much more energetic radiation.[87] In bean roots and at least some mammals,[88] however, experiments showed that the RBE of intracellular THO was not abnormal. Damage per unit dose (i.e. per r.) was as expected on experience with much more energetic X and β rays.

140,000 r. had no measurable effect on cytochrome oxidase or succinate oxidase of Bacillus subtilis cells or lysates bombarded with electrons.[108] Dry, powdered ribonuclease and adenylpyrophosphatase required respectively 3.4×10^7 and 5.5×10^6 r. of gamma rays to inactivate them.[109] Soft X-rays were used to irradiate thin films of enzymes at the rate of 10^5 r./min. 37% of the enzyme activity remained after 30 min. of that irradiation.[110]

The radiation level at which healthy seeds retain 20-40% of their germination ability was measured, in pot tests, by adding[111] appropriate amounts of $NaH_2\,^{32}PO_4$. The critical dose was as low as 450 microcuries for "Express" peas, 300 for two tomato varieties and one carrot variety, and 150 for another carrot variety. Onion seeds were almost all killed by 50 microcuries. No control experiments were reported, which may account for the great radiation sensitivity implied. One-day-old seedlings of Cicer arietinum were treated with 450 r. at 30 r.min.$^{-1}$ from an X-ray therapy source,[113] a nd then kept at 24±1° for one hour. Some vaguely-described chromatography then indicated that the radiation had caused a marked

increase in alanine concentration. Glutamate and threonine were not separated from each other. Their combined concentration had risen markedly. The concentration of 4-aminobutyrate had doubled. The median lethal radiation dose from THO drunk by rats over two months at 57-77 rep/day was an accumulated dosage of 3000-3700 rep.[114] Algae were grown in various levels of THO, ^{32}P, ^{90}Sr-^{90}Y or ^{35}S, and then sub-cultured in non-radioactive inorganic nutrient solutions.[115] Growth rates decreased as radiation dosage rose, and the reduced growth continued in the sub-culture. 5-40 mC/ml. THO (1600-13,000 rep/day) gave no appreciable inhibition in one day but thereafter growth rate slowed with increased accumulated dose. There was a marked slowing of growth after 72 hr. at the highest dose rate.

Sometimes stimulation by low doses of radiation has been noted.[117] The beta and gamma rays from uranium and potassium stimulated the germination of some species, at very low dose levels.[118] Radon in the air speeded sprouting and growth of oats.[119] ^{45}Ca accelerated sprouting of oats at 12° but not at higher temperatures.[120]

Often, heavy doses of radiation do not inhibit enzymes <u>in vivo</u>, and may even activate them.[116] Doses up to 5000 r. on rats did not inhibit seven important enzymes, nor oxidative phosphorylation in liver mitochondria.[116] However, 50 r. markedly depressed the oxidative phosphorylation in spleen mitochondria, in similar experiments.[116] Among living organisms, seeds are relatively radioresistant, especially when dry.[116] It seems that inactivation of en-

zymes is not involved in death from radiation. The primary lesion in radiation damage remains a mystery; at the rate of 25 eV to break one C-H bond, even a dose of 2×10^6 r. could result in breaking of only 0.003% of such bonds in an organism. It has been suggested, though,[121] that 32 eV could be sufficient to break from 40 to 90 bonds, in small molecules, owing to chain reactions.

Doses of 2×10^6 rep are needed to sterilize food.[122] The giant unicellular alga Acetabularia mediterranea[126] can recover from a dose of 500,000 r.

Increased water contents in barley seeds decreased radiosensitivity in the range of water contents between normal and 20%, but soaking increased the sensitivity so that LD_{50} at 20% water content was fifteen times that at 40% water content.[123] (LD_{50} is the dose to kill 50% of a statistically significant population.) Wheat seeds given 8×10^5 r. of ^{60}Co gamma radiation before moistening germinated to give small seedlings without cell division or DNA synthesis, but fixing carbon from carbon dioxide into sugar phosphates, sucrose, amino-acids and aliphatic acids.[124] 180 kV X-rays were used to irradiate wheat:[125] 1000 r. on air-dry seeds or 12 hr. after wetting had no effect on growth; but this dose given after 24 hr. had some inhibitory effect, and if given 48 hr. after wetting its effect was markedly adverse. Doses as large as 36,000 r. inhibited but did not kill the germinating wheat, no matter when the dose was given. 400 r./day for five days had about the same effect as 1000 r. in one dose after two days. The same

worker reported that 40,000 r. did not damage the leaflets of a moss. X and gamma rays had no effects on the chlamydospores of four species of rust fungus at dose levels below 1×10^5 r., the germination percentage remaining at the control figure of 97%. Two species germinated 60% after doses of 2×10^5 r. but two others germinated 0° (ref. 127). Maize seeds irradiated at 1000 r./min. still germinated 20% after 15,000 r. (control germination 100%).[128] Soybean plants in which the specific activity of ^{14}C was 0.4-1.2 millicuries/g. carbon grew and flowered normally, but set inviable seed.[129] Opium poppy seeds in which the ^{14}C specific activity was 445 microcuries/g. carbon germinated slowly and later stopped growing;[129] but these adverse effects were less marked if the seeds were kept at 7° for four days before germination. Rice seeds required[130] more than 1.6×10^6 rep to prevent germination, when bombarded with 1 MeV cathode rays, or ^{60}Co gamma rays, before wetting. 10^5 rep caused only a slight decrease in germination percentage, and negligible loss of thiamine or riboflavin.

Spedding[1] soaked 10 mustard seeds, as used in the present work, in THO (5 curies/ml.) for 60 min., after which ordinary water was added. They germinated 90%, which was comparable with the control test, 92% germination in a 100-seed sample. For his model from which to calculate the dose, Spedding assumed a mustard seed to be a sphere of radius 2 mm. surrounded by a layer of 5 microliters of THO. On this model it was calculated that the seed received[1]

36,000 rep (per day - Spedding, pers. comm.) The effect of this dose would probably be genetical rather than physiological, it was said.[1] That conclusion, based on no quoted evidence, could scarcely be held with confidence in the light of the above literature survey, but it could be true. Another model seemed more appropriate for the present work, namely a seed which had fully imbibed 0.1 C. of THO (5 curies/ml.) This represents more truly the state of affairs when a seed is treated with 1/50 ml. THO and then kept at 0° for many days. Assuming complete absorption of the beta rays, an approximate formula for the radiation dose is:[131]

$$D = \frac{55AE}{3v}$$

where D = the dose rate (r./day)

E = E_{max} of the betas, in eV (18×10^3 eV for tritium, ref. 107)

A = the activity in the system, in curies.

v = the volume (ml.) of the system.

Taking A = 0.1 curie and v = 0.1 ml., $D = 3 \times 10^5$ r./day, which is one order more than Spedding's result based on the water's being all on the surface of the seed. The actual situation is of course between the two extremes. Although the dose rate is high enough to make physiological aberrations seem likely, the experiment quoted above indicates otherwise.

The writer is grateful for kind permission to quote here unpublished results of W. J. H. Baillie. Batches of dry seeds, of

the species (Sinapis alba) used in this thesis, were irradiated for about two days at various measured distances from a ^{60}Co source, so as to expose each batch of seeds to a known radiation dose. The seeds were then moistened and kept 25 days under conditions which normally lead to germination in two days. Radiation doses were measured in rads. For the present purpose the rad may be taken as equivalent to the roentgen, nearly enough, because there is in any case considerable uncertainty in applying these results to seeds wet at 0° with THO, i.e. the conditions of irradiation in the work for this thesis. After doses of 0.2, 0.5 and 1.0 megarad, batches of seeds germinated 99%, 99% and 98%, respectively. After 2.0 megarad the germination was 20%, and after 5.0 or 10 megarad, 0%.

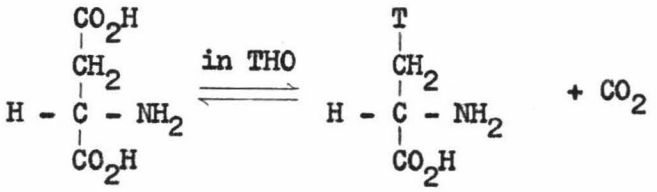
The above survey shows that experiments on seeds using the "THO method" will probably not entail significant radiation damage below dose levels around 10^6 r. On the estimate calculated above of the dose rate in the experiments for this thesis, namely 3×10^5 r./day, it seems, then, that results will be of somewhat limited value in experiments where the seeds were imbibed with THO for longer than 3 days. Fortunately, this limitation has little impact on the formulation of conclusions, because, as will be seen, most of the important conclusions are based on results from experiments

Interpretation

Care is needed in forming conclusions from the experimental results of the THO method. If THO is introduced into a living organism and tritium is incorporated into citric, isocitric, malic and succinic acids, one can conclude with certainty that these compounds are involved in metabolic changes; but one may only suggest that the citric acid (i.e. Krebs) cycle is operating. On the other hand, if none of these acids becomes labelled it will probably be a waste of time to embark on a conventional enzymological search for the enzymes of the Krebs cycle in that organism.

This example shows how a simple preliminary experiment with THO could be very appropriate as a prelude and guide to much more protracted experiments.

The position of the label in a tritiated molecule may help in deducing what reaction caused the labelling. For instance, alanine will be labelled at the α-carbon by the reverse of oxidative deamination, but at the β-carbon by the β-decarboxylation of aspartic acid:



Some metabolism may not be detected by the THO method because it does not entail the formation of any C-T bonds. Hydrolyses of peptide or glycosidic links are examples. Thus even if an experiment with the THO method yields only one tritiated metabolite, it need not be true that this compound is the sole one involved in metabolic activity.

The true role of tritium incorporation as an index of metabolism is supplementary to that of other methods. The THO method by no means supersedes others, but forms a valuable adjunct to them.

CLASSIFICATION OF KNOWN ENZYMATIC REACTIONS

For each type of reaction (redox, group transfer, isomerisation, etc), individual reactions will be listed in the three classes defined above. For convenience, the definitions are reiterated:

A. Reactions in which non-exchangeable incorporation of tritium from THO is expected to occur.

B. Reactions which are not expected to entail non-exchangeable incorporation of tritium.

C. Reactions of which the mechanisms are not well enough known for a confident prediction to be made as to whether tritium would be non-exchangeably incorporated.

In a few cases, reactions have been moved from one category to another because of experiments which have given results different from those expected.

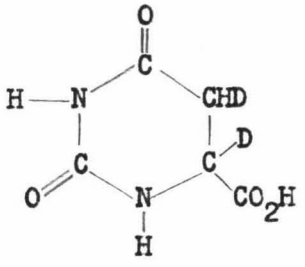
Where applicable, the numbering of the Commission on Enzymes[20] is given.

Reversibility is generally assumed. By and large, experimental evidence on this is lacking, and the assumption may well be false in some cases. However, the purpose here is to list all those metabolites which could become labelled. Therefore, in the absence of evidence that the assumption is false, it is the most appropriate one to adopt here. The THO method could be used as a test for irreversibility in vivo.

Metabolites expected to become non-exchangeably labelled are marked with an asterisk. A question mark next to an asterisk denotes doubt as to whether the compound, when isolated, would be non-exchangeably labelled. The reasons, in different cases, for this doubt are:

1. The position of equilibrium in carbonyl $\rightleftharpoons$ enol tautomerism, and the rate of interconversion of the tautomers, both vary immensely between different cases[28]. It is found[28] that the specific rate k of enolisation is roughly proportional to the equilibrium constant K = [enol form] ÷ [carbonyl form]. In some cases, hydrogen which can be exchanged out of a compound

by washing with water is not in practice removed at any considerable rate. What was very likely 4,5-dideutero-orotic acid[30], when purified from 95% D_2O contained 1.50 atoms of deuterium per molecule, decreasing to only 1.44 on recrystallising. It seemed[30] that this was a case of enolisation so slow that enolisable hydrogen was still present to a large extent, unexchanged, in the washed compound.



Again, glucose 6-phosphate labelled at carbon 2 with isotopic hydrogen does not rapidly lose this label[32]. In general, no confident prediction is possible about the rate of enolisation, i.e. about the survival of the label. Topper[32] converted glucose 6-phosphate to fructose 6-phosphate by incubation for 15 hr. at 26° with glucosephosphate isomerase (5.3.1.9) in neutral 95-99.5% D_2O. The osazone of the product contained no deuterium. Treatment after incubation had not been such as to exchange out the deuterium which was found to be on C-1 of fructose 6-phosphate. These results can be interpreted[60] as proving that, under the conditions of

Topper's experiment at least, the hexoses contain 7 non-exchangeable hydrogen atoms.

2. There are many well-authenticated cases[31] of enzymatic reactions at meso carbon atoms, CX_2YZ, which distinguish between the two chemically identical groups X. A famous example is the ethanol dehydrogenation[30] in which the two α-hydrogens of ethanol are discriminated between. Some discussion of this appears below in the comments in lists of enzymatic reactions. Meantime, it is useful, for the present purpose of predicting tritium incorporation from THO, to distinguish between two types of enzymatic reaction at meso carbon atoms.

(a) Reactions leading to a new centre of asymmetry

e.g. 4-aminobutyrate + CO_2 = L-glutamate

Where such reactions produce only one enantiomorph, it seems that in the conversion of the meso centre CX_2YZ to a new centre of asymmetry, the two groups X must have been discriminated between. Otherwise, it is very hard to imagine how only one enantiomorph could result.

(b) Reactions not leading to a new centre of asymmetry

e.g. $Z\text{-}CH_2OH - 2H = Z\text{-}CHO$

Although in some cases which have been examined[31] there is discrimination between the two chemically identical groups X on CX_2YZ, there seems, in contrast to the position in category (a), to be no compelling reason why all reactions in this category (b) should

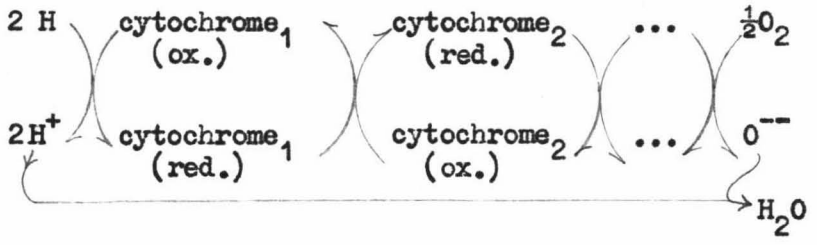
involve such discrimination. Thus, there is doubt as to which compounds will become labelled, depending on whether or not there is discrimination, and this latter is not usually known, the appropriate experiments not having been done yet.

3. In some known enzymatic reactions, the mechanism is not known. The non-reductive, non-hydrolytic cleavage of arginosuccinate (4.3.2.1) is one of many such. Whether or not fumarate and arginosuccinate become labelled if this reaction proceeds in THO depends on whether there is exchange of the relevant protons with the THO. This cannot be confidently predicted so a question mark is appended to the asterisks on these compounds. Obviously, tritium incorporation could be applied as an excellent means of studying the mechanisms of such reactions.

Reactions on which only $NADH_2$ or $NADPH_2$ is labelled are classed in B.

Most biochemists would, presumably, support the assumption that, when a metabolite is being reduced by $NADH_2$ or $NADPH_2$ in a living organism, the hydrogen being newly attached to the metabolite comes ultimately from water. Certainly this is, in the absence of any evidence to the contrary, the most reasonable assumption. It has been adopted here. This is so notwithstanding the demonstration by Vennesland[22,80], for alcohol dehydrogenation and its reverse, of direct hydrogen transfer from ethanol to NAD and from $NADH_2$ onto

acetaldehyde. Many experiments since then (ref. 81, p.268) have given similar results, for a wide range of hydrogen transfers to NAD(P) and from NAD(P)H_2. Despite these experiments on NAD(P), at the next step in the hydrogen transfer chain <u>in vivo</u>, i.e. flavoproteins, the hydrogen atoms being transferred are exchanged with water (ref. 81, p.272). Also, widely-favoured theories of cytochromes' functions present a scheme of hydrogen and electron transport essentially (e.g. ref. 44,p.371) so:



The number and order of cytochromes is irrelevant; the essential assertion of any such scheme is, for our present purpose, that hydrogen removed from metabolites is released as <u>protons</u>. Therefore, given reversibility of hydrogen transport, tritium from THO will be incorporated by the metabolite which is being oxidised or reduced. In fact schemes of this type are not universally believed (ref. 81, p.263). Experiments <u>in vivo</u> with THO in a functioning cytochrome system might provide useful evidence. In this connection, see section 1.9.3 of the List.

In addition to hydrogen exchange with THO at the flavoproteins, and possibly at the cytochromes, some substrate : NAD(P) oxidoreductase

reactions may be expected to label metabolites. $NAD(P)H_2$ is, in neutral solution (ref. 81, p.367), $NAD(P)H + H^+$. The experiments referred to two paragraphs above proved that it is the hydrogen bound covalently to $NAD(P)H^+$, and not the proton, which is transferred to or from carbon of alcohols. However, in reactions of the type

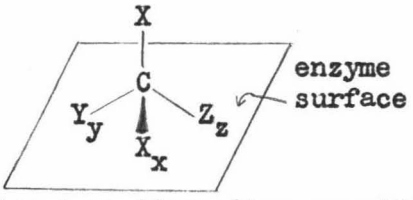
if the stoichiometry quoted in the Enzyme List[20] is correct, only one $NAD(P)H_2$ "molecule" is involved, meaning that of the two hydrogen atoms added to the double bond, one is a proton, i.e. in equilibrium with THO.

When a metabolite is being oxidised and there is a (net) movement of hydrogen out of it, experiment is in most cases needed to tell whether enough hydrogen (tritium) moves in the other direction to label the metabolite.

A comment on 1.1.1.1 and 1.1.1.2, relevant to several other reactions also

There is no doubt that the alcohols will be labelled (at the α-carbon). It appears eminently reasonable to predict that the aldehyde will therefore also be labelled. However, unequivocal experiments have shown conclusively[22] that in at least some enzymatic

dehydrogenations of alcohols, this is not so. Many chemists find trouble in believing that the two similar groups X on a "meso carbon atom" CX_2YZ, i.e. in this case the two α-hydrogen atoms of a primary alcohol, can be distinguished. However, the "3-point contact" theory of Ogston[23] provides a plausible explanation. Briefly, it rests on the fact that while (say) ethanol is a symmetrical molecule, the enzyme for its dehydrogenation may well not be. Discrimination between the α-hydrogens of ethanol becomes possible if the ethanol molecule is "held" on the enzyme by specific "active sites" for methyl, hydroxyl and hydrogen, in a particular steric order. The diagram shows this for the general case.



The letters x, y, z denote active sites specific for the groups X,Y,Z respectively.

It may be too much to extrapolate and assert that all alcohol dehydrogenase reactions are stereospecific in this way. Therefore the aldehyde is marked*? in 1.1.1.1,2.

It is appropriate to point out that reactions of symmetrical compounds to give only one enantiomorph of an unsymmetrical product are explicable at present by "3-point contact" and no other theory, as far as the author is aware. This is important in making predictions about labelling of metabolites in TH0, e.g. see 4.1.1.15.

1 REDOX REACTIONS

1.1 Acting on the CH-OH group of H donors

1.1.1 With NAD or NADP as acceptor

A 1.1.1.1 Alcohol* + NAD = aldehyde*? or ketone + $NADH_2^*$

1.1.1.a Allyl alcohol* + NADP = acrolein*? + $NADPH_2^*$

1.1.1.2 Alcohol* + NADP = aldehyde*? + $NADPH_2^*$

1.1.1.3 L-homoserine* + NAD(P) = L-aspartate β-semialdehyde* + $NAD(P)H_2^*$

1.1.1.4 2,3 butyleneglycol* + NAD = acetoin + $NADH_2^*$

1.1.1.6 Glycerol* + NAD = dihydroxyacetone + $NADH_2^*$

1.1.1.7 1,2-propanediol 1-phosphate* + NAD = acetol phosphate + $NADH_2^*$

1.1.1.b 1,2-propanediol* + NADP = L-lactaldehyde*? + $NADPH_2^*$

1.1.1.8 L-glycerol 3-phosphate* + NAD = dihydroxyacetone phosphate + $NADH_2^*$

1.1.1.9 Xylitol* + NAD = D-xylulose + $NADH_2^*$

1.1.1.9 Ribitol* + NAD = D-ribulose + $NADH_2^*$

1.1.1.10 Xylitol* + NADP = L-xylulose + $NADPH_2^*$

1.1.1.11 D-arabitol* + NAD = D-xylulose + $NADH_2^*$

1.1.1.11 D-mannitol* + NAD = D-fructose + $NADH_2^*$

1.1.1.12 L-arabitol* + NAD = L-xylulose + $NADH_2^*$

1.1.1.13 L-arabitol* + NAD = L-ribulose + $NADH_2^*$

1.1.1.14 L-iditol* + NAD = L-sorbose + $NADH_2^*$

1.1.1.14 D-glucitol* + NAD = D-fructose + $NADH_2^*$

1.1.1.15 D-iditol* + NAD = D-sorbose + $NADH_2^*$

1.1.1.15 Xylitol* + NAD = L-xylulose + $NADH_2^*$

1.1.1.15 L-glucitol* + NAD = L-fructose + $NADH_2^*$

1.1.1.16 Galactitol* + NAD = D-tagatose + $NADH_2^*$

1.1.1.17 D-mannitol 1-phosphate* + NAD = D-fructose 6-phosphate + $NADH_2^*$

1.1.1.d D-mannonate* + NAD = D-fructuronate*? + $NADH_2^*$

1.1.1.e D-altronate* + NAD = D-tagaturonate*? + $NADH_2^*$

1.1.1.18 myo-inositol* + NAD = 2-oxo-myo-inositol + $NADH_2^*$

1.1.1.19 L-gulonate* + NADP = D-glucuronate*? + $NADPH_2^*$

1.1.1.20 L-gulono-γ-lactone* + NADP = D-glucurono-γ-lactone*? + $NADPH_2^*$

1.1.1.21 Polyol* + NADP = aldose*? + $NADPH_2^*$

We do not know whether the stereospecificity will be as in 1.1.1.1.

1.1.1.22 UDP glucose* + 2NAD + $*H_2O$ = UDP glucuronate + $2NADH_2^*$

1.1.1.23 L-histidinol* + 2NAD = L-histidine + $2NADH_2^*$

1.1.1.24 Quinate* + NAD = 5-dehydroquinate + $NADH_2^*$

1.1.1.25 Shikimate* + NADP = 5-dehydroshikimate + $NADPH_2^*$

1.1.1.26 Glycollate* + NAD = glyoxylate*? + $NADH_2^*$ See comment on 1.1.1.21.

1.1.1.26 D-glycerate* + NAD = hydroxypyruvate + $NADH_2^*$

1.1.1.27 L-2-hydroxymonocarboxylate* (e.g. L-lactate) + NAD
= 2-oxomonocarboxylate (e.g. pyruvate) + $NADH_2^*$

1.1.1.28 D-lactate* + NAD = pyruvate + $NADH_2^*$

1.1.1.30 D-3-hydroxybutyrate* + NAD = acetoacetate + $NADH_2^*$

1.1.1.31 3-hydroxyisobutyrate* + NAD = methylmalonate semialdehyde*? + $NADH_2^*$

1.1.1.32,33 Mevalonate* + NAD(P) = mevaldate*? + $NAD(P)H_2^*$

1.1.1.34 Mevalonate* + CoA + 2NADP = 3-hydroxy-3-methylglutaryl-CoA + $2NADPH_2^*$

1.1.1.35 L-3-hydroxyacyl-CoA* + NAD = 3-oxo-acyl-CoA + $NADH_2^*$

1.1.1.36 D-3-hydroxyacyl-CoA* + NADP = 3-oxo-acyl-CoA + $NADPH_2^*$

1.1.1.37 L-malate* + NAD = oxaloacetate + $NADH_2^*$

1.1.1.38,39,40 L-malate* + NAD(P) = pyruvate + CO_2 + NAD(P)H_2*

1.1.1.41,42 L_s-isocitrate* + NAD(P) = 2-oxoglutarate + CO_2 + NAD(P)H_2*

1.1.1.43 6-phospho-D-gluconate* + NAD = 6-phospho-2-oxo-D-gluconate + $NADH_2$*

1.1.1.44 6-phospho-D-gluconate* + NADP = D-ribulose-5-phosphate*? + $NADPH_2$* + CO_2

1.1.1.45 L-gulonate* + NAD = L-xylulose*? + $NADH_2$* + CO_2

1.1.1.46 L-arabinose* + NAD = L-arabono-γ-lactone + $NADH_2$*

1.1.1.47 β-D-glucose* + NAD(P) = D-glucono-δ-lactone + NAD(P)H_2*

1.1.1.48 D-galactose* + NAD = D-galactono-γ-lactone + $NADH_2$*

1.1.1.49 D-glucose-6-phosphate* + NADP = D-glucono-δ-lactone 6-phosphate + $NADPH_2$*

1.1.1.50 3-α-hydroxysteroid* + NAD(P) = 3-oxosteroid + NAD(P)H_2*

1.1.1.51 3(or 17)-β-hydroxysteroid* + NAD(P) = 3(or 17)-oxosteroid + NAD(P)H_2*

1.1.1.53 20-dihydrocortisone* + NADP = cortisone + $NADPH_2$*

1.1.1.f 3-hydroxypropionate* + NAD = malonate semialdehyde*? + $NADH_2$*

1.1.1.g D-glycerate* + NAD(P) = tartronate semialdehyde*? + NAD(P)H_2*

1.1.1.h 4-hydroxybutyrate* + NAD = succinic semialdehyde*? + $NADH_2$*

1.1.1.i Oestradiol* + NAD = oestrone + $NADH_2$*

1.1.1.j Testosterone* + NAD = Δ^5-androstene-3,17-dione + $NADH_2$*

1.1.1.k Testosterone* + NADP = Δ^5-androstene-3,17-dione + $NADPH_2$*

1.1.1.l Pyridoxin* + NADP = pyridoxal*? + $NADPH_2$*

1.1.1.m 10-hydroxydecanoate* + NAD = 10-oxodecanoate + $NADH_2$*

B 1.1.1.5 Acetoin + NAD = diacetyl + $NADH_2$*

1.1.2 H accepted by a cytochrome

A 1.1.2.1 L-glycerol 3-phosphate* + oxidized cyto.c = dihydroxyacetone phosphate + reduced cyto c.*? See comment on 1.9.3.1.

1.1.2.2 D-mannitol* + cyto. (ox.) = D-fructose + cyto. (red.)*?

1.1.2.3 L-lactate* + cyto.c (ox.) = pyruvate + cyto. c (red.)*?

1.1.2.4 D-2-hydroxyacid* (e.g. D-lactate) + cyto. c (ox.) = 2-oxoacid (e.g. pyruvate) + cyto. c (red.)*?

1.1.3 H accepted by O_2

A 1.1.3.1 Glycollate* + O_2 = glyoxylate*? + H_2O_2

1.1.3.a An aromatic 1^{o} alcohol* + O_2 = an aromatic aldehyde*? + H_2O_2

1.1.3.b L-gulono-γ-lactone* + O_2 = L-xylohexulonolactone + H_2O_2

1.1.3.c D-galactose* + O_2 = D-galacto-hexodialdose*? + H_2O_2

1.1.3.2 L-lactate* + O_2 = acetate + CO_2 + H_2O_2

1.1.3.4 β-D-glucose* + O_2 = D-glucono-δ-lactone + H_2O_2

C 1.1.3.3 L-malate*? + O_2 = oxaloacetate + (?)*?

1.1.3.6 Cholesterol*? + O_2 = Δ^4-cholestene-3-one + (?)*?

1.1.99 Artificial acceptors (indophenol, pyocyanine etc.)

A 1.1.99.1 Choline* + acceptor = betaine aldehyde + reduced acceptor

1.1.99.a D-2-hydroxyacid* + acceptor (ox.) = D-2-oxoacid + acceptor (red.)

1.1.99.2 L-2-hydroxyglutarate* + acceptor = 2-oxoglutarate + reduced acceptor

1.1.99.3 D-gluconate* + acceptor = 2-oxo-D-gluconate + reduced acceptor

1.1.99.4 2-oxo-D-gluconate* + acceptor = 2,5-dioxo-D-gluconate + reduced acceptor

1.2 Acting on carbonyl group of H donor

1.2.1 NAD(P) as acceptor

A 1.2.1.1 Formaldehyde* + NAD + H_2^*O = formate*? + $NADH_2^*$

1.2.1.a Malonate semialdehyde* + NAD(P) = malonate + $NAD(P)H_2^*$

1.2.1.9 D-glyceraldehyde 3-phosphate* + NADP + H_2^*O = D-3-phosphoglycerate + $NADPH_2^*$

1.2.1.b Succinate semialdehyde* + NAD(P) = succinate + $NAD(P)H_2^*$

1.2.1.10 Aldehyde* + CoA + NAD = acyl-CoA + $NADH_2^*$

1.2.1.c Glyoxylate* + CoA + NADP = oxalyl-CoA + $NADPH_2^*$

1.2.1.11 L-aspartate β-semialdehyde* + phosphate + NADP = L-β-aspartylphosphate + $NADPH_2^*$

1.2.1.12,13 D-glyceraldehyde 3-phosphate* + phosphate + NAD(P) = D-1,3-diphosphoglycerate + $NAD(P)H_2^*$

1.2.1.14 IMP* + NAD + H_2O = xanthosine 5'-phosphate + $NADH_2^*$

1.2.1.e 4-aminobutyraldehyde* + NAD = 4-aminobutyrate + $NADH_2^*$

1.2.1.f Glutarate semialdehyde* + NAD = glutarate + $NADH_2^*$

C 1.2.1.d Malonate semialdehyde*? + CoA + NAD(P) = acetyl-CoA*? + CO_2 + $NAD(P)H_2^*$

1.2.2 H accepted by a cytochrome

A 1.2.2.1 Formate* + cyto. b_1 (oxidized) = CO_2 + cyto. b_1 (reduced)

C 1.2.2.2 Pyruvate + cyto. (ox.) = acetate + CO_2 + cyto. (red.)*?

1.2.3 H accepted by O_2

A 1.2.3.1 Aldehyde* + H_2O + O_2 = acid + H_2O_2

1.2.3.2 Xanthine* + H_2O + O_2 = urate + H_2O_2

B 1.2.3.3 Pyruvate + phosphate + O_2 = acetylphosphate + CO_2 + H_2O_2

1.2.3.4 Oxalate + O_2 = $2CO_2$ + H_2O_2

1.2.4 H accepted by lipoate

B 1.2.4.1 Pyruvate + oxidized lipoate = 6-S-acetylhydrolipoate + CO_2

1.2.4.2 2-oxoglutarate + oxidized lipoate = 6-S-succinylhydrolipoate + CO_2

1.2.99 Artificial acceptors

C 1.2.99.1 Uracil*? + methylene blue = barbiturate + leuco-methylene blue

1.3 Acting on the CH-CH group of donors

1.3.1 H accepted by NAD or NADP

C 1.3.1.1,2 4,5-dihydro-uracil*? + NAD(P) = uracil + $NAD(P)H_2$*

A 1.3.1.a 4,5-α-dihydrocortisone* + NADP = cortisone*? + $NADPH_2$*

1.3.1.b 3,5-cyclohexadiene-1,2-diol* + NADP = catechol*? + $NADPH_2$*

1.3.1.3 4,5-β-dihydrocortisone* + NADP = cortisone*? + $NADPH_2$*

1.3.2 H accepted by a cytochrome

A 1.3.2.1,2 Acyl-CoA* + cyto.c (ox.) = 2,3-dehydroacyl-CoA*? + cyto.c (red.)*?

1.3.2.3 L-galactono-γ-lactone* + cyto.c (ox.) = L-ascorbate + cyto.c (red.)*?

1.3.3 H accepted by O_2

A 1.3.3.1 4,5-L-dihydro-orotate* + O_2 = orotate*? + H_2O_2 (?) (ref.30)

1.3.99 Artificial acceptors

C 1.3.99.1 Succinate*? + phenazine alkylsulphate (ox.) = fumarate*? + phenazine alkylsulphate (red.)

A 1.3.99.a A 3-ketosteroid* + acceptor (ox.) = a Δ^1-3-ketosteroid*? + acceptor (red.)

1.3.99.b A 3-ketosteroid* + acceptor (ox.) = a Δ^4-3-ketosteroid*? + acceptor (red.)

1.4 Acting on the CH-NH_2 group of donors

1.4.1 H accepted by NAD or NADP

A 1.4.1-5 An L-α-aminoacid* + H_2O + NAD(P) = a 2-oxo-acid + NH_3 + NAD(P)H_2

1.4.1.6 5-aminovalerate* + NAD = D-proline*? + $NADH_2$

1.4.3 H accepted by O_2

A 1.4.3.1 D-aspartate* + H_2O + O_2 = oxaloacetate + NH_3 + H_2O_2

1.4.3.2 An L-aminoacid* + H_2O + O_2 = a 2-oxo-acid + NH_3 + H_2O_2

1.4.3.3 A D-aminoacid* + H_2O + O_2 = a 2-oxo-acid + NH_3 + H_2O_2

1.4.3.4 A monoamine* + H_2O + O_2 = an aldehyde*? + NH_3 + H_2O_2

1.4.3.5 Pyridoxamine* + H_2O + O_2 = pyridoxal*? + NH_3 + H_2O_2

1.4.3.6 A diamine* + H_2O + O_2 = an aminoaldehyde*? + NH_3 + H_2O_2

1.5 Acting on the C-NH group of donors

1.5.1 H accepted by NAD or NADP

A 1.5.1.1 L-proline* + NAD(P) = Δ^1-pyrroline-2-carboxylate + NAD(P)H_2

1.5.1.1 L-pipecolate* + NAD(P) = Δ^1-piperidine-2-carboxylate + NAD(P)H_2

1.5.1.3 5,6,7,8-tetrahydrofolate* + NAD(P) = 7,8-dihydrofolate*? + NAD(P)H_2*

1.5.1.4 7,8-dihydrofolate* + NADP = folate*? + $NADPH_2$*

1.5.1.5 5,10-methylenetetrahydrofolate* + H^+ + NADP = 5,10-methenyltetrahydrofolate*? + H_2O + $NADPH_2$*

1.5.3 H accepted by O_2

A 1.5.3.3 Spermine* + H_2O + O_2 = $H_2N.(CH_2)_3.CH.(CH_2)_3.CHO$*? + $H_2N.(CH_2)_3.NH_2$ + H_2O_2

C 1.5.3.1,2 An N-methyl-L-aminoacid*? + H_2O + O_2 = an L-aminoacid*? + HCHO*? + H_2O_2

1.6 Acting on $NADH_2$ or $NADPH_2$ as donor

1.6.1 H accepted by NAD or NADP

B 1.6.1.1 $NADPH_2^*$ + NAD = NADP + $NADH_2^*$

1.6.2 H accepted by a cytochrome

C 1.6.2.1,2 $NADH_2^*$ + a cyto. (ox.) = NAD + a cyto. (red.) *?

1.6.2.3 $NADPH_2^*$ + cyto. c (ox.) = NADP + cyto. c (red.) *?

1.6.4 H accepted by a disulphide

B 1.6.4.1 $NADH_2^*$ + L-cystine = NAD + 2 L-cysteine

1.6.4.2 $NAD(P)H_2^*$ + glutathione (ox.) = NAD(P) + glutathione (red.)

1.6.4.3 $NADH_2^*$ + lipoamide = NAD + dihydro-lipoamide

1.6.5 H accepted by a quinone or related compound

B 1.6.5.1 $NAD(P)H_2^*$ + a quinone = NAD(P) + a diphenol

1.6.5.2 $NAD(P)H_2^*$ + a naphthoquinone = NAD(P) + a naphthohydroquinone

1.6.5.3 $NAD(P)H_2^*$ + ubiquinone = NAD(P) + dihydro-ubiquinone

1.6.5.4 $NAD(P)H_2^*$ + ascorbate (ox.) = NAD(P) + ascorbate

1.6.6 H accepted by a nitrogenous group

A 1.6.6.a $NADPH_2^*$ + GMP = NADP + IMP* + NH_3

B 1.6.6.1,2,3 $NAD(P)H_2^*$ + nitrate = NAD(P) + nitrite + H_2O

1.6.6.4 $2NAD(P)H_2^*$ + nitrite = $2NAD(P)$ + NH_2OH + H_2O

1.6.6.5 $NAD(P)H_2^*$ + 2 nitrite = NAD(P) + 2NO + $2H_2O$

1.6.6.6 $NADH_2^*$ + hyponitrite = NAD + NH_2OH

1.6.6.7 $NADPH_2^*$ + dimethylaminoazobenzene = NADP + dimethyl-*p*-phenylenediamine + aniline

1.6.99 Other acceptors

B 1.6.99.1 $NADPH_2^*$ + methylene blue = NADP + leuco-methylene blue

1.7 Acting on other nitrogenous groups as donors

1.7.3. H accepted by O_2

A 1.7.3.1 $CH_3CH_2NO_2^*$ + H_2O + O_2 = $CH_3CHO^{*?}$ + HNO_2 + H_2O_2

C 1.7.3.2 N-acetylindoxyl + O_2 = N-acetylisatin + (?)

1.7.3.3 $Urate^{*?}$ + O_2 = unidentified $products^{*?}$

1.7.99 Other acceptors

B 1.7.99.a $2NO + 2H_2O$ + acceptor (ox.) = 2 nitrite + acceptor (red.)

B 1.7.99.1 NH_3 + acceptor = NH_2OH + acceptor (red.)

1.7.99.2 N_2 + acceptor = 2NO + acceptor (red.)

1.8 Acting on sulphur groups of donors

1.8.1 H accepted by NAD or NADP

B 1.8.1.1 Cysteamine + NAD + H_2O = cystamine disulphoxide + $NADH_2^*$

1.8.1.2 H_2S + 3NADP + $3H_2O$ = sulphite + $NADPH_2^*$

1.8.3 H accepted by O_2

B 1.8.3.1 Sulphite + O_2 + H_2O = sulphate + H_2O_2

1.8.3.2 4R:CR'.SH + O_2 = 2R:CR'.S.S.CR':R + $2H_2O$

1.8.4 H accepted by a disulphide

B 1.8.4.1 2 glutathione (red.) + homocystine = glutathione (ox.) + 2 homocysteine

1.8.6 H accepted by a nitrogenous group

C 1.8.6.1 2 glutathione + polyolnitrate = glutathione (ox.) + nitrite + unidentified product.

1.9 Acting on haem groups of donors

1.9.3 H accepted by O_2

C 1.9.3.a 4 cyto. C_4 or C_5 (red.)*? + O_2 = 4 cyto. (ox.) + $2H_2O$

1.9.3.1 4 cyto. c (red.)*? + O_2 = 4 cyto. c (ox.) + $2H_2O$

1.9.6 H accepted by a nitrogenous group

C 1.9.6.1 A cyto. (red.)*? + nitrate = cyto. (ox.) + nitrite

The nature of reduced cytochromes is not settled[81].

1.10 Acting on diphenols and related substances as donors

1.10.3 H accepted by O_2

B 1.10.3.1 2 o-diphenol + O_2 = 2 o-quinone + $2H_2O$

1.10.3.2 2 p-diphenol + O_2 = 2 p-quinone + $2H_2O$

1.10.3.3 2 L-ascorbate + O_2 = 2 dehydroascorbate + $2H_2O$

1.11 Acting on H_2O_2 as acceptor

A 1.11.1.3 Palmitic acid* + $2H_2O_2$ = 1-pentadecanol*? + CO_2 + $3H_2O$

B 1.11.1.1 $NADH_2^*$ + H_2O_2 = NAD + $2H_2O$

1.11.1.2 $NADPH_2^*$ + H_2O_2 = NADP + $2H_2O$

1.11.1.8 Iodide + H_2O_2 = iodine + $2H_2O$

1.11.1.a 2 glutathione (red.) + H_2O_2 = glutathione (ox.) + $2H_2O$

1.11.1.6 $2H_2O_2$ = O_2 + $2H_2O$

C 1.11.1.5 2 cyto.c (red.)*? + H_2O_2 = 2 cyto.c (ox.) + $2H_2O$

1.11.1.4 L-tryptophan + H_2O_2 = (unknown) + $2H_2O$

1.11.1.7 (a great variety of donors) + H_2O_2 $\overset{\text{peroxidase}}{=}$ donor (ox.) + $2H_2O$

1.98 H_2 used as a reductant

C 1.98.1.1 Primary reaction not yet known

1.99 Other reactions using O_2 as oxidant

1.99.1 Hydroxylase reactions (not yet fully understood)

A 1.99.1.12 $R.CH_2.O.C_6H_4.R'^{*} \longrightarrow RCHO^{*?} + HO.C_6H_4.R'$

C 1.99.1.1 $Ar\text{-}NH_2^{*?} \xrightarrow{NADPH_2}$ hydroxylated product

1.99.1.2 L-phenylalanine$^{*?} \xrightarrow{NADH_2}$ L-tyrosine

1.99.1.3 Nicotine$^{*?} \longrightarrow$ 6-hydroxynicotinate

1.99.1.4 L-tryptophan$^{*?} \longrightarrow$ 6-hydroxytryptophan

1.99.1.5 L-kynurenine$^{*?} \xrightarrow{NADPH_2}$ 3-hydroxykynurenine

1.99.1.6 Steroid$^{*?} \longrightarrow$ 11-α-hydroxysteroid

1.99.1.7 Steroid$^{*?} \longrightarrow$ 11-β-hydroxysteroid

1.99.1.8 Steroid$^{*?} \longrightarrow$ 6-β-hydroxysteroid

1.99.1.9 Steroid$^{*?} \xrightarrow{NADPH_2}$ 17-α-hydroxysteroid

1.99.1.10 11-deoxycorticosterone$^{*?} \longrightarrow$ 19-hydroxy-11-deoxycorticosterone

1.99.1.11 Steroid$^{*?} \xrightarrow{NADPH_2}$ 21-hydroxysteroids

1.99.1.13 Squalene$^{*?} \xrightarrow{NADPH_2}$ lanosterol$^{*?}$

1.99.1.14 p-hydroxyphenylpyruvate$^{*?} \longrightarrow$ homogentisate + CO_2

1.99.2 Oxygenase reactions (not yet fully understood)

C 1.99.2.1 Unsaturated fat*? + O_2 = peroxide of the unsaturated fat

1.99.2.2 Catechol*? + O_2 = cis-cis-muconate

1.99.2.3 Protocatechuate*? + O_2 = 3-carboxy-cis-cis-muconate

1.99.2.a Catechol + O_2 = 2-hydroxy-muconate semialdehyde*?

1.99.2.b 3-hydroxyanthranilate + O_2 = 2-amino-3-carboxy-muconate semialdehyde*?

1.99.2.c L-tryptophan + O_2 = formyl-kynurenine*?

1.99.2.4 Gentisate*? + O_2 = 3-maleylpyruvate

1.99.2.5 Homogentisate*? + O_2 = 4-maleylacetoacetate

1.99.2.6 myo-inositol*? + O_2 = D-glucuronate

2 GROUP TRANSFER REACTIONS

2.1 Transferring one-carbon groups

2.1.1 Methyl transfers

In a double labelling experiment using methionine labelled with ^{14}C and ^{3}H in the methyl group, it was found[33] that in the transmethylation providing C-24 of ergosterol, 86-91% of the tritium stayed on the radiocarbon. There is no telling what degree of exchange would occur in the methyl group during other transmethylations, however.

C 2.1.1.1 S-adenosylmethionine*? + nicotinamide = S-adenosylhomocysteine + N-methylnicotinamide*?

2.1.1.2 S-adenosylmethionine*? + guanidinoacetate = S-adenosylhomocysteine + creatine*?

2.1.1.3 Dimethylthetin*? + L-homocysteine = S-methylthioglycollate + L-methionine*?

2.1.1.4 S-adenosylmethionine*? + N-acetylserotonin = S-adenosylhomocysteine + N-acetyl-5-methoxytryptamine*?

2.1.1.5 Betaine*? + L-homocysteine = dimethylglycine + L-methionine*?

2.1.1.a S-adenosylmethionine*? + catechol = S-adenosylhomocysteine + guiacol*?

2.1.1.b S-adenosylmethionine*? + nicotinate = S-adenosylhomocysteine + N-methylnicotinate*?

2.1.1.c S-adenosylmethionine*? + histamine = S-adenosylhomocysteine + 1-methylhistamine*?

2.1.1.d S-adenosylmethionine*? + a thiol = S-adenosylhomocysteine + a thioether*?

2.1.1.e S-adenosylmethionine*? + L-homocysteine = S-adenosylhomocysteine + methionine*?

2.1.2 Hydroxymethyl, formyl and related transfers

Tritium might exchange onto the hydroxymethyl group during transfer.

A 2.1.2.1 L-serine*? + tetrahydrofolate = glycine* + 10-hydroxymethyltetrahydrofolate*?

B 2.1.2.4 N-formiminoglycine + tetrahydrofolate = glycine + 5-formiminotetrahydrofolate

2.1.2.5 N-formimino-L-glutamate + tetrahydrofolate = L-glutamate + 5-formiminotetrahydrofolate

C 2.1.2.2 5'-phosphoribosyl-N-formylglycineamide + tetrahydrofolate = 5'-phosphoribosylglycineamide + 5,10-methenyltetrahydrofolate + H_2O

2.1.2.a N-formyl-L-glutamate*? + tetrahydrofolate = L-glutamate + 5-formyltetrahydrofolate*?

2.1.2.3 5'-phosphoribosyl-5-formamido-4-imidazole-carboxamide + tetrahydrofolate = 5'-phosphoribosyl-5-amino-4-imidazole-carboxamide + 5,10-methenyltetrahydrofolate + H_2O

2.1.3 Carboxyl and carbamoyl transfers

A 2.1.3.1 2-methylmalonyl-CoA*? + pyruvate = propionyl-CoA* + oxaloacetate

B 2.1.3.2 Carbamoylphosphate + L-aspartate = orthophosphate + N-carbamoyl-L-aspartate

2.1.3.3 Carbamoylphosphate + L-ornithine = orthophosphate + L-citrulline

2.2 Transferring aldehydic or ketonic residues

C 2.2.1.1 Transketolase-catalysed reactions, e.g. D-sedoheptulose-7-phosphate*? + D-glyceraldehyde 3-phosphate*? = D-xylulose 5-phosphate*? + D-ribose 5-phosphate*?

2.2.1.2 D-sedoheptulose 7-phosphate*? + D-glyceraldehyde 3-phosphate*? = D-erythrose 4-phosphate*? + D-fructose 6-phosphate*?

2.3.1 Acyl transfers

B 2.3.1.1 Acetyl-CoA + an L-aminoacid = CoA + N-acetylaminoacid

2.3.1.2 Acetyl-CoA + imidazole = CoA + N-acetylimidazole

2.3.1.3 Acetyl-CoA + 2-amino-2-deoxy-D-glucose = CoA + 2-acetylamino-2-deoxy-D-glucose

2.3.1.4 Acetyl-CoA + 2-amino-2-deoxy-D-glucose 6-phosphate = CoA + 2-acetylamino-2-deoxy-D-glucose 6-phosphate

2.3.1.5 Acetyl-CoA + arylamine = CoA + N-acetylarylamine

2.3.1.6 Acetyl-CoA + choline = CoA + O-acetylcholine

2.3.1.7 Acetyl-CoA + carnitine = CoA + O-acetylcarnitine

2.3.1.8 Acetyl-CoA + orthophosphate = CoA + acetylphosphate

2.3.1.a Acetyl-CoA + L-aspartate = CoA + N-acetyl-L-aspartate

2.3.1.b Acetyl-CoA + a β-D-galactoside = CoA + a 6-acetyl-β-D-galactoside

2.3.1.c Butyryl-CoA + orthophosphate = CoA + butyrylphosphate

2.3.1.d Acyl-CoA + a 1,2-diglyceride = CoA + a triglyceride

2.3.1.10 Acetyl-CoA + H_2S = CoA + thioacetate

2.3.1.11 Acetyl-CoA + thioethanolamine = CoA + S-acetyl-thioethanolamine

2.3.1.12 Acetyl-CoA + dihydrolipoate = CoA + S-6-acetylhydrolipoate

2.3.1.13 Acyl-CoA + glycine = CoA + N-acylglycine

2.3.1.14 Phenylacetyl-CoA + L-glutamine = CoA + α-N-phenylacetyl-L-glutamine

2.3.1.15 Acyl-CoA + L-glycerol 3-phosphate = CoA + monoglyceride phosphate

C 2.3.1.16 Acyl-CoA*? + acetyl-CoA = CoA + 3-oxo-acyl-CoA

2.3.1.17 2 acetyl-CoA*? = CoA + acetoacetyl-CoA

2.3.2 Aminoacyl transfers

B 2.3.2.1 L(or D)-glutamine + D-glutamyl-R = NH_3 + 5-glutamyl-D-glutamyl-R

2.4 Glycosyl transfers

2.4.1 Hexosyl transfers

B 2.4.1.1 $(\alpha\text{-}1,4\text{-glucosyl})_n$ + orthophosphate = $(\alpha\text{-}1,4\text{-glucosyl})_{n-1}$ + α-D-glucose 1-phosphate

2.4.1.2 $(\alpha\text{-}1,4\text{-glucosyl})_n$ + $(\alpha\text{-}1,6\text{-glucosyl})_m$ = $(\alpha\text{-}1,4\text{-glucosyl})_{n-1}$ + $(\alpha\text{-}1,6\text{-glucosyl})_{m+1}$

2.4.1.3 $(\alpha\text{-}1,4\text{-glucosyl})_n$ + D-glucose = $(\alpha\text{-}1,4\text{-glucosyl})_{n-1}$ + maltose

2.4.1.4 $(\alpha\text{-}1,4\text{-glucosyl})_n$ + D-fructose = $(\alpha\text{-}1,4\text{-glucosyl})_{n-1}$ + sucrose

2.4.1.5 $(\alpha\text{-}1,6\text{-glucosyl})_n$ + D-fructose = $(\alpha\text{-}1,6\text{-glucosyl})_{n-1}$ + sucrose

2.4.1.6 Maltose + D-glucose = D-glucose + α-1,3-glucosyl-glucose

2.4.1.7 α-D-glucosyl-1-R + HO-R' = α-D-glucosyl-1-R' + HO-R
(ROH and R'OH represent various ketoses, L-arabinose, phosphate or arsenate)

2.4.1.8 Maltose + orthophosphate = β-D-glucose 1-phosphate + D-glucose

2.4.1.9 (β-1,2-fructosyl)$_n$ + D-glucose = (β-1,2-fructosyl)$_{n-1}$ + sucrose

2.4.1.10 (β-2,6-fructosyl)$_n$ + D-glucose = (β-2,6-fructosyl)$_{n-1}$ + sucrose

2.4.1.18 Amylose = amylopectin

2.4.1.19 1,4-glucan = cyclic dextrin

2.4.2 Pentosyl transfers

B 2.4.2.1 Purine nucleoside + orthophosphate = α-D-ribose 1-phosphate + purine

2.4.2.2 Adenosine + orthophosphate = adenine + D-ribose 1-phosphate

2.4.2.3 Uridine + orthophosphate = uracil + D-ribose 1-phosphate

2.4.2.4 Thymidine + orthophosphate = thymine + 2-deoxy-D-ribose 1-phosphate

2.4.2.5 D-ribosyl-base + base' = D-ribosyl-base' + base

2.4.2.6 2-deoxy-D-ribosyl-base + base' = 2-deoxy-D-ribosyl-base' + base

2.4.2.7 AMP + pyrophosphate = adenine + 5 phospho-α-D-ribosyl-pyrophosphate

2.4.2.8 IMP + pyrophosphate = hypoxanthine + 5-phospho-α-D-ribosyl-pyrophosphate

2.4.2.9 UMP + pyrophosphate = uracil + 5-phospho-α-D-ribosyl-pyrophosphate

2.4.2.10 Orotidine-5'-phosphate + pyrophosphate = orotate + 5-phospho-α-D-ribosyl-pyrophosphate

2.4.2.11 Nicotinateribonucleotide + pyrophosphate = nicotinate + 5-phospho-α-D-ribosyl-pyrophosphate

2.4.2.12 Nicotinamideribonucleotide + pyrophosphate = nicotinamide + 5-phospho-α-D-ribosyl-pyrophosphate

2.4.2.13 ATP + L-methionine + H_2O= orthophosphate + pyrophosphate + S-adenosylmethionine

2.4.2.14 5-phospho-β-D-ribosylamine + pyrophosphate + L-glutamate = L-glutamine + 5-phospho-α-D-ribosyl-pyrophosphate

2.5 Alkyl (or related group) transfers

C 2.5.1.1 Dimethylallylpyrophosphate*? + isopentenylpyrophosphate*? = pyrophosphate + geranylpyrophosphate*?

A lengthy discussion of measurements on this reaction and related ones leads to a postulated mechanism[34] which would, in THO, label isopentenylpyrophosphate at C-2.

2.5.1.2 Thiamine + pyridine = heteropyrithiamine + thiazole

2.5.1.a 2-methyl-4-amino-5-hydroxymethylpyrimidine pyrophosphate + 4-methyl-5-(2'-phospho-ethyl)-thiazole = pyrophosphate + thiamine monophosphate

2.5.1.b S-adenosyl-methionine = 5'-(methylthio)-adenosine + 2-amino-γ-butyrolactone

2.5.1.c Galactose 6-sulphate residue of porphyran - sulphate = 3,6-anhydrogalactose residue

2.6 Transfers of nitrogenous groups

2.6.1 Amino transfers

Transamination entails exchange of the α-hydrogen of the amino-acids with hydrogen of water[35,36]. The rate of exchange of the α-hydrogen of aspartate is much greater than the actual rate of transamination between aspartate and α-oxoglutarate[37]. However, as expected, no exchange occurs without the appropriate enzyme[36].

This means that amino-acids which become tritiated in enzymatic transaminations will retain the label after killing and extraction of the organism by the methods used for this thesis (see "Methods", below).

Plausible mechanisms for transamination have been composed[38,39]. These involve formation of a Schiff's base between the aldehyde group of the obligatory coenzyme pyridoxal phosphate and the α-amino group of the amino-acid. Exchange of the α-hydrogen is explained by these mechanisms. The later discovery[40] that exchange of β-hydrogen is also important does not[41] necessitate any revision. However, a degradation product of a transaminase included pyridoxal phosphate bound by its aldehyde group[42]. If this is the case <u>in vivo</u>, then of course that aldehyde group will not be available for Schiff's base formation with amino-acids in transamination. The mechanism of enzymatic transamination is, then, still in doubt. There is no doubt, however, about the experimental labelling of amino-acids in the presence of transaminases.

A

2.6.1.1 L-aspartate* + 2-oxoglutarate = oxaloacetate + L-glutamate*

2.6.1.2 L-alanine* + 2-oxoglutarate = pyruvate + L-glutamate*

2.6.1.3 L-cysteine* + 2-oxoglutarate = mercaptopyruvate + L-glutamate*

2.6.1.4 Glycine* + 2-oxoglutarate = glyoxylate + L-glutamate*

2.6.1.5 L-tyrosine* + 2-oxoglutarate = <u>p</u>-hydroxyphenylpyruvate + L-glutamate*

2.6.1.6 L-leucine* + 2-oxoglutarate = 2-oxoisocaproate + L-glutamate*

2.6.1.7 L-kynurenine* + 2-oxoglutarate = o-aminobenzoylpyruvate + L-glutamate*

2.6.1.8 2,5-diaminovalerate* + 2-oxoglutarate = 2-oxo-5-aminovalerate + L-glutamate*

2.6.1.9 L-histidinolphosphate* + 2-oxoglutarate = imidazoleacetol-phosphate + L-glutamate*

2.6.1.5 L-phenylalanine* + 2-oxoglutarate = phenylpyruvate + L-glutamate*

2.6.1.10 D-aspartate* + 2-oxoglutarate = oxaloacetate + D-glutamate*

2.6.1.10 D-aspartate* + pyruvate = oxaloacetate + D-alanine*

2.6.1.11 α-N-acetyl-L-ornithine* + 2-oxoglutarate = N-acetyl-L-glutamate γ-semialdehyde + L-glutamate*

2.6.1.12 L-alanine* + a 2-oxoacid = pyruvate + an L-aminoacid*

2.6.1.13 L-ornithine* + a 2-oxoacid = L-glutamate γ-semialdehyde + an L-aminoacid*

2.6.1.a N-succinyl-L-diaminopimelate* + 2-oxoglutarate = N-succinyl-2-amino-6-oxo-L-pimelate + L-glutamate*

2.6.1.b L-alanine* + malonate semialdehyde = pyruvate + β-alanine*

2.6.1.c 4-aminobutyrate* + 2-oxoglutarate = succinate semialdehyde + L-glutamate*.

2.6.1.14 L-asparagine* + a 2-oxoacid = 2-oxosuccinamate + an aminoacid*

2.6.1.15 L-glutamine* + a 2-oxoacid = 2-oxoglutaramate + an aminoacid*

C 2.6.1.16 L-glutamine + D-fructose 6-phosphate*? = 2-amino-2-deoxy-D-glucose 6-phosphate*? + L-glutamate

2.6.2 Amidino transfers

B 2.6.2.1 L-arginine + glycine = L-ornithine + guanidinoacetate

2.6.3 Oximino transfers

B 2.6.3.1 (Pyruvateoxime or D-glucoseoxime) + (acetone or acetaldehyde) = (pyruvate or D-glucose) + (acetoxime or acetaldoxime)

2.7 Transfers of phosphorus-containing groups

2.7.1. Phosphate transferred to an alcohol

B 2.7.1.1-42 All these kinase-catalysed reactions fall in B. General reaction: Z phosphate (usually ATP) + Y-OH = Z + Y-O-phosphate

2.7.2 Phosphate transferred to carboxyl

B 2.7.2.1 ATP + acetate = ADP + acetylphosphate

2.7.2.2 ATP + NH_3 + CO_2 = ADP + carbamoylphosphate

2.7.2.3 ATP + D-3-phosphoglycerate = ADP + D-1,3-diphosphoglyceric acid

2.7.2.4. ATP + L-aspartate = ADP + 4-phospho-L-aspartate

2.7.3 Phosphate transferred onto nitrogen

B 2.7.3.1 ATP + guanidinoacetate = ADP + phosphoguanidino-acetate

2.7.3.2 ATP + creatine = ADP + phosphocreatine

2.7.3.3 ATP + L-argine = ADP + L-phosphoarginine

2.7.4 Phosphate transferred onto a phospho-group

B 2.7.4.1 ATP + $(phosphate)_n$ = ADP + $(phosphate)_{n+1}$

2.7.4.2 ATP + 5-phosphomevalonate = ADP + 5-pyrophosphomevalonate

2.7.4.3 ATP + AMP = ADP + ADP

2.7.4.4 ATP + a nucleoside monophosphate = ADP + a nucleoside diphosphate

2.7.4.5 ATP + deoxyCMP = ADP + deoxyCDP

2.7.4.5 ATP + CMP = ADP + CDP

2.7.4.6 ATP + a nucleoside diphosphate = ADP + a nucleoside triphosphate

2.7.5 Phospho-transfers with regeneration of donors (apparently catalysing intramolecular transfers)

B 2.7.5.1 D-glucose 1,6-diphosphate + D-glucose 1-phosphate = D-glucose 6-phosphate + D-glucose 1,6-phosphate

2.7.5.2 2-acetylamino-2-deoxy-D-glucose 1,6-diphosphate + 2-acetylamino-2-deoxy-D-glucose 1-phosphate = 2-acetylamino-2-deoxy-D-glucose 6-phosphate + 2-acetylamino-2-deoxy-D-glucose 1,6-diphosphate

2.7.5.3 D-2,3-diphosphoglycerate + D-2-phosphoglycerate = D-3-phosphoglycerate + D-2,3-diphosphoglycerate

2.7.5.4 D-1,3-diphosphoglyceric acid + D-3-phosphoglycerate = D-3-phosphoglycerate + D-2,3-diphosphoglycerate

2.7.6. Pyrophospho-transfers

B 2.7.6.1 ATP + D-ribose 5-phosphate = AMP + 5-phospho-α-D-ribosyl-pyrophosphate

2.7.6.2 ATP + thiamine = AMP + thiamine pyrophosphate

2.7.7 Nucleotidyl transfers

B 2.7.7.1 ATP + NMN = pyrophosphate + NAD

2.7.7.2 ATP + FMN = pyrophosphate + FAD

2.7.7.3 ATP + pantetheine 4'-phosphate = pyrophosphate + dephospho-CoA

2.7.7.4 ATP + sulphate = pyrophosphate + adenylylsulphate

2.7.7.5 ADP + sulphate = orthophosphate + adenylylsulphate

2.7.7.6 4 nucleoside triphosphates + RNA_n = 4 pyrophosphate + RNA_{n+4}

2.7.7.7 4 deoxynucleoside triphosphates + DNA_n = 4 pyrophosphate + DNA_{n+4}

2.7.7.8 A nucleoside diphosphate + RNA_n = orthophosphate + RNA_{n+1}

2.7.7.9 UTP + α-D-glucose 1-phosphate = pyrophosphate + UDPglucose

2.7.7.10 UTP + α-D-galactose 1-phosphate = pyrophosphate + UDP-galactose

2.7.7.11 UTP + α-D-xylose 1-phosphate = pyrophosphate + UDPxylose

2.7.7.12 UDPglucose + α-D-galactose 1-phosphate = α-D-glucose 1-phosphate + UDPgalactose

2.7.7.13 GTP + α-D-mannose 1-phosphate = pyrophosphate + GDPmannose

2.7.7.14 CTP + ethanolamine phosphate = pyrophosphate + CDPethanolamine

2.7.7.15 CTP + choline phosphate = pyrophosphate + CDPcholine

2.7.7.16 The ribonuclease reaction : transfer of the 3'-phosphate of a pyrimidine nucleotide residue of a polynucleotide from the 5'-position of the adjoining nucleotide to the 2'-position of the pyrimidine nucleotide itself, forming a cyclic nucleotide. The pancreatic enzyme (but not the leaf enzyme) also catalyses the transfer of the phosphate group from the 2'-position in the cyclic nucleotide to water; the overall reaction brings about the depolymerisation of RNA

2.7.8 Transfers of other substituted phospho-groups

B 2.7.8.1 CDPethanolamine + 1,2-diglyceride = CMP + a phosphatidyl-ethanolamine

2.7.8.2 CDPcholine + 1,2-diglyceride = CMP + a phosphatidylcholine

2.8 Transfers of sulphur-containing groups

2.8.1. Sulphur transfers

B 2.8.1.1 Thiosulphate + cyanide = sulphite + thiocyanate

2.8.1.2 3-mercaptopyruvate + cyanide = pyruvate + thiocyanate

2.8.2 Sulphate transfers

B 2.8.2.1 3'-phosphoadenylylsulphate + a phenol = adenosine 3',5'-diphosphate + an arylsulphate

2.8.2.2 3'-phosphoadenylylsulphate + a 3-β-hydroxysteroid = adenosine 3',5'-diphosphate + a steroid 3-β-sulphate

2.8.3 CoA transfers

B 2.8.3.1 Acetyl-CoA + propionate = acetate + propionyl-CoA

2.8.3.2 Acetyl-CoA + oxalate = acetate + oxalyl-CoA

2.8.3.3 Acetyl-CoA + malonate = acetate + malonyl-CoA

2.8.3.4 Acetyl-CoA + butyrate = acetate + butyryl-CoA

2.8.3.5 Succinyl-Co A + a 3-oxo-acid = succinate + a 3-oxo-acyl-CoA

2.8.3.6 Succinyl-CoA + 3-oxo-adipate = succinate + 3-oxo-adipyl-CoA

3 HYDROLYSES

3.1 Acting on ester bonds

3.1.1 Carboxylic ester hydrolyses

B 3.1.1-20 All hydrolyses of carboxylic esters and lactones fall in B.

3.1.2 Thioester hydrolyses

B 3.1.2.1-9 All these reactions fall in B.

3.1.3 Phosphoric monoester hydrolyses

B 3.1.3.1-17 All fall in B.

3.1.4 Phosphoric diester hydrolyses

B 3.1.4.1-8 All fall in B.

3.1.5 Triphosphoric monoester hydrolyses

B 3.1.5.1 (Deoxy-)GTP + H_2O = (deoxy-)guanosine + triphosphate

3.1.6 Sulphuric ester hydrolyses

B 3.1.6.1-5

3.2 Acting on glycosyl compounds

3.2.1 Glycoside hydrolyses

B 3.2.1.1-31

3.2.2 Hydrolyses of N-glycosyl compounds

B 3.2.2.1-6

3.2.3 Hydrolyses of S-glycosyl compounds

B 3.2.3.1 Merosinigrin + H_2O = allylisothiocyanate + D-glucose

3.3 Acting on ether bonds

3.3.1 Thioether hydrolyses

B 3.3.1.1 S-adenosyl-L-homocysteine + H_2O = adenosine + L-homocysteine

3.4 Acting on peptide bonds

B All 33 reactions in this section fall in B.

3.5 Acting on C-N bonds other than peptide bonds

3.5.1 In linear amides

B 3.5.1.1-12

3.5.2 In cyclic amides

B 3.5.2.1-6

3.5.3 In linear amidines

B 3.5.3.1-6

3.5.4 In cyclic amidines

B 3.5.4.1-10 A possible exception is 3.5.4.8

C 3.5.4.8 4-aminoimidazole*? + H_2O = unidentified product*? + NH_3

3.5.99 In other compounds

A 3.5.99.1 Riboflavin + H_2O = lumichrome + ribitol*

B 3.5.99.2 Thiamine + H_2O = 2-methyl-4-amino-5-hydroxymethylpyrimidine + 4-methyl-5-(2'hydroxyethyl)-thiazole

3.6 Acting on acid anhydride bonds

3.6.1 In phosphoryl-containing anhydrides

B 3.6.1.1-10 (Most are analogous to ATP + H_2O = $\textcircled{P}_1$ + ADP)

3.7 Acting on C-C bonds

3.7.1 In ketonic compounds

A 3.7.1.1 Oxaloacetate + H_2O = oxalate + acetate*

3.7.1.2 4-fumarylacetoacetate* + H_2O = acetoacetate* + fumarate

Acetyl-CoA enolises very slowly[78]; it is assumed that acetoacetyl-CoA will too.

3.8 Acting on halide bonds

3.8.1 In C-halide compounds

C 3.8.1.1 $CH_2BrCl + H_2O = HCHO^{*?} + HBr + HCl$

3.8.2 In P-halide compounds

B 3.8.2.1 Di-isopropylphosphofluoridate + H_2O = di-isopropylphosphate + HF

3.9 Acting on P-N bonds

B 3.9.1.1 Phosphocreatine + H_2O = creatine + orthophosphate

4 LYASE REACTIONS (i.e. non-hydrolytic removal of groups, leaving double bonds)

4.1 C-C lyase reactions

4.1.1 Carboxy-lyase reactions

A 4.1.1.1 A 2-oxo-acid = an aldehyde* + CO_2

4.1.1.2 Oxalate = formate* + CO_2

4.1.1.6 cis-aconitate$^{*?}$ = itaconate* + CO_2

4.1.1.7 Benzoylformate = benzaldehyde* + CO_2

4.1.1.8 Oxalyl-CoA + H_2O = formic acid* + CoA + CO_2

4.1.1.9 Malonyl-CoA* = acetyl-CoA* + CO_2

4.1.1.10 Aminomalonate$^{*?}$ = glycine* + CO_2

4.1.1.11 L-aspartate$^{*?}$ = β-alanine* + CO_2

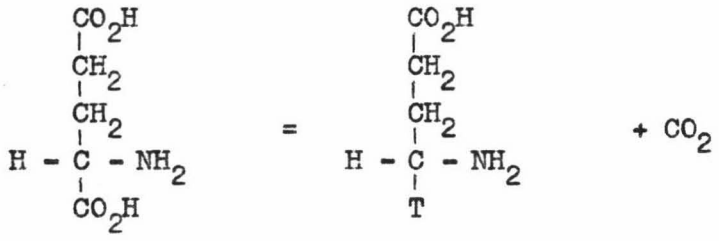
4.1.1.12 L-aspartate* = L-alanine* + CO_2

4.1.1.13 L-carbamoylaspartate$^{*?}$ = carbamoyl-β-alanine* + CO_2

4.1.1.14 L-valine$^{*?}$ = isobutylamine* + CO_2

4.1.1.15 L-glutamate$^{*?}$ = 4-aminobutyrate* + CO_2

The decarboxylation of L-glutamate to 4-aminobutyrate will tritiate the product at a "meso carbon atom":



L-glutamate 4-aminobutyrate

It appears that, if the reverse reaction is to give only L-glutamate (and no D-glutamate) then it must be specifically that hydrogen atom which was newly attached in the forward reaction, i.e. the tritium atom, which is removed in the carboxylation. L-glutamate enzymatically decarboxylated by glutamate α-decarboxylase[29], in D_2O, gave 4-aminobutyrate with only one deuterium atom incorporated, on what was the α-carbon of glutamate. This product, when incubated in ^1H_2O with the enzyme, lost its deuterium. The authors concluded that only one enantiomorph of monodeutero-4-aminobutyrate had formed. They also prepared the other enantiomorph. Configuration is retained in the reaction[102], the entering deuterium atom positionally replacing the carboxyl group.

4.1.1.15 4-methylene-L-glutamate*? = 2-methylene-4-aminobutyrate* + CO_2

4.1.1.16 L-3-hydroxyglutamate*? = 3-hydroxy-4-aminobutyrate* + CO_2

4.1.1.17 L-ornithine*? = putrescine* + CO_2

4.1.1.18 L-lysine*? = cadaverine* + CO_2

4.1.1.19 L-arginine*? = agmatine* + CO_2

4.1.1.20 meso-2,6-diaminopimelate*? = L-lysine* + CO_2

4.1.1.21 5'-phosphoribosyl-5-amino-4-imidazolecarboxylate = 5'-phosphoribosyl-5-aminoimidazole* + CO_2

4.1.1.22 L-histidine*? = histamine* + CO_2

4.1.1.24 p(or o)-aminobenzoate = aniline* + CO_2

4.1.1.23 Orotidine 5'-phosphate = UMP* + CO_2

4.1.1.25 L-tyrosine*? = tyramine* + CO_2

4.1.1.26 3,4-dihydroxy-L-phenylalanine*? = dihydroxyphenylethylamine* + CO_2

4.1.1.27 L-tryptophan*? = tryptamine* + CO_2

4.1.1.28 5-hydroxy-L-tryptophan*? = 5-hydroxytryptamine* + CO_2

4.1.1.29 L-cysteinesulphinate*? = hypotaurine* + CO_2

4.1.1.30 N-(L-pantothenoyl)-L-cysteine*? = pantetheine* + CO_2

4.1.1.c 4'-phospho-N-(L-pantethenoyl)-L-cysteine = pantetheine 4'-phosphate* + CO_2

4.1.1.d Uroporphyrinogen-III* = coproporphyrinogen* + $4CO_2$

4.1.1.33 ATP + 5-pyrophosphomevalonate* = ADP + orthophosphate + CO_2 + isopentenylpyrophosphate*

4.1.1.a 3-oxo-L-gulonate = L-xylulose* + CO_2

4.1.1.b UDPglucuronate = UDPxylose* + CO_2

4.1.1.f 2 3-phospho-D-glycerate* = D-ribulose 1,5-diphosphate* + CO_2 + H_2O

B 4.1.1.3 Oxaloacetate = pyruvate + CO_2

4.1.1.4 Acetoacetate = acetone + CO_2

4.1.1.5 (+)-2-acetolactate = (-)-acetoin + CO_2

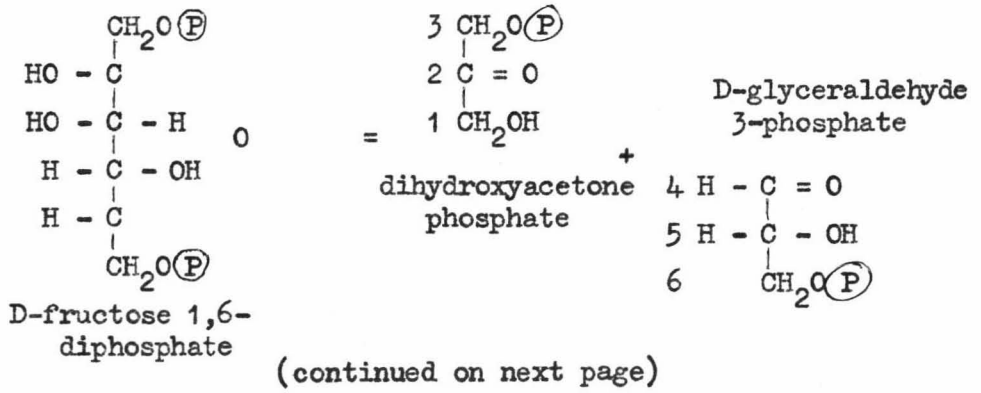
C 4.1.1.31 Orthophosphate + oxaloacetate = H_2O + phospho-enolpyruvate*? + CO_2

4.1.1.e Pyrophosphate + oxaloacetate = orthophosphate + CO_2 + phosphoenolpyruvate*?

4.1.1.32 GTP + oxaloacetate = GDP + phospho-enolpyruvate*? + CO_2

4.1.2 Aldehyde-lyase reactions

Confident predictions should perhaps not be offered about labelling in aldolase-catalysed reactions. It is probably necessary to resort to experiment. The aldolase-catalysed step in the Embden-Meyerhof glycolytic sequence has been studied using a purified enzyme preparation in THO[43]. The reaction is[44]:



(continued on next page)

A 4.1.2.a 2-oxo-pantoate = 2-oxo-isovalerate* + formaldehyde

4.1.2.b Fructose 1,6-diphosphate = dihydroxyacetone phosphate* + D-glyceraldehyde 3-phosphate

A 4.1.2.2 Erythrulose 1-phosphate = dihydroxyacetone phosphate* + formaldehyde

When the two product compounds, free of tritium, were incubated in THO with no enzyme, and then isolated and washed, neither was tritiated. However, the aldolase-catalysed breakdown of fructose 1,6-diphosphate entailed incorporation of one atom of hydrogen from water onto C-1 of dihydroxyacetone 3-phosphate. Only a very small degree of incorporation into fructose diphosphate occurred, and this was attributed to traces of triose phosphate isomerase[43].

Labelling in other aldolase-catalysed reactions is predicted on the basis of that result; but experiment might show them to be different.

Incidentally, the isolation[43] of dihydroxyacetone 3-phosphate stably labelled at C-1 is another example of an enolisable hydrogen which is exchanged out at only a very slow rate. Pyruvate has, however, been assumed in 4.1.2.1 and elsewhere to have no non-exchangeable hydrogen.

4.1.2.3 Ribose 5-phosphate = erythrulose 1-phosphate* + formaldehyde

4.1.2.5 L-threonine = glycine* + acetaldehyde

4.1.2.6 L-allothreonine = glycine* + acetaldehyde

4.1.2.7 A ketose 1-phosphate = dihydroxyacetone phosphate* + an aldehyde

4.1.2.8 Indole 3-glycerolphosphate = indole + D-glyceraldehyde 3-phosphate*

4.1.2.9 D-xylulose 5-phosphate + orthophosphate = acetylphosphate* + D-glyceraldehyde 3-phosphate

B 4.1.2.1 2-oxo-4-hydroxybutyrate = pyruvate + formaldehyde

4.1.2.c 6-phospho-2-oxo-3-deoxy-D-gluconate = pyruvate + D-glyceraldehyde 3-phosphate

4.1.2.4 2-deoxy-D-ribose 5-phosphate = D-glyceraldehyde 3-phosphate + acetaldehyde

C 4.1.2.d 7-phospho-2-oxo-3-deoxy-D-arabinoheptonate + orthophosphate = phosphoenolpyruvate*? + D-erythrose 4-phosphate + H_2O

4.1.2.e 8-phospho-2-oxo-3-deoxy-D-octonate + orthophosphate = phosphoenolpyruvate*? + D-arabinose 5-phosphate + H_2O

4.1.2.f p-hydroxymandelonitrile = p-hydroxybenzaldehyde*? + HCN

4.1.3.a ATP + citrate + CoA = ADP + orthophosphate + acetyl-CoA*? + oxaloacetate

4.1.2.10 Mandelonitrile*? = benzaldehyde*? + HCN

4.1.3 Ketoacid-lyase reactions

A 4.1.3.4 3-hydroxy-3-methylglutaryl-CoA* = acetyl-CoA* + acetoacetate

4.1.3.5 3-hydroxy-3-methylglutaryl-CoA* + CoA = acetyl-CoA* + H_2O + acetoacetyl-CoA

4.1.3.6 Citrate* = acetate* + oxaloacetate

4.1.3.7 Citrate* + CoA = acetyl-CoA* + H_2O + oxaloacetate

C 4.1.3.1 L_s - isocitrate*? = succinate*? + glyoxylate*?

4.1.3.2 L-malate*? + CoA = acetyl-CoA*? + H_2O + glyoxylate*?

4.1.3.3 N-acetylneuraminate*? = 2-acetylamino-2-deoxy-D-mannose*? + pyruvate

4.2 C-O lyase reactions

4.2.1 Hydro-lyase reactions

The stereospecificity, with regard to "indistinguishable" groups, of these enzymatic reactions, is discussed under the citric acid cycle, below.

A 4.2.1.2 L-malate* = fumarate + H_2O

4.2.1.3,4 Citrate* = cis-aconitate + H_2O

4.2.1.3 Isocitrate* = cis-aconitate + H_2O

4.2.1.5 D-arabonate* = 2-oxo-3-deoxy-D-arabonate + H_2O

4.2.1.6 D-galactonate* = 2-oxo-3-deoxy-D-galactonate + H_2O

4.2.1.7 D-altronate* = 2-oxo-3-deoxy-D-altronate + H_2O

4.2.1.8 D-mannoate* = 2-oxo-3-deoxy-D-mannoate + H_2O

4.2.1.a L-arabonate* = 2-oxo-3-deoxy-L-arabonate* + H_2O

4.2.1.b 2-amino-2-deoxy-D-gluconate* + H_2O = 2-oxo-3-deoxy-D-gluconate*? + NH_3 + H_2O

4.2.1.9 2,3-dihydroxyisovalerate* = 2-oxoisovalerate + H_2O

4.2.1.10 5-dehydroquinate = 5-dehydroshikimate* + H_2O

4.2.1.11 D-2-phosphoglycerate* = phospho-enolpyruvate + H_2O

4.2.1.12 D-6-phosphogluconate* = 2-oxo-3-deoxy-6-phospho-D-gluconate + H_2O

4.2.1.13 L-serine* + H_2O = pyruvate + NH_3 + H_2O

4.2.1.14 D-serine* + H_2O = pyruvate + NH_3 + H_2O

4.2.1.15 L-homoserine* + H_2O = 2-oxobutyrate* + NH_3 + H_2O

4.2.1.16 L-threonine* + H_2O = 2-oxobutyrate + NH_3 + H_2O

4.2.1.17 An L-3-hydroxyacyl-CoA* = a 2,3-trans-enoyl-CoA* + H_2O

4.2.1.17 An L-3-hydroxyacyl-CoA*# = a 3,4-trans-enoyl-CoA + H_2O

4.2.1.18 3-hydroxy-3-methylglutaryl-CoA* = trans-3-methylgutaconyl-CoA*? + H_2O

4.2.1.19 D-erythro-imidazoleglycerol phosphate* = imidazoleacetol phosphate + H_2O

4.2.1.20 L-serine* + indole* = L-tryptophan*? + H_2O

On the face of it, inter- or intramolecular dehydration is possible in this reaction. In fact the α-hydrogen of serine is exchanged with water during the reaction[45] which is strong evidence for the intramolecular mechanism.

B 4.2.1.24 2 5-aminolaevulinate = porphobilinogen + $2H_2O$

4.2.1.c Malonate semialdehyde = acetylene monocarboxylate + H_2O

4.2.1.1 H_2CO_3 = CO_2 + H_2O

C 4.2.1.21 L-serine*? + L-homocysteine = cystathionine*? + H_2O

4.2.1.22 L-serine*? + H_2S = L-cysteine*? + H_2O

4.2.1.23 L-serine*? + CH_3SH = S-methylcysteine*? + H_2O

4.2.99 Other C-O lyase reactions

A 4.2.99.2 O-phosphohomoserine* + H_2O = threonine* + phosphate

Conducting this enzymatic reaction in D_2O, and in THO, showed[49] that the newly-formed threonine molecule has incorporated exactly 2 hydrogen atoms from water; one of these being on the α-carbon. Residual O-phosphohomoserine was heavily labelled.

C 4.2.99.1 Hyaluronate = n 3(β -D-gluco-3,4-en-urono)-2-acetylamino-2-deoxy-D-glucose

4.3 C-N lyase reactions

4.3.1 Ammonia-lyase reactions

A 4.3.1.1 L-aspartate* = fumarate + NH_3

This reaction has[46] similar stereospecificity to the "fumarase" reaction in the citric acid cycle. The result is no labelling of fumarate.

4.3.1.2 L-threo-3-methylaspartate* = mesaconate + NH_3

4.3.1.3 L-histidine* = urocanate + NH_3

4.3.1.a L-phenylalanine* = trans-cinnamate + NH_3

If the stereospecificity of the enzyme is as for the "aspartase" reaction (4.3.1.1) then trans-cinnamate will not become labelled in this reaction.

4.3.1.b β -alanyl-CoA* = acrylyl-CoA*? + NH_3

4.3.2 Amidine-lyase reactions

C 4.3.2.1 L-arginosuccinate*? = fumarate*? + L-arginine

4.3.2.2 Adenylosuccinate*? = fumarate*? + AMP

As pointed out above, conducting these reactions (preferably with the pure enzymes) in THO should provide valuable information on their mechanism. This is true of many other reactions listed, too.

4.4 C-S lyase reactions

A 4.4.1.1 L-cysteine* + H_2O = pyruvate + NH_3 + H_2S

4.4.1.2 L-homocysteine* + H_2O = 2-oxobutyrate* + NH_3 + H_2S

4.4.1.3 S-dimethyl-β-propiothetin* = acrylate*? + dimethylsulphide

4.4.1.4 An S-alkyl-L-cysteinesulphoxide* = 2-aminoacrylate + an alkylsulphenate

B 4.4.1.5 S-lactoyl-glutathione = glutathione + methylglyoxal

4.5 C-halide lyase reactions

C 4.5.1.1 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane*? = 1,1-dichloro-2,2-bis-(p-chlorophenyl)-ethylene + HCl

In vitro, with no enzyme, and ethoxide added, this reaction gives no labelling[47]. It is just possible, however, that in vivo another (E_1 CB) mechanism might apply; in which case there would be labelling of the DDT.

5 ISOMERISATIONS

5.1 Racemase and epimerase reactions

5.1.1 Acting on aminoacids and their derivatives

Enzymatic racemisation of L-glutamate in THO gives tritiated glutamate[48].

A 5.1.1.1 L-alanine* = D-alanine*

5.1.1.2 L-methionine* = D-methionine*

5.1.1.3 L-glutamate* = D-glutamate*

5.1.1.4 L-proline* = D-proline*

5.1.1.a L-hydroxyproline* = D-allohydroxyproline*

5.1.1.5 L-lysine* = D-lysine*

5.1.1.6 L-threonine* = D-threonine*

5.1.1.7 2,6-LL-diaminopimelate* = meso-diaminopimelate*

5.1.2 Acting on hydroxyacids

A 5.1.2.2 L-mandelate* = D-mandelate*

5.1.2.1 L-lactate* = D-lactate*

5.1.2.3 L-3-hydroxybutyryl-CoA* = D-3-hydroxybutyryl-CoA

5.1.3 Acting on carbohydrates and derivatives

A 5.1.3.1 D-ribulose 5-phosphate* = D-xylulose 5-phosphate* (ref. 89)

5.1.3.2 UDPglucose* = UDPgalactose*

5.1.3.3 α-aldose* = β-aldose

B 5.1.3.a L-ribulose 5-phosphate = D-xylulose 5-phosphate.

This enzymatic reaction reached equilibrium in THO without any T having been incorporated[89].

5.1.3.b UDP-L-arabinose = UDP-D-xylulose

This reaction is classed in B on the strength of the similar 4-epimerisation 5.1.3.a which entails no incorporation.

5.1.3.c UDP-D-glucuronate = UDP-D-galacturonate

Again by analogy with 5.1.3.a, no labelling is expected. Experiment might, of course, prove otherwise.

5.1.3.d UDP-2-acetylamino-2-deoxy-D-glucose = UDP-2-acetylamino-2-deoxy-D-galactose

Once again, the evidence quoted under 5.1.3.a is relied on; but not very confidently, as these sugars are obviously different from ketopentose 5-phosphates!

A 5.1.99.a D-methylmalonyl-CoA* = L-methylmalonyl-CoA*

The hydrogen atom on the asymmetric centre exchanged with tritium when[90] the reaction was carried out in THO. It would presumably be slowly exchangeable in ^1H_2O after isolation, reasoning from the lability of the α-hydrogens of malonate, which allows preparation of the well-known sodio derivative.

5.2 Cis-trans isomerase reactions

B 5.2.1.a 3-maleylpyruvate = 3-fumarylpyruvate

This belongs in B because experiments in D_2O gave, surprisingly, no incorporation[92].

C 5.2.1.1 Maleate*? = fumarate*?

5.2.1.2 4-maleylacetoacetate*? = 4-fumarylacetoacetate

5.2.1.3 All-trans-retinene*? = 11-cis-retinene*?

5.3 Intramolecular redox reactions

5.3.1 Aldose $\rightleftharpoons$ ketose

A 5.3.1.1 D-glyceraldehyde 3-phosphate = dihydroxyacetone phosphate*

Dihydroxyacetone phosphate was incubated with triose phosphate isomerase[50] in THO. Almost one hydrogen from water was stably bound per molecule of equilibrium product, which is 96% dihydroxyacetone phosphate[51].

5.3.1.12 D-glucuronate* = D-fructuronate

5.3.1.2 D-erythrose* = D-erythrulose*

5.3.1.3 D-arabinose* = D-ribulose*

5.3.1.4 L-arabinose* = L-ribulose*

5.3.1.a D-arabinose 5-phosphate* = D-ribulose 5-phosphate*

5.3.1.5 D-xylose* = D-xylulose*

5.3.1.b L-rhamnose* = L-rhamnulose*

5.3.1.6 D-ribose 5-phosphate* = D-ribulose 5-phosphate*

5.3.1.7 D-mannose* = D-fructose*

5.3.1.8 D-mannose 6-phosphate* = D-fructose 6-phosphate*

5.3.1.9 D-glucose 6-phosphate* = D-fructose 6-phosphate*
See ref. 91

5.3.1.10 2-amino-2-deoxy-D-glucose 6-phosphate* $\overset{+H_2O}{\rightleftharpoons}$ D-fructose 6-phosphate* + NH_3

5.3.1.11 2-acetylamino-2-deoxy-D-glucose 6-phosphate* + $2H_2O$ = D-fructose 6-phosphate* + NH_3 + acetate

5.3.2 Keto- $\rightleftharpoons$ enol-

B 5.3.2.1 Keto-phenylpyruvate = enol-phenylpyruvate

5.3.3 Transposing C=C bonds

B 5.3.3.2 Dimethylallyl pyrophosphate* = isopentenyl pyrophosphate*

Mevalonic acid-2-^{14}C-5,5-d_2 was converted to squalene with a microsomal preparation[55]. The labelling in the resultant squalene strongly suggested that in the reaction 5.3.3.2 there had not been the expected indiscriminate exchange of protons with the solvent. This could be explained[34] by shielding of the protons from water during intramolecular proton transfer.

A 5.3.3.3 Vinylacetyl-CoA* = crotonyl-CoA*

B 5.3.3.1 Δ^5-3-ketosteroids = Δ^4-3-ketosteroids

The enzymatic conversion of 5-androsten-3,17-dione to 4-androsten-3,17-dione in D_2O labelled neither compound![54]

Intramolecular hydride shifts is a possible explanation. It would be rather difficult to discriminate experimentally that postulated mechanism from the other, namely transfer of protons shielded from water so that no exchange can occur.

5.4 Intramolecular transfers

5.4.1 Transferring acyl groups

B 5.4.1.1 2-lysolecithin = 3-lysolecithin

5.4.2 Transferring phosphoryl groups

B 5.4.2.1 2-phospho-D-glycerate = 3-phospho-D-glycerate

5.4.99 Transferring other groups

A 5.4.99.1 L-threo-3-methylaspartate* = L-glutamate*

5.4.99.2 2-methylmalonyl-CoA* = succinyl-CoA*

5.5 Intramolecular lyase reactions

A 5.5.1.1 (+)-4-carboxymethyl-4-hydroxy-isocrotonolactone* = cis-cis-muconate*?

6 LIGASE REACTIONS

6.1 Forming C-O bonds

6.1.1 Aminoacid-RNA ligase reactions

B 6.1.1.1-11 ATP + Z-CO_2H (an aminoacid) + sRNA = AMP + pyrophosphate + A-CO-sRNA

6.2 Forming C-S bonds

6.2.1 Acid-thiol ligase reactions

B 6.2.1.1-7 NucleosideTP + acid + CoA = NucleosideMP + pyrophosphate + acyl-CoA

6.3 Forming C-N bonds

6.3.1 Acid-ammonia ligase reactions

B 6.3.1.1 ATP + L-aspartate + NH_3 = ADP + orthophosphate + L-asparagine

6.3.1.2 ATP + L-glutamate + NH_3 = ADP + orthophosphate + L-glutamine

6.3.1.3 ATP + glycine + 5'-phosphoribosylamine = ADP + orthophosphate + 5'-phosphoribosyl-glycineamide

6.3.4.5 ATP + L-citrulline + L-aspartate = AMP + pyrophosphate + L-arginosuccinate

6.3.5 C-N ligases (glutamine as donor)

B 6.3.5.2 ATP + xanthosine 5'-phosphate + L-glutamine = AMP + pyrophosphate + GMP + L-glutamate

6.3.5.1 ATP + deamido-NAD + L-glutamine = AMP + pyrophosphate + NAD + L-glutamate

6.3.5.3 ATP + 5'-phosphoribosyl-formylglycineamide + L-glutamine = 5'-phosphoribosyl-formylglycineamidine + L-glutamate

6.3.4 Other C-N ligase reactions

B 6.3.4.1 ATP + xanthosine 5'-phosphate + NH_3 = AMP + pyrophosphate + GMP

6.3.4.2 ATP + UTP + NH_3 = ADP + orthophosphate + CTP

6.3.4.3 ATP + formate + tetrahydrofolate = ADP + orthophosphate + 10-formyltetrahydrofolate

6.3.4.4 GTP + IMP + L-aspartate = GDP + orthophosphate + adenoylsuccinate

6.3.2 Acid-aminoacid ligase reactions (peptide syntheses)

B 6.3.2.1 ATP + L-pantoate + β-alanine = AMP + pyrophosphate + L-pantothenate

6.3.2.2 ATP + L-glutamate + L-cysteine = ADP + orthophosphate + γ-L-glutamyl-L-cysteine

6.3.2.3 ATP + γ-L-glutamyl-L-cysteine + glycine = ADP + orthophosphate + reduced glutathione

6.3.2.4 ATP + D-alanine + D-alanine = ADP + orthophosphate + D-alanyl-D-alanine

6.3.2.6 ATP + 5'-phosphoribosyl-4-carboxy-5 amino-imidazole + L-aspartate = ADP + orthophosphate + 5'-phosphoribosyl-4-(N-succinocarboxamide)-5-aminoimidazole

6.3.2.a ATP + L-histidine + β-alanine = AMP + pyrophosphate + carnosine

6.3.2.7 ATP + UDP-muramyl-L-alanyl-D-glutamate + L-lysine = ADP + orthophosphate + UDP-muramyl-L-alanyl-D-glutamyl-L-lysine

C 6.3.2.5 CTP + 4'-phospho-L-pantothenate + L-cysteine = unidentified products of CTP breakdown + 4'-phospho-L-pantothenoyl-L-cysteine

6.3.3 Cyclo-ligase reactions

B 6.3.3.1 ATP + 5'-phosphoribosyl-formyl-glycineamide = ADP + orthophosphate + 5'-phosphoribosyl-5-aminoimidazole

6.4 Forming C-C bonds

A 6.4.1.4 ATP + 3-methylcrotonoyl-CoA* + CO_2 = ADP + orthophosphate + 3-methylglutaconoyl-CoA*

B 6.4.1.1 ATP + pyruvate + CO_2 = ADP + orthophosphate + oxaloacetate

6.4.1.2 ATP + acetyl-CoA + CO_2 = ADP + orthophosphate + malonyl-CoA

6.4.1.3 ATP + propionyl-CoA + CO_2 = ADP + orthophosphate + methylmalonyl-CoA

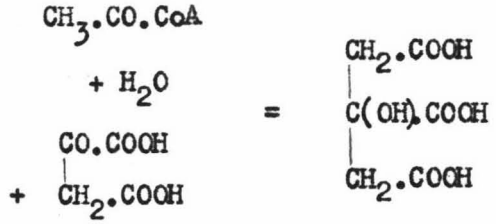
LABELLING EXPECTED IN METABOLIC PATHWAYS

As has been shown immediately above, many metabolites will become labelled by incorporation of tritium from THO during reactions. It remains to consider a further means of labelling, namely the carrying of tritium from one compound to another along a metabolic pathway, even in the absence of further reactions entailing incorporation of tritium from THO. If a compound A is being converted to E by the metabolic pathway A → B → C → D → E, and the first step entails tritium incorporation, say A + THO → B^*, then the tritium <u>may</u> be carried along with the carbon, acting as a conventional tracer comparable to isotopic carbon. However, the label may also be <u>lost</u> between B and E, depending on the nature of those steps along the pathway. We must therefore consider each pathway in detail. It will not surprise us if we are unable to make confident predictions in every case, because too little is at present known about the mechanism of many biochemical reactions - witness the number of reactions which had to be placed in category <u>C</u> in the list of known enzymatic reactions. The reader is again reminded that in such cases of doubt experiments using THO will very likely increase our knowledge of the mechanisms.

There are some pathways which cannot here be considered. Tracer experiments have often revealed that a certain amino-acid

is a precursor of a certain alkaloid. Data of that kind are obviously insufficient for our purpose now. The survey of pathways given here will be confined to those of which the steps are known in some detail[52,81].

One point to be observed is with regard to exchangeable hydrogen. It might be said that some molecules, e.g. pyruvate and oxaloacetate, have not a single non-exchangeable hydrogen. This might be an appropriate statement when we are considering in which compounds tritium could be detected after killing an organism, extracting compounds from it, and fractionating the extracts (although the <u>rate</u> of loss of label is the crucial factor, and not always easily predictable). But in the living organism, while metabolism is proceeding in THO, such molecules will, of course, <u>not</u> lose their exchangeable tritium. That means they could pass that tritium along a pathway to a non-exchanging position in another compound. An example of this possibility is the condensation reaction whereby acetate is taken into the citric acid cycle (enzymatic reaction no. 4.1.3.7):



Even if labelled from previous reactions, or merely from enolisation, acetyl-CoA and oxaloacetate would probably not be detected as labelled, by the method used for this thesis (see "Methods", below), owing to loss of tritium by enolisation during the extraction and chromatography. The point being made here is, however, that if pyruvate or oxaloacetate contained tritium bonded to carbon at the moment of condensation to citrate in THO, this tritium might well be fixed at a non-exchanging position in citrate.

Lack, possibility and confident expectancy of labelling will be indicated in the pathway maps by no asterisk, an asterisk plus a question mark, and an asterisk, respectively, placed on each hydrogen atom. Note that these refer to labelling detected in the extracted, chromatographed compounds. For each pathway, the steps will be considered in turn, with reference where appropriate to the list of enzymatic reactions. Then the possibility of "passing along" of tritium (discussed above) will be examined. Finally, possible loss of labels in the extraction and chromatography will be considered, leading at last to the assignment of asterisks with the meaning defined in the present paragraph (which is also their meaning in the list of enzymatic reactions).

Instantly exchangeable labels (e.g. H on O, S and N) will be ignored unless otherwise specified.

Reversibility will be usually assumed. As previously remarked, this is not an assertion of universal reversibility, but is merely the best working hypothesis in the absence of contrary evidence. The THO method could give information on whether reactions *in vivo* were reversible or not.

I. The Embden-Meyerhof Glycolytic Sequence

(Note: a vertical stick represents a hydroxyl group)

Reaction ia (2.7.1.1) No labelling.

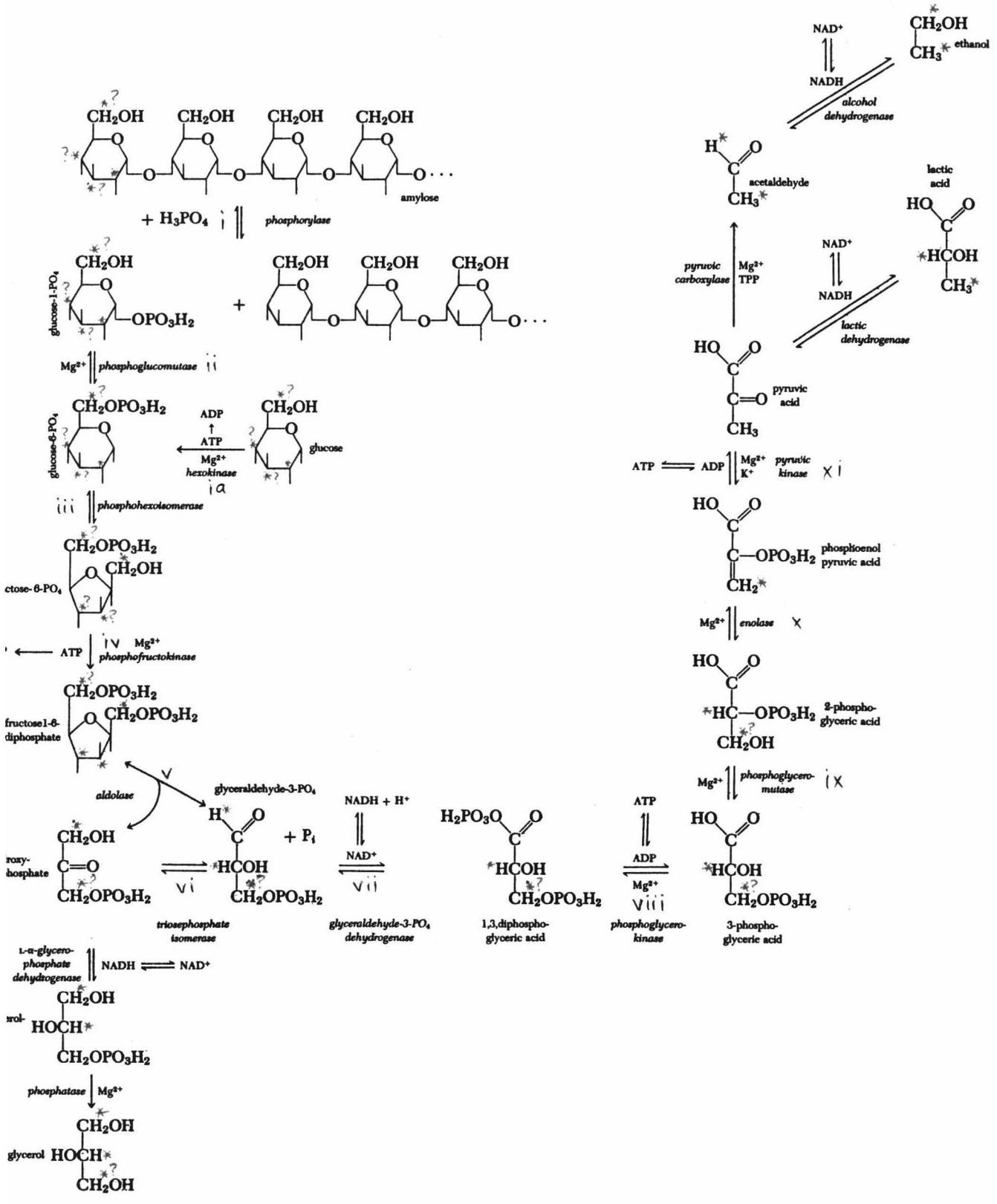
i (2.4.1.1) No labelling.

ii (2.7.5.1) The mechanism is interesting (ref. 44, p.462) but not expected to cause labelling.

iii (5.3.1.9) Topper[32] carried out this enzymatic reaction by incubation of glucose 6-phosphate with the purified enzyme in D_2O for 15 hr. at 26°. When isolated, fructose 6-phosphate was labelled at C-1 and glucose 6-phosphate was lebelled at C-2. This result, incidentally, constitutes a case of enolisation so slow that the enolisable hydrogen is not lost during isolation. Later work confirmed Topper's result and showed[91] that fructose 6-phosphate tritiated at C-1 is converted by the enzyme to glucose 6-phosphate labelled at C-2. Thus, while incorporation occurs, there is also transfer of tritium from C-1 to C-2 of the same molecule. At low temperatures the transfer is much more rapid than the incorporation.

PLATE I

Glycolysis and Alcoholic Fermentation

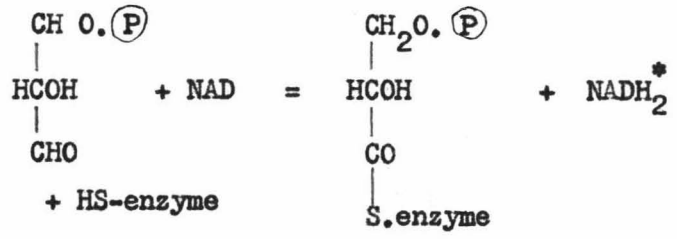


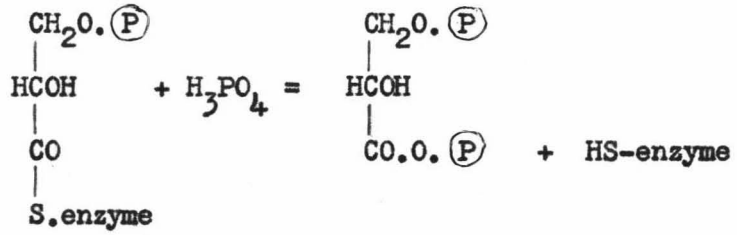
iv (2.7.1.11) No labelling.

v This reaction is catalysed by aldolase (4.1.2.b). When incubated in THO with no enzyme, dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate incorporated no tritium, but the enzymatic splitting of fructose 1,6-diphosphate in THO to produce these two compounds entailed the incorporation of one tritium atom onto C-1 (i.e. the carbon having no phosphate attached) of dihydroxyacetone phosphate[43]. Only a very little labelling of fructose 1,6-diphosphate occurred in that experiment, and was attributed to traces of contaminating triosephosphate isomerase.

vi (5.3.1.1) Specifically the hydrogen on C-1 of dihydroxyacetone (3-)phosphate other than that labilised by aldolase is exchanged in the interconversion of the triosephosphates by triosephosphate isomerase[50].

vii (1.2.1.12) All reactions from vii onwards must be multiplied by two, because all triosephosphate is reacted via glyceraldehyde phosphate. Reaction vii is believed (ref. 81, p.569) to proceed:





Clearly the reverse of the first of these steps will label glyceraldehyde phosphate at the aldehyde carbon.

viii (2.7.2.3) No labelling.

ix (2.7.5.3) The mechanism is analogous to that of step ii. No labelling.

x (4.2.1.11) The reverse reaction labels 2-phospho-D-glycerate at C-2.

xi (2.7.1.40) Pyruvate becomes labelled in the methyl group.

Reversibility throughout seems an acceptable assumption. New labelling occurs at steps iii, v, vi, vii, x and xi, as has just been described in detail. Which compounds will be tritiated after extraction, washing and chromatography, depends then on:

(a) the extent to which labels will be carried back "against the current", i.e. in the direction pyruvate ⟶ glucose. This cannot be predicted without data on the rate constants and throughput rates of each reaction. If there were little or no throughput of carbon from glucose to pyruvate, as might happen in a shortage of glucose or if the pathway(s) removing pyruvate went slow, then tritium would probably find its way back very quickly. But if the throughput were fast, at each step backwards there would be an appreciable dilution of the specific activity of the label being passed back.

It is convenient in this pathway to begin discussion of labelling at the last step. Pyruvate is labelled in the methyl group by reaction xi, and also probably by keto-enol tautomerism anyway. This label could be passed right back to the start of the sequence (but see the previous paragraph). Usually it would probably not get further back than step v, as step iv is almost irreversible (ref. 44, p.461). Therefore an asterisk is to be placed on this label back past step iv; and, to stress the point of the previous paragraph, it might not go that far back.

2-phospho-D-glycerate is labelled in step x. Similar remarks to those just made are applicable.

Step vi exchanges one of the hydrogens on what has been defined here as C-1 of dihydroxyacetone phosphate, and step v exchanges the other. Therefore glyceraldehyde phosphate will be labelled on the aldehyde carbon. That also is the result of step vii. When step vi occurs with its enzyme only, glyceraldehyde phosphate does not become labelled, owing to the enzyme's stereospecificity[50]; but when the whole pathway is operating in vivo, glyceraldehyde 3-phosphate will become labelled at C-1 and C-2. This is because both the hydrogens on what we are calling C-1 of dihydroxyacetone phosphate will be tritium, by the operation concurrently of the stereospecific enzymes of steps v and vi. Fructose 1,6-diphosphate will therefore be labelled at C-3 and C-4. Steps iii labels fructose 6-phosphate at C-1 and glucose 6-phosphate at C-2. These labels will carry forward and possibly back.

(b) Finally we consider what labels will be lost during the analysis of the killed organism. Probably pyruvate will enolise fast enough to lose its tritium. Contrary to what might be expected, hydrogen on C-2 of fructose 6-phosphate and on C-1 of glucose 6-phosphate exchanges out relatively slowly, as was mentioned above under step iii. If, however, the extract containing these labelled compounds was, for some reason, alkaline, the enolisation would of course be accelerated.

The pathway chart shows which hydrogens will be tritium in the isolated samples of each compound. The question marks are the unavoidable result of uncertainty in the matters of rates of enolisation and back-carrying of labels.

Some reactions which can be undergone by the pyruvate produced by the glycolytic sequence are 1.1.1.27 (giving labelled lactate), 4.1.1.1 (giving labelled acetaldehyde), 4.1.1.1 + 1.1.1.1 (giving labelled ethanol), or to feed the pyruvate into the citric acid (Krebs) cycle, conversion to acetyl-CoA by 1.2.4.1 and 2.3.1.12.

II. The Citric Acid Cycle (Krebs cycle)

This metabolic pathway is believed[93] to act in all respiring tissues of all animals, from protozoa to mammals. Also there is[93] strong evidence that this cycle is active in many respiring plants. It is essentially a means for oxidising acetyl-CoA which may arise

from any or all of three big classes of metabolites: carbohydrates, fats and proteins. If the source of acetyl-CoA is the glycolytic sequence, it will be tritiated in the acetyl group on arrival, because pyruvate is tritiated in that sequence. This tritium will not be lost from pyruvate <u>in vivo</u>, the cellular water being of course THO. Acetyl-CoA supplied from the "fatty acid spiral" will also be tritiated in the acetyl group, because the step which splits out the acetyl group from the fatty acid is (2.3.1.16):

$$CH_3(CH_2)_y.COCH_2CO.SCoA + CoA.SH$$
$$= CH_3(CH_2)_yCO.SCoA + CH_3^*CO.SCoA$$

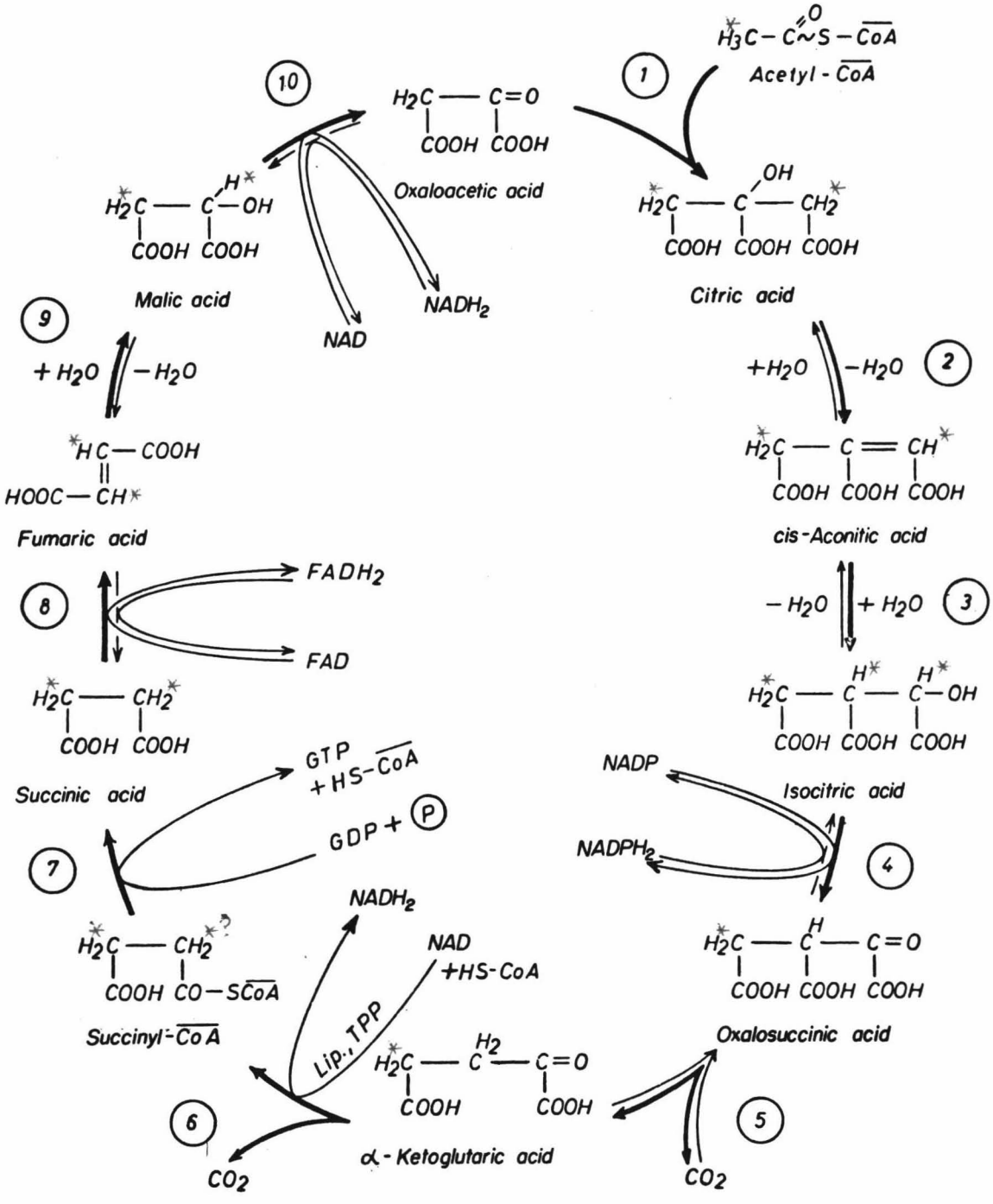
On the other hand, pyruvate obtained by deamination of alanine will probably not be labelled in the methyl, so the acetyl group might not in that case carry tritium into the citric acid cycle. By contrast, <u>transamination</u> of alanine, which also is a possible means of supplying acetyl units, entails exchange of the β-hydrogen of alanine with water[40] and will therefore supply labelled acetyl groups.

A significant aspect of the Krebs cycle is that the equilibrium in the oxidative decarboxylation of α-oxoglutarate is so far in the direction of succinate as to make the operation of the cycle essentially unidirectional (ref. 44, p.509). The introduction of new acetyl units also is highly exergonic (ref. 44, p.506).

The net reaction for the breakdown of acetyl-CoA can be written as follows:

$$CH_3—C(=O)\sim S\overline{CoA} + 3H_2O = 2CO_2 + 8[H] + HS\overline{CoA}$$

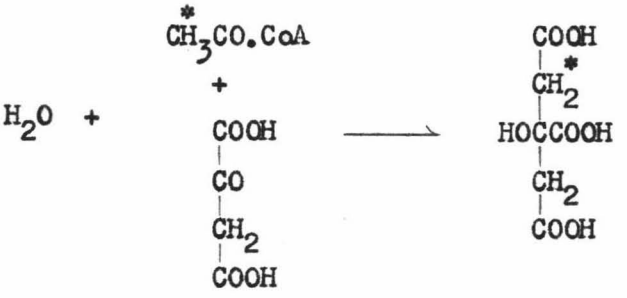
No oxygen is taken up in this oxidation; instead, water is added and dehydro-



Only <u>new</u> labels incorporated at each step will be shown in the consideration of each individual step; then "passing along" of labels will be considered. Finally loss of labels in the extract will be examined.

<u>Reaction 1</u> (4.1.3.7) As explained immediately above, the acetyl-CoA may be supplied tritiated or not, depending on its source. Usually it will be, and this is taken as the basis for the present treatment.

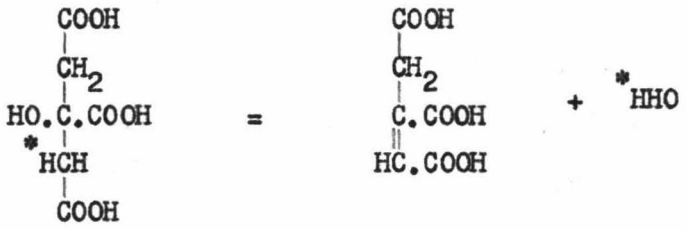
The condensing enzyme was shown fairly conclusively[77] not to catalyse enolisation of acetyl-CoA in D_2O. Better experiments[78], in THO, confirmed this. As mentioned above, tritiated acetyl-CoA will be assumed.



Little or no enolisation of oxaloacetate occurs in this reaction[77,100].

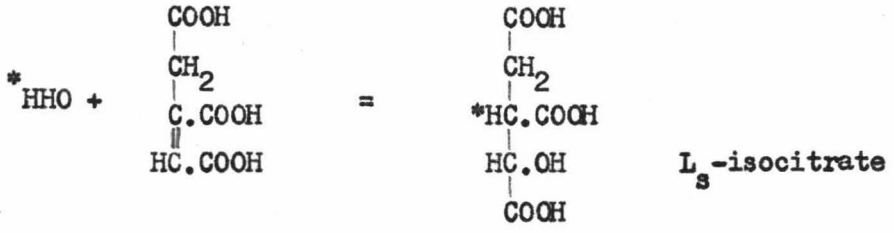
<u>2</u> (4.2.1.3) The enzyme distinguishes between the two "indistinguishable" ends of the citrate molecule. The proof of this makes a fascinating story[93,25,94]. It is so far beyond doubt that it will not be given here, but its conclusion merely quoted. The consequence is that a specific hydrogen on a specific methylene

group of citrate is exchanged with water.



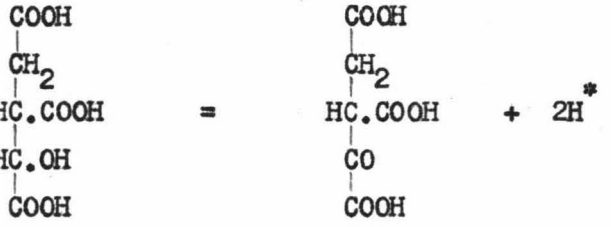
Cis-aconitate is not labelled in this reaction[25].

3 (4.2.1.3)

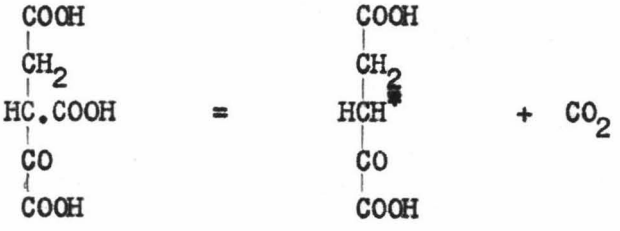


Cis-aconitate is not labelled by this reaction[25].

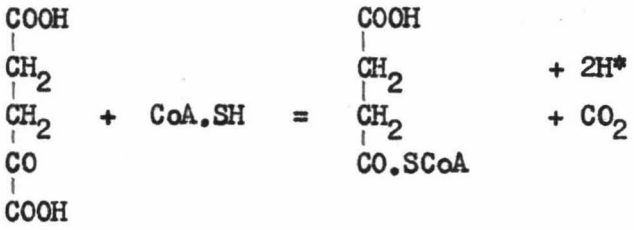
4 (1.1.1.42)



5 (1.1.1.42)

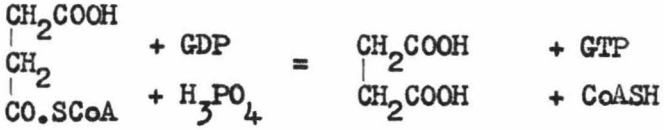


6 (1.2.4.2) + (2.3.1.12)?



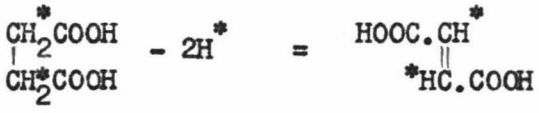
No new labelling.

7 (6.2.1.4)

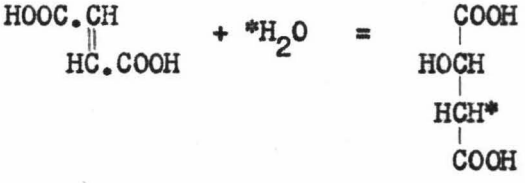


No new labelling.

8 (1.3.99.1)

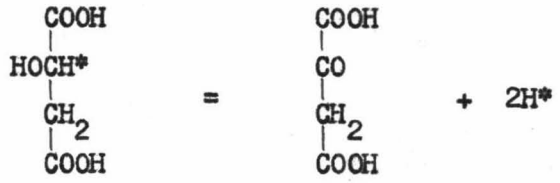


9 (4.2.1.2)



Trans addition of water occurs[95,96]. In D_2O no deuterium is incorporated into fumarate, and only one atom of deuterium into malate[26]. Only the one enantiomorph of malate is produced.

10 (1.1.1.37)



The hydrogen removed in the several oxidation reactions was shown, in the introductory remarks before the list of enzymatic reactions, to be in steady-state equilibrium with THO.

"Passing along" of labels

Owing to their supposed near-irreversibility, steps 1 and 7 will be taken as effectively preventing the passage backwards of labels.

Since the two methylene groups of succinate are both labelled (step 7), the methylene group of oxaloacetate will be labelled, so that when tritiated acetyl-CoA reacts in step 1, the product will be citrate in which all the non-exchangeable hydrogen is label. The stereospecificity of steps 2 and 3 will not, therefore, prevent the labelling of cis-aconitate in vivo, and in fact all members of the cycle will receive tritium passed along from the methylene groups of citrate. The result of that fact, and the labelling which occurs in steps 3,4,5,8 and 9, is that in vivo every intermediate of the cycle will be labelled.

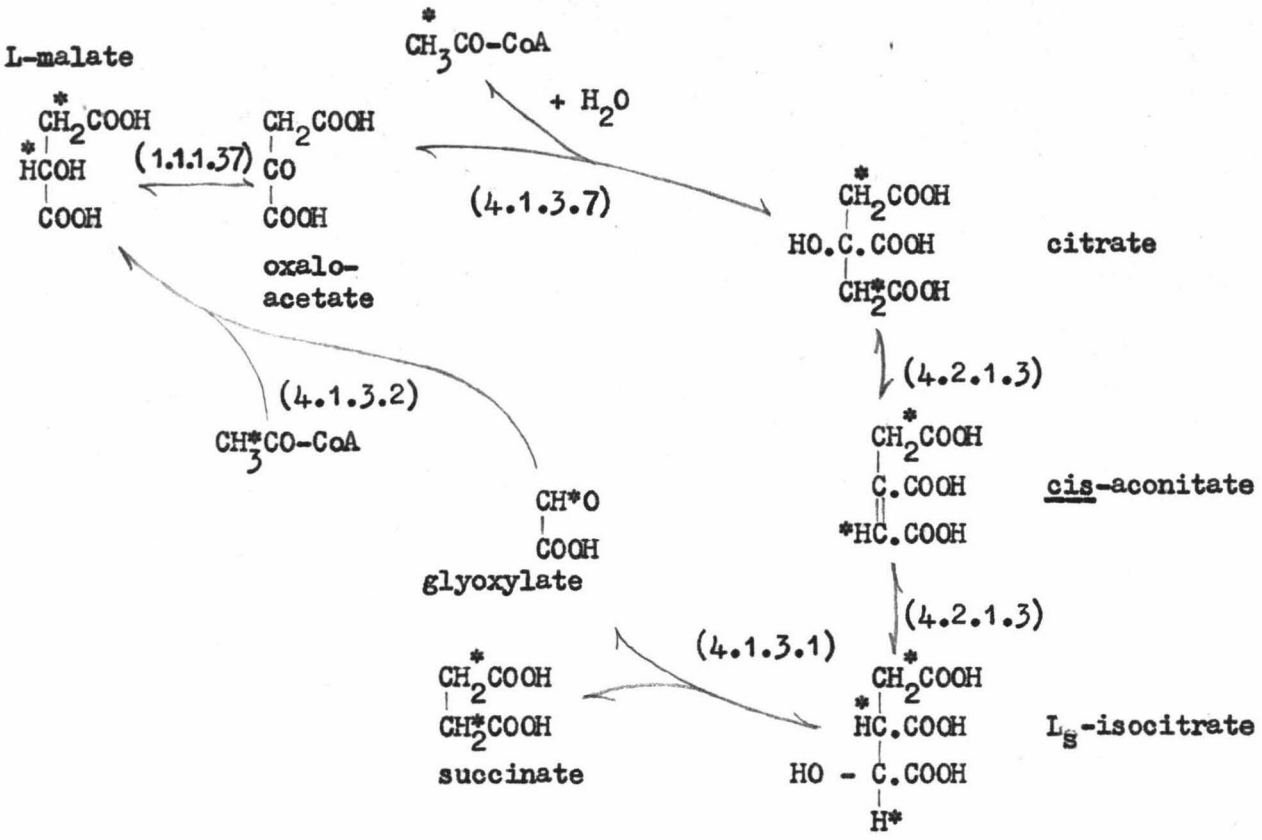
Loss of labels after extraction

Oxaloacetate, oxalosuccinate, succinyl-CoA and α-oxoglutarate will probably lose by enolisation the label on carbon adjacent to an oxo-group. It must also be considered that in practice α-oxo-acids can decompose (e.g. in chromatography[101]). Decarboxylation of α-oxo-acids will lay open to slow exchange any label on what had been the β-carbon of the α-oxo-acid.

The types of reasoning used in predicting which metabolites will become tritiated, and on which carbon atoms, have been set out rather fully in the analyses of the glycolytic sequence and the Krebs cycle. For the subsequent pathways the reasoning will not be written down here so fully; mostly just its results will be given.

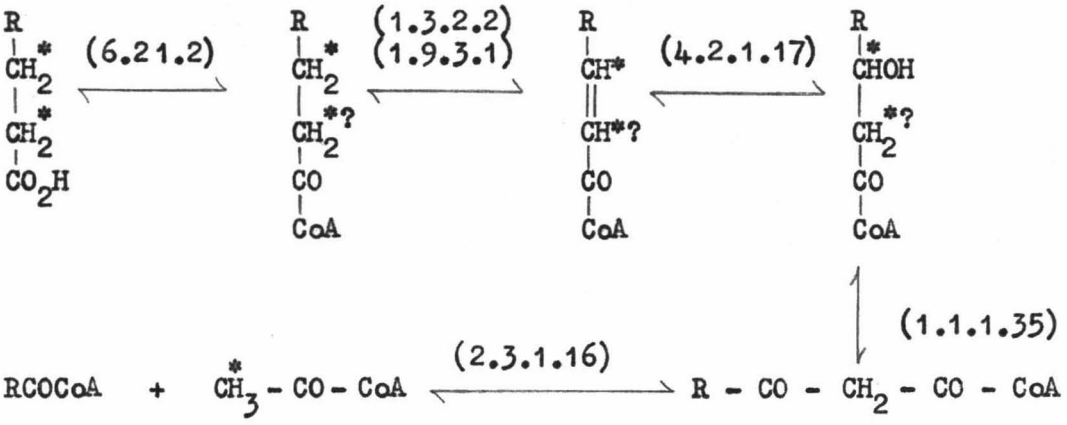
III. The Glyoxylate By-pass

This "modified Krebs cycle" occurs in plants and micro-organisms. It is a means for converting two acetyl groups into a C_4 acid. Seeds rich in fatty acids might degrade them to acetyl-CoA, convert that to succinate, and then via oxaloacetate and phosphoenolpyruvate obtain any of the metabolites of the Embden-Meyerhof sequence (ref. 81, p.557). Tritiated acetyl groups are assumed (see the discussion of this point, under the Krebs cycle, above).



Note: randomisation occurs at succinate, all four α-hydrogens of which are indistinguishable by any means imaginable (provided all the succinate is not held locked on a stereospecific enzyme site). The effect of that randomisation traced back to citrate means all non-exchangeable hydrogen on isocitrate, aconitate and citrate is label, regardless of the stereospecificity of "aconitase" (4.2.1.3). This is made doubly sure by the fact that all hydrogen on carbon which goes to make citrate in the condensation is, in vivo, label.

IV. Fatty acid breakdown ("the spiral")



The question marks express doubt about rates of enolisation in the extract.

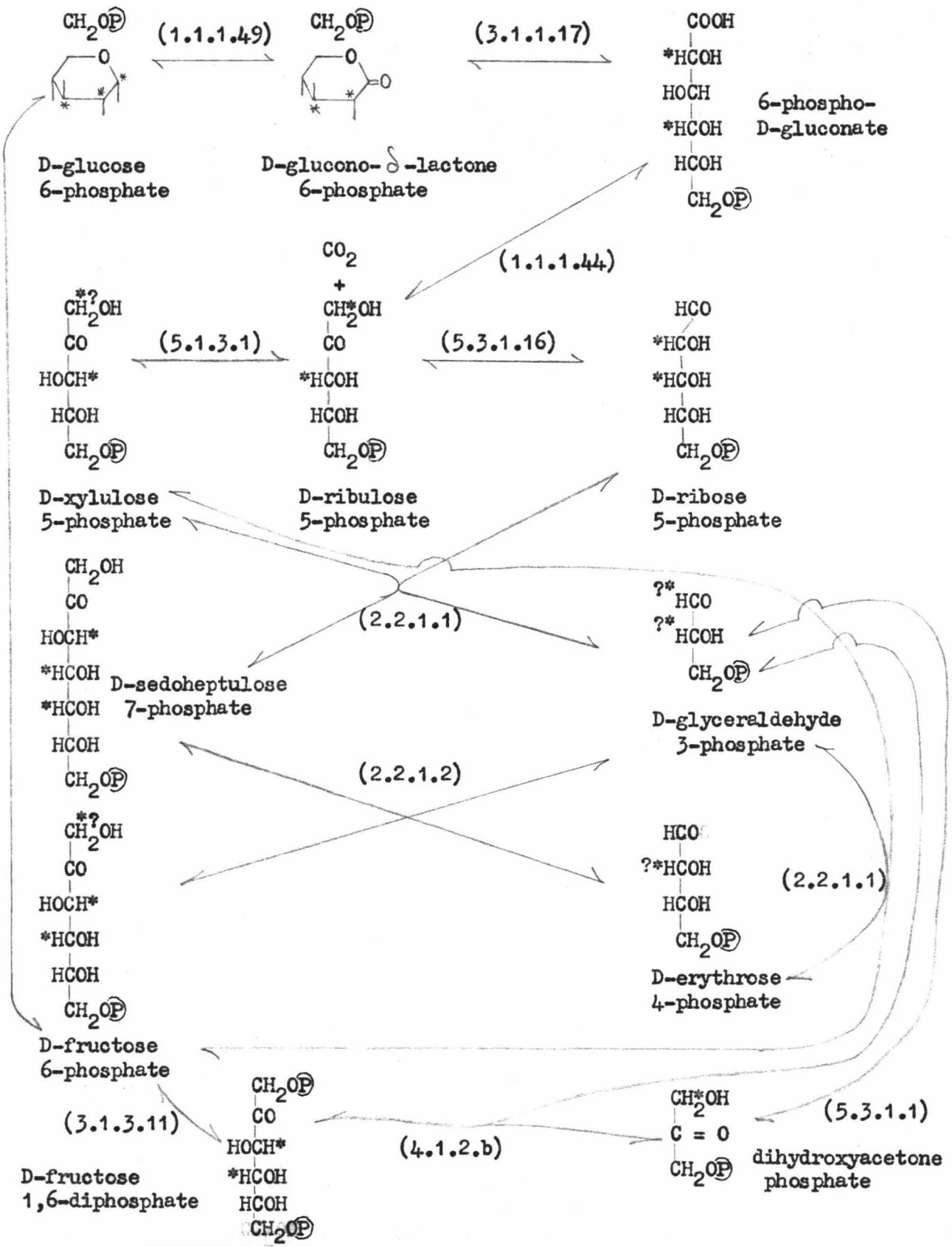
V. Fatty acid synthesis

Schoenheimer et al[57] showed that unsaturated and saturated fatty acids, the former more than the latter, incorporated deuterium from labelled water, during their biosynthesis in whole mice. This was confirmed[61] in liver slices with THO, when more tritium was found on odd- than even-numbered carbons of the fatty acids, contrary to expectations[97]. The detailed mechanisms of fatty acid biosynthesis cannot at present be written, in the opinion of Kosower[34]. Malonyl-CoA is the starting compound, gained e.g. by carboxylation of acetyl-CoA. The enzymes of the later steps behave as one particle[97]. No reaction scheme will be presented here as the matter is in some doubt. A discussion of this and one possible pathway is given in ref. 81, p.558.

Glucose-6-P
NADP
NADPH2
Gluconolactone-P
Gluconic acid-P
NAD
NADPH2
[3-Keto-gluconic acid -P]
CO2
Ribulose-5-P
Rib-P
Xyl-P
C5
C5
C5
C3
C7
C6
C4
C6
C3
"C2"
"C3"
"C2"

PLATE III

VI. The Hexose Monophosphate Shunt



Owing to lack of knowledge on the mechanisms of reactions (2.2.1.1) and (2.2.1.2) there is uncertainty about where some compounds will be tritiated. The positions of some labels shown are based on the questionable assumption that (2.2.1.1) and (2.2.1.2) entail similar hydrogen shifts to (4.1.2.b), the mechanism of which is well known[43], as is[50] that of (5.3.1.1).

In summary, the exact pattern of labelling in this pathway cannot be predicted, but the following compounds are confidently expected to be labelled:

D-glucose 6-phosphate

D-glucono-δ-lactone 6-phosphate

6-phospho-D-gluconate

D-ribulose 5-phosphate

D-xylulose 5-phosphate

D-ribose 5-phosphate

dihydroxyacetone phosphate

D-fructose 1,6-phosphate

D-sedoheptulose 7-phosphate

The following might be labelled:

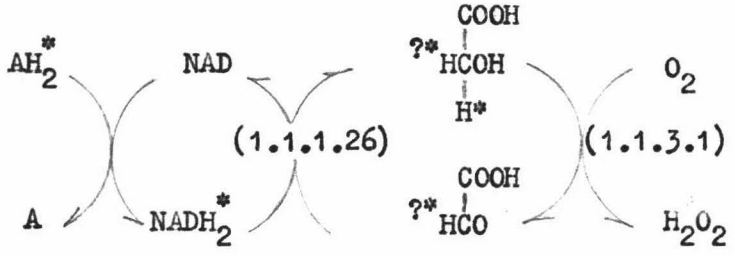
D-erythrose 4-phosphate

D-glyceraldehyde 3-phosphate

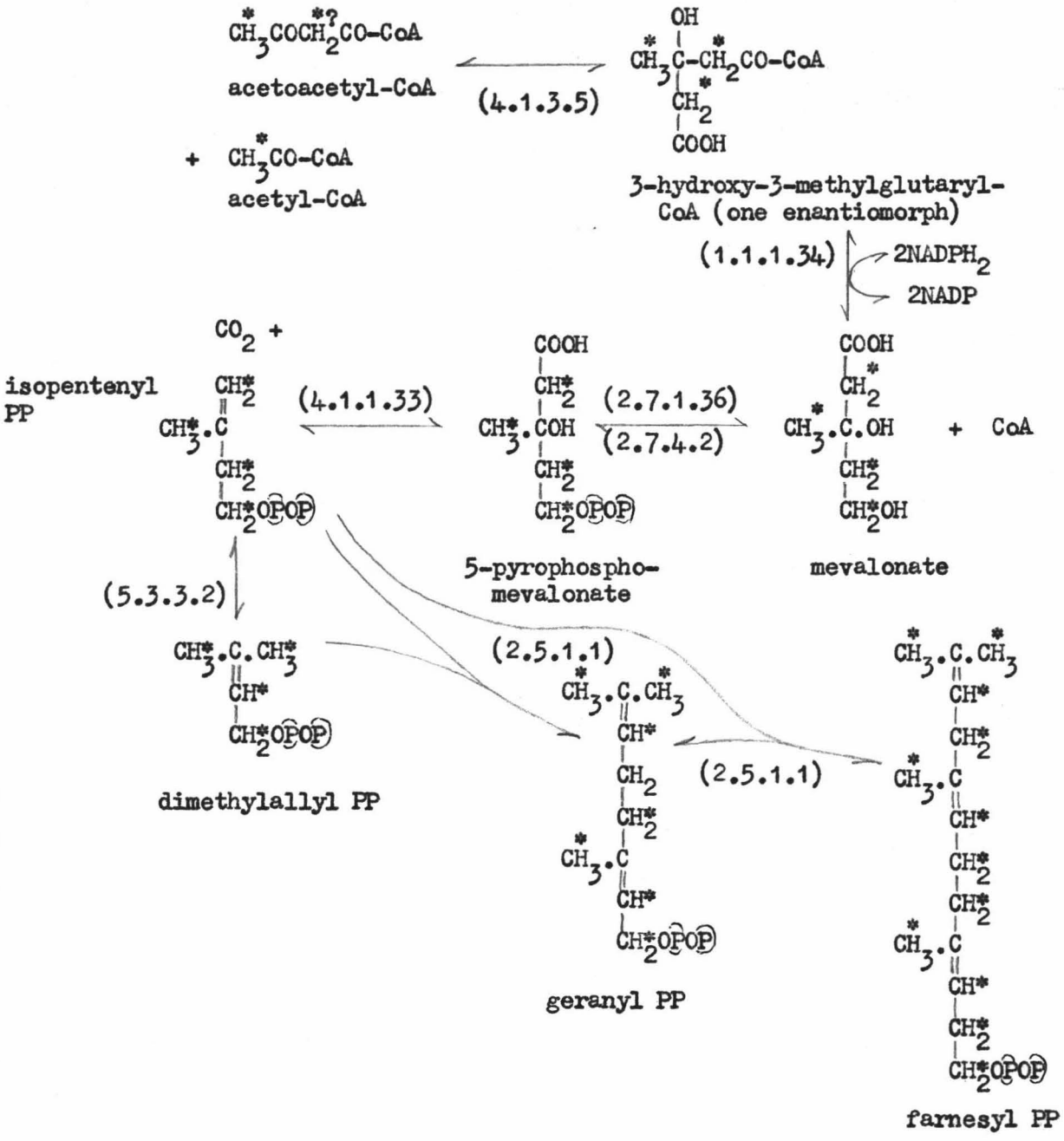
VII. A hydrogen-transport pathway

In certain plants over 50% of the respiration is by this system[98]. AH_2 represents various substrates[98]. Whether or not glyoxylate becomes labelled depends on the stereospecificity of the enzymes reducing and oxidising it.

Glyoxylate and glycollate might be wholly or partly lost by evaporation from extracts.



VIII. Synthesis of Isoprenoids



Divergent, imperfectly known pathways now lead to

(a) many terpenes

(b) ubiquinone, rubber, phytol, tocopherol, vitamin K and carotenoids

(c) by tail-tail linking, all-trans squalene.

These pathways could be studied by the "THO method". They include some unusual reaction types. Particularly baffling is the enzyme (1.99.1.13) cyclising squalene which of course has in the chemist's view no functional groups for the enzyme to "grasp", to a highly stereospecific product, lanosterol. How the several asymmetric centres of lanosterol are uniquely determined in configuration is a question which tritium incorporation might help to answer.

IX. The Urea Cycle

Only one reaction in this pathway looks like a candidate for causing labelling, viz. the cleavage of arginosuccinate (4.3.2.1). As that reaction's mechanism is unknown and not readily predictable, it cannot be said whether the urea cycle will entail tritium incorporation.

X. Purine Nucleotide Synthesis

The D-ribose 5-phosphate will presumably come from the hexosemonophosphate shunt or the photosynthesis cycle. In either case it will be labelled, and will retain that label right through to actual nucleotide formation. As for possible labelling in the purine moiety, this will occur if the two carbons donated by the folic acid pool bring tritium with them, as they might; but this is uncertain. Impossible to predict, then, is the extent of labelling of intermediates along the pathway.

XI. Pyrimidine Nucleotide Synthesis

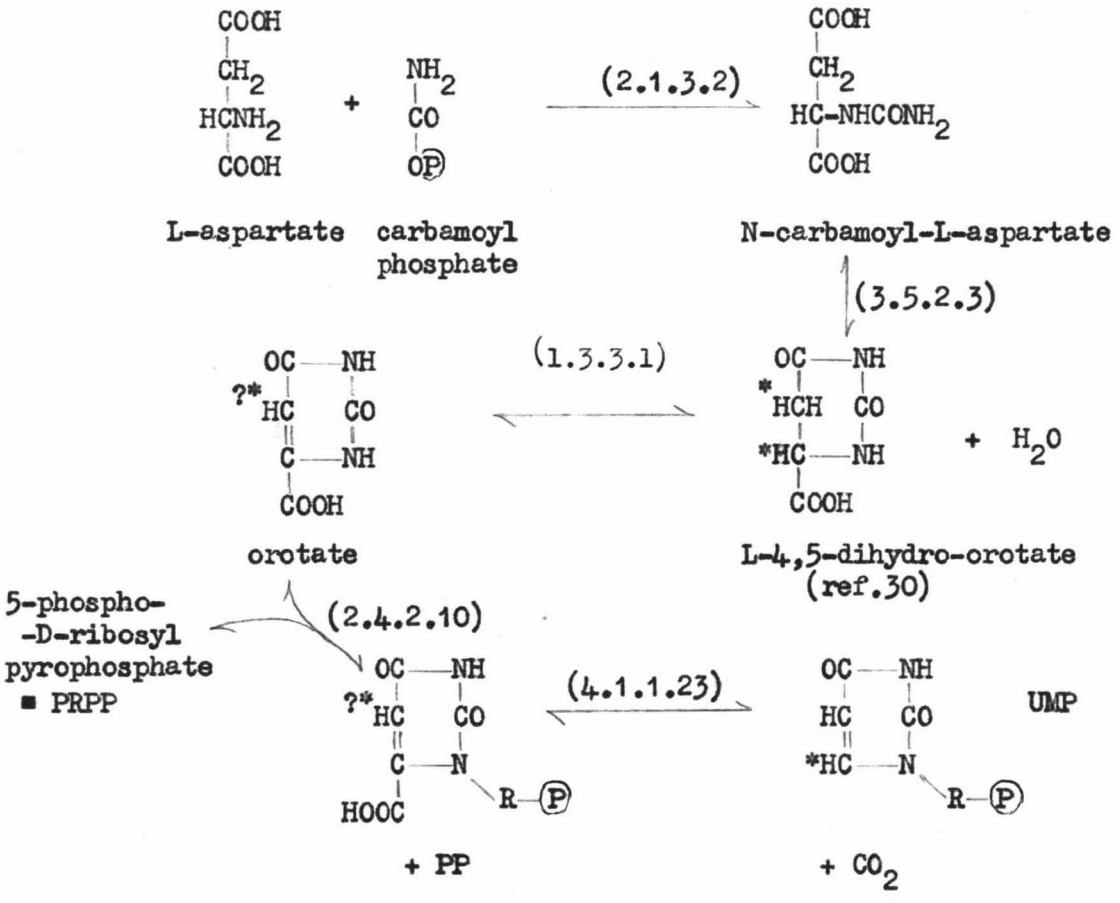
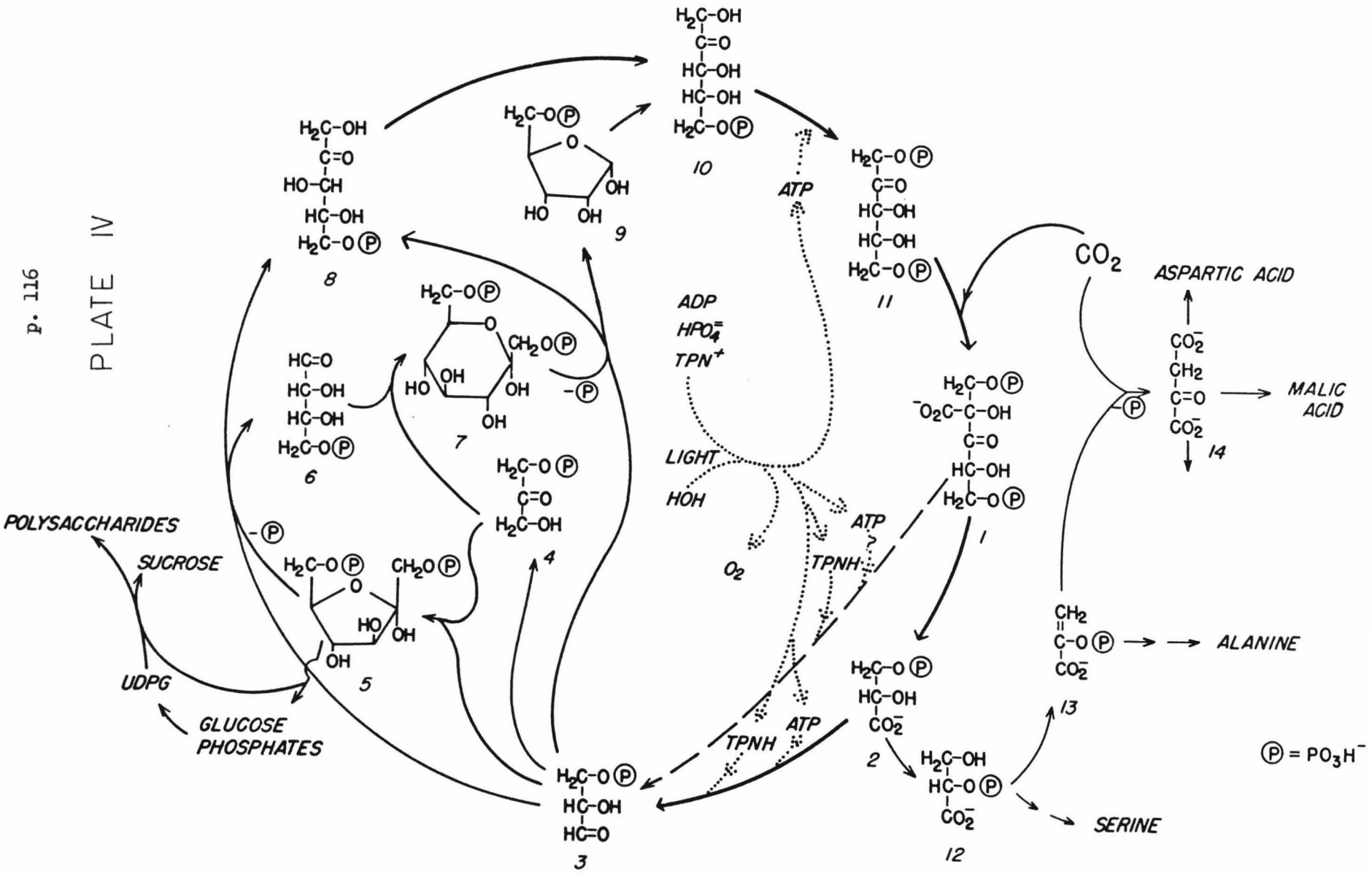


PLATE IV


POLYSACCHARIDES
SUCROSE
UDPG
GLUCOSE PHOSPHATES
ADP
HPO4=
TPN+
LIGHT
HOH
O2
ATP
TPNH
CO2
ASPARTIC ACID
MALIC ACID
ALANINE
SERINE
Ⓟ = PO3H−
1
2
3
4
5
6
7
8
9
10
11
12
13
14

XII. Photosynthesis of Sucrose

The overall equation is:

$$12CO_2 + 11H_2O = C_{12}H_{22}O_{11} + 12O_2$$

One would deduce from the equation that THO will provide all the hydrogen attached to carbon in this process. In fact the proximal hydrogen donor is in relatively rapid equilibrium with water, as was shown by introducing THO vapour into soybean leaves[60].

The main pathway of carbon is[19] a reductive sugar phosphate cycle strikingly similar to the hexose monophosphate shunt, and involving very similar enzymes. For that reason alone, a precise prediction of which intermediates will be labelled at which carbon atoms cannot be essayed. The matter is under experimental study[60]. On the other hand, as in the hexose monophosphate shunt, some of the mechanisms are well known. Without making risky predictions about positions or extents of labelling, we can confidently expect to become labelled:

D-glyceraldehyde-3Ⓟ

dihydroxyacetone-Ⓟ

D-fructose 1,6-di Ⓟ

D-fructose-6Ⓟ

D-xylulose-5Ⓟ

D-ribulose-5Ⓟ

D-glucose-6Ⓟ

D-ribose-5Ⓟ

Sucrose

D-sedoheptulose-7Ⓟ

D-erythrose-4Ⓟ

D-sedoheptulose 1,7-diⓅ

D-ribulose 1,5-diⓅ

3-Ⓟ-D-glycerate

1,3-diⓅ-D-glycerate

UDP-glucose

fructose

D-glucose-1Ⓟ

In short, every intermediate of the cycle will very likely be labelled.

Some important pathways have been omitted from this account. But several have been analysed for the first time; and those which have been re-examined have, for the first time, been looked into thoroughly. It would be tedious to specify here the errors and omissions in this task of previous writers[1,2]. Future analyses of the kind will, it is suggested, prevent most mistakes by using the modes of reasoning described here.

MATERIALS

Seeds: White mustard (Sinapis alba) seeds were bought soon after harvest and kept in a desiccator containing silica gel. Germination tests, initially and every few months, gave at least 170 of 200 seeds sprouting in two days at room temperature (i.e.85% germination) on set filter paper in Petri dishes.

Fern spores: A pinna of Cyathea medullaris (the giant tree fern or mamaku), showing ripe sori, was laid on a sheet of paper overnight. Dehisced sporangia were separated from spores by "surface sifting": it was found that agitating the mixture on a sloping sheet of unfilled writing paper caused the sporangia to separate, travelling more quickly down the slope. The spores were inspected under a microscope and no contaminants could be observed.

Sapwood: A Pinus radiata tree, trunk diameter c. $\frac{1}{2}$ m., was laid open with an axe to the third annual ring from the outside. From a large chip of the sapwood thus removed was cut with a scalpel, immediately before use, a piece roughly cubic, of side 2 mm.

Sperm: Bull semen was kindly supplied, frozen, by Dr D. Fielden, Awahuri.

THO: The Radiochemical Centre, Amersham, supplied phials of 5 ml. (25 curies).

Ethanol: Technical abs. EtOH was kept in a polythene wash bottle.

Deionized water: Distilled water was passed slowly (3 ml./min.) down a column of Biodemineralit, a mixed resin exchanging H^+ for cations and OH^- for anions. The product was kept in a polythene wash bottle.

Chromatography paper: The sole type used was Whatman no. 4, which is similar to Whatman no.1 but was found to give doubly fast development, and is known to give improved separation of some compounds[132].

Metal ions such as Ca^{++} are present in this paper and cause tailing or even origin sticking of sugar phosphates[132]. These metals therefore had to be chelated to prevent this trouble. The sheets of paper were set up as for descending chromatography, about ten sheets hanging from each side of the trough. About $\frac{1}{2}$-1 l. of $\frac{1}{2}\%$ oxalic acid (B.D.H.) was allowed to descend, followed by 20-40 l. of deionized water. After drying, the paper was cut to size.

This acid washing incidentally further increases considerably the speed of development of chromatograms.

Chromatography solvents:

(1) Phenol (redistilled, collecting fraction b.p. 180-182°) 100 g : deionized water 39 g. Kept at 4° (ref. 19).

(2) (a) 3750 ml n-butanol (B.D.H.)
253 ml deionized water.

(b) 1760 ml propionic acid (B.D.H.)
2240 ml deionized water.

Immediately before use, solvent (2) was made up by mixing equal volumes of (a) and (b)[132]. More (b) was added dropwise if necessary for production of a one-phase system.

(3)	Acetone (technical)	4 v.	
	distilled water	1 v.	(Ref. 134)
(4)	n-butanol (B.D.H.)	3 v.	
	pyridine (B.D.H.)	2 v.	
	distilled water	1 v.	
	Kept at 4°.		(refs. 135,136)
(5)	i-propanol (B.D.H.)	80 v.	
	.880 ammonia	5 v.	
	deionized water	15 v.	(ref. 2)
(6)	n-propanol (B.D.H.)	50 v.	
	cineole (B.D.H.	50 v.	
	98% formic acid (B.D.H.)	20 v.	
	deionized water	to saturation	(ref. 2)

Chromatogram Sprays: For amino acids, 0.3% ninhydrin in ethanol (aerosol can, Sigma Biochemical Corp.) was most convenient. The ninhydrin /copper/ s-collidine spray of Moffat and Lytle[137] was found to be much more specific in its colour reactions, even discriminating leucine from isoleucine; but was much less convenient and rarely needed.

For organic acids, (1) 0.1% mercurochrome in ethanol[138],

or (2) 2,6 dichlorophenolindophenol 0.1% in abs. EtOH (ref. 2).

For sugars (1) 0.5 ml saturated aq. $AgNO_3$ was added to 50 ml acetone. Any precipitate was redissolved by adding the minimum amount of water. The chromatogram was sprayed lightly with this solution, and then with a second, made by making 2.5 ml of 40% aq. NaOH up to 50 ml with abs. EtOH. When the spots have appeared, a 5% aq. $Na_2S_2O_3$ spray is applied to lighten the background. This is the method of Trevelyan et al[139] as modified by Anet and Reynolds[140], and by Bailey[141]. This spray was less convenient to use than the other one described below; also the silver nitrate solution did not keep well. The worst detraction, however, was the positive reaction with residual phenol from chromatography solvent no.1.

(2) 0.9 g aniline + 1.66 g phthalic acid in 100 ml water-saturated n-BuOH (Merck, in aerosol cans).

Scintillator for autography: Technical toluene was purified by running slowly (1 ml./min.) down a 5 x 30 cm column packed by the slurry method with chromatographic alumina (in the lower 20 cm) and finely powdered silica gel[1]. This purification is needed to remove sulphur compounds which quench fluorescence.

To $2\frac{1}{2}$ l. of purified toluene was added 6 g p-diphenylbenzene (B.D.H.). The concentration is not critical, as fluorescence efficiency does not vary sharply with concentration of phosphor[142].

Scintillator for counting: 5.0 g p-diphenylbenzene was dissolved in 1.0 l. sulfur-free toluene (B.D.H.) and 0.010 g. 9,10 diphenylanthracene added (a "wavelength shifter", to make the wavelength of the fluorescence match more closely the response curve of the photomultiplier).

Photographic films: Kodak "Blue Brand" 14 x 17 in. screen-type X-ray film was used, as directed by Wilson[143], but gave way during the work to its successively faster modifications, "FE-101" and "Royal Blue".

Amino acids: "Shandon" 0.01 M solutions in 10% i-propanol were used.

Sugars: 1% solutions in 10% i-propanol were made up.

METHODS AND RESULTS

Outline of Procedure

In this paragraph is given a brief outline of the experimental procedure on mustard seeds, which is described in full below. In each experiment, mustard seeds were kept at 0° wet with a film of THO for the desired time, after which they were killed by homogenising in ethanol. This also extracted some metabolites. A water extract was then made. The separate extracts were fractionated by two-dimensional paper chromatography. Tritiated spots on the chromatograms were detected by scintillation autography, and identified by co-chromatography with authentic known compounds.

Treatment of Seeds

When seeds were to be wet for more than 24 hr., they were used unmodified; but for shorter runs than 24 hr., a very small area of each seed's testa was removed with a scalpel[1] to allow freer ingress of THO. For the longer runs, it was decided that risk of fungal invasion would outweigh any slight improvement in rate of imbibition resulting from removal of part of the testa.

Even after the longest run, where seeds were wet for 26 weeks, a seed showed no fungal mycelium on dissection and examination under a binocular microscope.

Five seeds were used for each run. The finding of Spedding[1] was confirmed, that this number of seeds gives a suitable concentration

of metabolites in the extracts. On the other hand, the criticism might be levelled that the statistics of such experiments are poor. This is not, however, an important fault; and in the several cases where more than one run was done for the same time interval, the results were identical.

In a 10 ml. conical centrifuge tube with rubber stopper, the seeds were thermally equilibrated for $\frac{1}{2}$ hr. before addition of THO. Within four hours of wetting with THO at 24°, mustard seeds are conducting many metabolic reactions (results of Spedding[1], confirmed by the writer). Runs for four hours at 13°, 10°, 5° and 0° revealed that metabolism at 0° is much slower but by no means "frozen". 0° was therefore fixed on for all later experiments, as metabolic aberrations due to the cold were thought more likely to reveal themselves at 0° than at higher temperatures, but 0° was not so cold as to inhibit all metabolism. Thermostatting was achieved by placing the tubes in a Dewar flask full of crushed, wet ice. For runs of over 24 hr., a very large (6 l.) Dewar was used, fitted with a lid and kept in a cold room (2-4°). The ice was renewed before it had all melted, i.e. within three weeks. In the six-month runs, the ice was inadvertently allowed to melt, and the temperature of the water in the Dewar rose to 2° for about a week before new ice was added. Temperatures above 0° were maintained in a thermostat with mercury-toluene thermoregulator, accurate to $\pm$ 0.2°.

Handling of THO

Health hazards can be eliminated with simple precautions in the handling of tritium, which is among the least hazardous of all radioisotopes[131]. Its radiation is extremely soft, it is diluted with many kilograms of protium if ingested, and its mean effective life in the body is very low, viz. 10 days[144]. The maximum permissible levels[144,145] are very high compared with those of other radioisotopes: they are 10^4 μC in the whole body, 10^3 μC deposition daily in the body, and 0.4 μC/ml. in drinking water.

On the other hand, geneticists believe that there is no threshold for mutations from irradiating rapidly dividing cells[144,146]. Also, despite the low "biological half-life" quoted above, there may possibly be some pools of hydrogen in the body which turn over much less quickly. Again, contamination of benches or other equipment might impair future work with them, and would be impossible to detect with conventional Geiger or scintillation monitors.

Therefore all manipulations of THO and tritiated seed extracts were done on a tray in a special fume cupboard with exhaust fan and running water installed for immediate washing-down in case of mishaps. Rubber gloves were worn. THO was transferred in disposable micropipettes attached to all-glass syringes. From the main stock of 5 ml. THO, working stocks of about 1 ml. were removed to small tubes with greased ground-glass stoppers, each in a test-tube lined with cotton wool and fitted with a rubber bung.

From a micropipette, two drops of THO were added to the pre-cooled seeds in their centrifuge tube, which was then stoppered and shaken gently if necessary to wet all seeds with a film of THO.

Oxygen Supply

The stopper of each tube was removed, every week or so, for a few minutes to replenish the oxygen supply in case that were necessary for oxidative metabolism.

Extraction

When the desired period of time had elapsed, the seeds were ground with c. 5 ml. ethanol for 4-5 min. in an all-glass Potter-Elvehjem tissue homogeniser. This was very confidently assumed to kill them. It was not judged necessary to resort to boiling ethanol.

Spedding[1] merely crushed the seeds as finely as possible with a glass rod, under ethanol. Some duplicate runs using that method were tried, and found to give very incomplete extraction of tritiated metabolites.

5 min. centrifugation at c. 3000 x g. afforded a clear supernatant, which was decanted into a pear-shaped flask of c. 20 ml. capacity and having a B14 socket. The residue was again extracted, as above.

Two water extracts were then made by the same method as the two ethanol extracts. Sometimes all four extracts were processed separately, with a view to possible elimination of the second extraction with either

solvent. It was always found, however, that all four extracts contained detectable amounts of tritiated compounds.

Throughout this project, ethanol and water extracts have been chromatographed separately, whereas Spedding usually combined them. The newer procedure has the disadvantage of doubling the number of chromatograms which have to be run, and the attendant processing of films and co-chromatography. However, the following advantages are claimed for:

1. If the water extracts are poured in with the ethanol extracts, a creamy suspension appears, of closely similar density to the solution and incompletely removed even by centrifuging at 20,000 x g for 20 min. This suspension would have a huge surface area and is expected to adsorb from solution at least some of the compounds which are intended to be chromatographed. A well-known case of such adsorption misled Gaffron's group into stating that the first product of carbon dioxide incorporation in photosynthesis is of very high molecular weight; only after a long altercation with Calvin's group was it discovered that radioactive phosphoglyceric acid had been adsorbed onto precipitated protein. That such an effect occurred in the present work was easily shown by centrifuging down a small sample of the creamy suspension, washing by re-suspension in 10 ml. ethanol and re-centrifuging, followed by similar washing with water. The residue, smeared on filter paper and exposed to film (see below) was intensely radioactive.

The nature of this suspension seems unimportant at present. One may surmise that it is a protein or polysaccharide thrown out of aqueous solution by the ethanol. Cajola[147] found mustard seeds to be 20% hydrophilic mucilage. But whatever its identity, its precipitation is to be avoided; and this is the first advantage of separate processing of ethanol and water extracts.

2. In the case of water extracts, salting-out in solvent (1) usually ruins the chromatogram if more than c. $\frac{1}{10}$ or $\frac{1}{5}$ of the extract is loaded onto one chromatogram. The result is bad tailing back to the origin, or even sticking of most compounds on the origin. A chromatogram of the water extract of a 4-week run, is shown in Plate VII as an example of incipient salting-out. Solvent (2) is less prone to salting-out, but cannot be run first (see below, "chromatography").

This fault is much less prominent with ethanol extracts; c. $\frac{2}{3}$ of the ethanol extract of five seeds can be run satisfactorily on one chromatogram, and the load-limiting factor usually turns out to be the merging of spots into each other; spot area is known to increase with the log of weight of compound in the spot[148].

Thus if ethanol extracts are treated separately, it is relatively easy to get good chromatograms of them, having well-separated and non-streaked spots.

3. Keeping the two extracts separate constitutes a preliminary fractionation which might well be useful; two compounds running to almost the same area, which might not be separated on a combined chromatogram, could conceivably be extracted separately, one in ethanol and the other in water. Also, to know (e.g.) that a compound is water-soluble but ethanol-insoluble is useful, and revealed by this method but not the previously-used one.

Each extract, in its flask, was connected onto a B14 cone on one arm of an inverted-Y-shaped yoke on a vacuum line. The extract and a receiver flask on the other free arm of the yoke having been frozen in liquid air, the pressure in the system was lowered to 5-15 μ Hg with a two-stage rotary oil pump. The yoke was then isolated from the vacuum line and the liquid air removed from around the extract. The solvent, and possibly other volatile compounds, sublimed over to the receiver. This freeze-drying was slow but automatic, needing no attention.

Freeze-dried extracts not immediately required were stoppered and stored at -20°.

CHROMATOGRAPHY

To load chromatograms, a drop of the appropriate solvent was introduced and the flask tilted to wet the walls thoroughly. This solution was taken up in a Pyrex capillary and spotted onto the

origins of chromatograms, using an air blower to keep the spot small (2-5 mm diameter). Hot air was never used. It would have allowed faster loading, but heating of extracts was thought likely to encourage decomposition and origin-sticking[132].

Graded loading

Of each extract a series of chromatograms was prepared, loaded to various extents. One benefit of this is seen in Plate VIII. Two of the graded range of chromatograms of the ethanol extract of a 10-week run are shown. In the one which was loaded only 5 times, spots U_3 and U_4 are seen to be separate; in the 20-load example, U_3 and U_4 are not obviously separate, but spots M and U_2 show up. Another illustration is given in Plate VII, which shows two chromatograms of the ethanol extract from a 2.5-week run. The more lightly-loaded one shows that the two spots Ala and GABA are in fact only two in number; the more heavily loaded chromatogram fails to show that clearly, but reveals the spots U_2 and fructose. In general, compounds of low concentration show up only on the heavily-loaded chromatograms; compounds of higher concentration, which tend to merge if of similar R_f's, are often revealed as separate spots on the lightly loaded chromatograms. There is, in other words, no single "correct" loading, and maximum information is gained only by the system of graded loading.

Another advantage is that several samples of each tritiated compound are usually gained; thus if the first guess in co-chromatography

proves wrong, one still has other samples available for further attempts.

Plate VI shows a chromatogram of the water extract from an 8-hour run which would lead one to think that there were two tritiated spots in the area marked "(iso)citric". The other chromatograms of the same extract, though less suitable for reproduction here, proved that this double spot was merely a curious anomaly. They showed that there was definitely one tritiated compound in this area of the chromatogram, and one only.

Perhaps the greatest benefit from the system of graded loading was that it virtually guaranteed the production of one good chromatogram; that is certainly not the case otherwise.

It will be seen that the system of chromatography standardised on this project involved many times more work than the simple system of combining ethanol and water extracts onto a single chromatogram. The author is, however, convinced that the extra work is worthwhile.

Descending chromatography was always used, in the interest of speed (the project involved at least 600 chromatograms in all). In the boxes available was room for a descent of c. 15 cm. This took only c. 2 hr. for solvent (1). These unusually small chromatograms showed satisfactory separation of spots; the benefits of running solvents further are largely offset by the larger spots resulting from the greater time available for diffusion.

Solvents (1) and (2) (see "Materials") were used to chromatograph seed extracts. Solvent (1) is of neutral pH and gives a very useful "group" separation - acids and bases travel with low R_f's, neutral compounds (amino-acids and sugars, in this case) with R_f's around .5, and lipids at the solvent front, i.e. R_f 1. Solvent (2) suppresses the ionization of acids such as citric and malic, so that they travel above the "neutral line" (see Plate IX). Solvent (1) always preceded solvent (2) because if solvent (2) were used first, traces of acid remaining on the paper would make solvent (1) behave like an acid (and not a neutral) solvent.

Pre-equilibration and thermostatting, reported as advantageous[132] when using solvent (1) were used for some dozens of chromatograms, but no detectable improvement resulted and so these precautions were discontinued.

After development in solvent (1) and drying overnight in a fume cupboard with exhaust fan, the chromatograms were developed in the second direction using solvent (2). This took c.1 hr. Pre-equilibration was known to be disastrous for this development[132] and so was not even tried. Another overnight drying was then given.

The dry chromatograms were stapled onto a used X-ray film and scanned under a UV lamp in a dark room. The solvent fronts were thus revealed and marked with pencil, as were any fluorescent or absorbent spots. If any such spots had then been found to be

tritiated, the fact of UV fluorescence or absorption would have been helpful in identifying them. The R_f (1,1) area, i.e. the corner diagonally opposite the origin, was cut off and dealt with separately.

DETECTION OF TRITIATED SPOTS

This was by the method of Wilson, which has been published[143] but will be briefly described here owing to its prime importance in this thesis.

Small pieces of filter paper bearing some tritium (e.g. solid residues) were stapled near two or three corners of the backing sheet to which the chromatograms had been stapled, which was then, in a shallow airtight tray, exposed to a very fast (screen-type X-ray) film, soaking all the while in an organic scintillator so as to convert the beta-radiation of tritium into light. After an exposure of 6 hr. - 6 wk., depending on the expected activity of the tritiated spots on the chromatogram, the film was swabbed free of phosphor and processed in the makers' developer and fixer. The film, when dried, was aligned with the chromatograms by placing the markers and their images in coincidence. Dark spots on the film then corresponded with tritiated compounds on the chromatograms. Very faint spots were seen more easily by oscillating the film slightly and viewing it at a low angle.

The process is, then, that of radio-autography as used for ^{14}C, ^{32}P, etc; but the very soft radiation of tritium is detected much more efficiently by this "scintillation autography".

The sensitivity is known[143] to permit detection of 0.1 μC on a spot 1 cm^2 with an exposure of 50 hr. Longer exposure up to 6 weeks was found to give continued improvement, but at a seriously decreasing rate, owing to reciprocity failure, which is inherent in the photographic process[149].

The exposure standardised in this work was one week, representing a compromise between sensitivity and speed of production.

One emulsion known, from long astronomical exposures, to give increased reciprocity, is Ilford Zenith Astro. Some plates of this were found to give much worse sensitivity in a 50 hr. exposure than the screen-type X-ray film used. Even worse were Kodak Tri-X, and Kodirex no-screen film. What is needed is an extremely fast film, sensitive to the blue and UV fluorescence but not, for preference, to the safelight which it is desirable to use during development; and giving increased reciprocity. Screen-type X-ray film fulfils all these requirements except the last.

Wavelength shifter, e.g. diphenyl hexatriene or 9,10-diphenylanthracene, was omitted except when an exposure of several weeks had failed to detect the tritium on a co-chromatogram. It gives an improvement of only 30% in sensitivity (Wilson, p. comm.) A similar improvement can be gained by using a sheet of

aluminium foil as a reflecting backing sheet for the chromatograms, instead of used film. This refinement was not resorted to.

Detection of tritiated lipids

Hydrophobic compounds such as triglycerides, fatty acids and sterols are known[132] to run as an unresolved mass at R_f 1 in solvents (1) and (2). Such compounds might well be soluble in the toluene used for scintillation autography. If they were, and scintillation autography were attempted, it would probably fail to detect them, as they would quite likely diffuse out of the paper and instead of showing up as a darkened area on the film over the "lipid corner" of the chromatogram, they would merely cause a general fogging of that film and all others using the same exposure tray thereafter. Therefore the lipid corner of each chromatogram was cut off before scintillation autography.

Quantitative results were not required, so the extraction procedure adopted was simply to place the piece of paper in a flat-bottomed glass bottle suitable for fitting into the scintillation head, and submerge it in 5.0 ml. of scintillating liquid. After occasional shaking for a day, the paper was discarded and the bottle counted in a non-refrigerated single-channel liquid scintillation counter without anti-coincidence circuit. The efficiency for ^3H was 4-7%. Count rates many standard deviations (σ) above background were obtained from ethanol extracts of seeds wet with THO for 12 hr.

or longer. The background count in each case was from the corresponding water extract. In no case did this fall more than 3σ outside the count rate from 5.0 ml. of scintillator.

Quantitative results were not aimed at, because the question was "After what period of time do lipids become labelled?" In practice this question was easy to decide on, as count rates were either within 3σ of background (i.e. 100-200 counts/min.) or many thousands of counts/min. In any case, the "lipid corner" is only vaguely defined, and an arbitrarily large piece of paper is removed.

Despite its unsophisticated nature compared with better liquid scintillation counters, the counter used was estimated to be several orders more sensitive than scintillation autography.

The negative results (viz. "lipids do not become labelled up to 12 hr.") are unambiguous, with the proviso that volatile compounds such as propionic acid would have been lost in the freeze-drying.

It must be added that if the lipid corner was subjected to scintillation autography in repeat runs of experiments which had given positive results in scintillation counting, in many cases the lipid corner showed dark on the film. An example is seen in Plate VIII, on the chromatogram of the ethanol extract of a 26-week run. This image, though only vaguely delineated in the direction of the origin, was <u>sharply</u> bounded by the solvent fronts.

Identification of Tritiated Compounds

In solvents (1) and (2), classes of compounds run to areas as shown in Plate IX.

Even more useful in identification is a "standard map" of positions to which common biochemical metabolites run in these solvents. That of Bassham[150] was supplemented by running other compounds. A very complete standard map was discovered later[19].

The confidence shown by some workers in R_f values as a means of identification (e.g. ref. 113) was not borne out in the present work. R_f values were found to vary greatly, e.g. .55 - .65 for 4-aminobutyric acid in solvent (1). Therefore, to add another compound to a standard map one must run it with several others already on the map, on the same chromatogram.

Even the relative position of compounds is not invariant. For instance, alanine sometimes ran ahead of 4-aminobutyric acid in solvent (1), as in the chromatogram of the ethanol extract of a 2-hr. run (Plate V), but in other cases ran behind it (Plate VII, ethanol extract of 2.5 week run).

The variations in R_f values necessitated the method of identification used here.

Co-chromatography

Unless the activity was low, in which case all the activity would be needed on the co-chromatogram, the whole spot was not cut out, but only the middle. This minimises the risk of including

overlapping spots, and leaves a sample of the radioactive spot for spraying with, say, ninhydrin in attempt to gain a clue to the nature of the radioactive spot. On account of the possibility of reaction by overlapping spots, however, not much faith could be put in any positive results from such spraying.

A typical identification proceeded as follows: a tritiated spot from an 8-hr. experiment, ethanol extract, ran to approximately where alanine appears on the standard map. It was ninhydrin-positive, and the fact that it had been extracted by ethanol confirmed that alanine was a likely guess for the compound's identity. Also alanine had been identified in extracts from 2-hr. and 4-hr. experiments. A detectable amount (2-5 μl. .01 M) of alanine was added to the excised piece of paper. The elution technique of Wilson and Calvin[133] was used to transfer the tritiated unknown and the unlabelled alanine onto the origin of a new piece of chromatography paper. The two-dimensional development was then carried out, as described above for seed extracts, using solvents (1) and (2). The tritium was located by scintillation autography, and the authentic alanine by spraying with ninhydrin. The two spots coincided in every respect, including irregularities of outline. This was weighty evidence for saying that the tritiated compound was alanine.

In the case of Krebs cycle acids, unwashed paper was used, as residual oxalic acid on washed paper raises the background in the spray test. These acids were very well resolved by solvents (1) and

(2), but co-chromatograms of them had to be dried for several days in a forced draught, or else the spray for acids would show a positive reaction with residual propionic acid all over the paper. The more sensitive 2,6 dichlorophenolindophenol spray was especially prone to this. A further difficulty was that even this more sensitive spray is an order or so less sensitive than ninhydrin is for amino-acids. This made it necessary to use 20-50 μl. of .01 M solution of the appropriate acid for each co-chromatogram. Such an amount gave quite a large spot on the developed chromatogram. These acids were relatively weakly labelled in seed extracts; and spreading a small amount of activity over a large spot often made it very hard to detect on the co-chromatogram. This is why citric and malic acids have been identified in relatively few cases. Solvents (5) and (6) were sometimes used.

It will be seen from table I that the identity of some spots has been only inferred, and not positively confirmed. The inference is often fairly confident, however; for instance, aspartic acid was labelled in very many experiments and so the spot labelled "Asp?" in the chromatogram of the 16-week run (Plate VIII), being in an area where aspartic acid is known to run, is quite likely to be that compound.

Spedding[1] used repeated co-chromatography in the <u>same</u> solvents to increase the level of confidence in identifications. The author thought it an improvement to use, in addition, solvents greatly

TABLE I: COMPOUNDS NON-EXCHANGEABLY TRITIATED BY MUSTARD SEEDS IN THO FOR VARIOUS TIMES, AT 0°

TIME; extract	GABA	Ala	Asp	(iso) citric	Glu	"M"	U_3	U_4	malic	fructose	lipids	solid residue	R_f's of other spots
10min) EtOH 20min) and 30min) H_2O												+ + +	
1.0hr. EtOH H_2O	2,1 1,1											+ 	
2.0hr. EtOH H_2O	2 1	2	 inf.	 inf.								 +	
4.0 hr. EtOH H_2O	1 1	1 1	 1	 1	 1							 +	
8.0hr. EtOH H_2O	2,1 1,1	2 1,2	 1,1	 1	 1							 +	
12hr. EtOH	2,1	1,1				*? (v.faint)					+	+	
24hr. H_2O	2,1	1,1	1,1	1	1,1				1			+	(.26; .42) - glyceric? 1 doubtful co-chromatogram
48hr. EtOH	1,1	1,1				+	+	+			+	+	
1.0wk. EtOH H_2O	2,1 1	2,1 1	 inf.	 1	 inf.	+			 1		+	 +	
2.5wk. EtOH	2	1				+		+		1	+		U_2 - (.81, .63)
4.0wk. EtOH H_2O	2,1 2	1 inf.	 inf.	 inf.	 inf.	+			 inf.		+	 +	glucose - 1 doubtful co-chromatogram
10wk. EtOH H_2O	2 1	1 1	 inf.	 inf.	 1	+	+	+			+	 +	
16wk. EtOH H_2O	1,1 1	1 1	 1	 inf. (v.faint)	 inf. (v.faint)	+	+	+ inf. (v.faint)			+	 +	
26 wk. EtOH H_2O	1,1 1,1	1 1	 1	 1	 inf. (v.faint)	+			 inf.	1,1	+	 +	U_5 (.40, .52) (.35, .48) - glyceric?

Explanatory note: A + sign indicates that a compound was tritiated. "inf." indicates that the identity of a compound was inferred from its position on the chromatogram. U designates a compound as yet unidentified. The numeral at the LHS of a column indicates the number of identifications by co-chromatography in solvents (1) and (2); at the RHS, in solvents (3) and (4). Abbreviations: see page 203

different from (1) and (2). This constitutes a much more strenuous test of the identity of a tritiated compound. Therefore amino-acids and sugars were, in many cases, also co-chromatographed in solvents (3) and (4). Pre-equilibration is essential with (3).

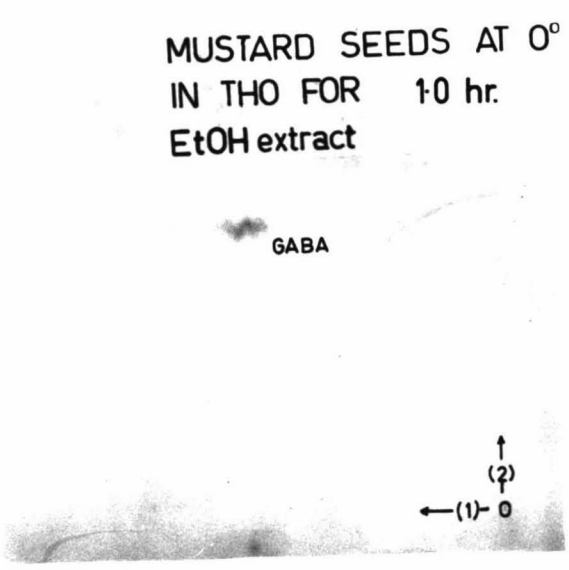
RESULTS

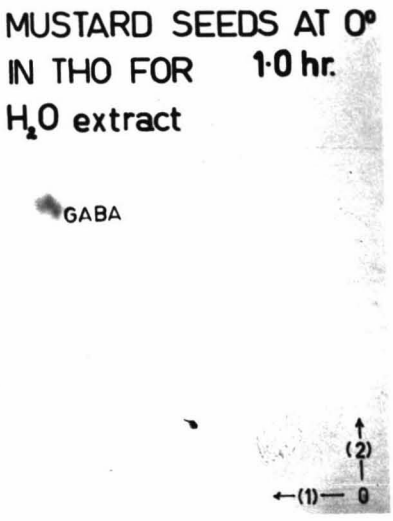
Selected scintillation autograms are shown in plates V-VIII. The complete scheme of tritiated compounds is shown in table I. Citric and isocitric acids cannot be separated by the methods used here, so the tritiated compound which co-chromatographed with citric acid may be citric or isocitric or both. It is therefore called "(iso)citric".

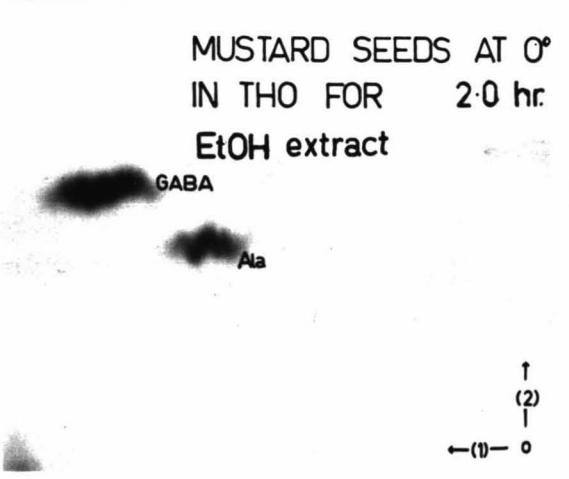
The large, diffuse, very highly tritiated origin spots reported by Spedding[1] never occurred in this work, probably because the bottle for solvent (1) was always washed and dried by the method which he found to prevent that trouble.

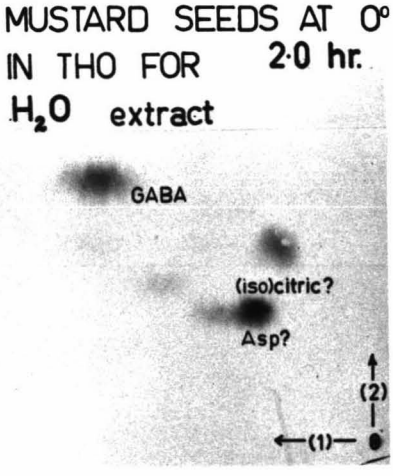
The first compound labelled is 4-aminobutyric acid. That experiment (i.e. a 1.0-hr. run) has been done three times, always with the same result; and the sole tritiated compound has been co-chromatographed thrice in solvents (1) and (2), and twice in solvents (3) and (4). It is very unusual and perhaps unique for a radiochemical experiment on a whole organism to give only one labelled product. By and large, compounds became labelled at c. $\frac{1}{8}$ the rate found[1] at normal temperatures.

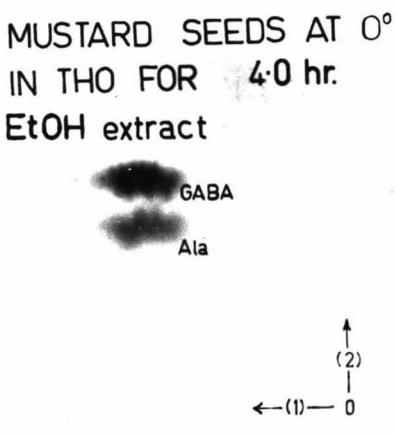
PLATE V


MUSTARD SEEDS AT 0°
IN THO FOR 1·0 hr.
EtOH extract
GABA
(2)
(1) 0


MUSTARD SEEDS AT 0°
IN THO FOR 1·0 hr.
H₂O extract
GABA
(2)
(1) 0


MUSTARD SEEDS AT 0°
IN THO FOR 2·0 hr.
EtOH extract
GABA
Ala
(2)
(1) 0


MUSTARD SEEDS AT 0°
IN THO FOR 2·0 hr.
H₂O extract
GABA
(iso)citric?
Asp?
(2)
(1) 0


MUSTARD SEEDS AT 0°
IN THO FOR 4·0 hr.
EtOH extract
GABA
Ala
(2)
(1) 0

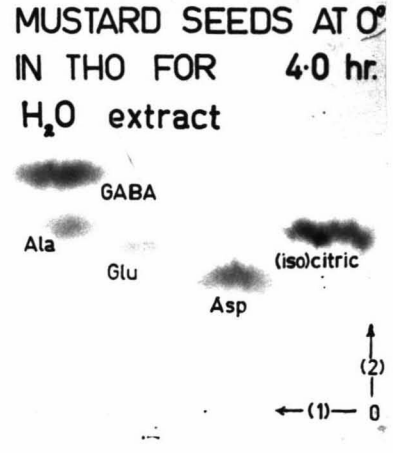
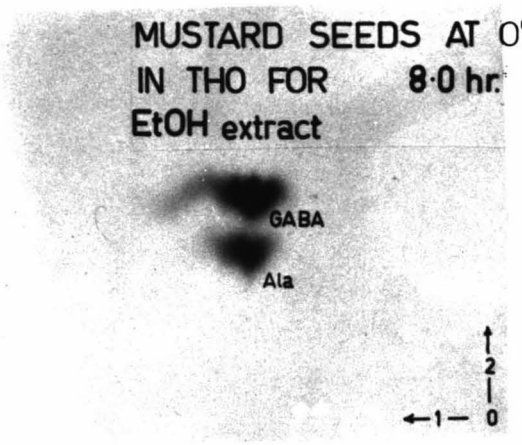
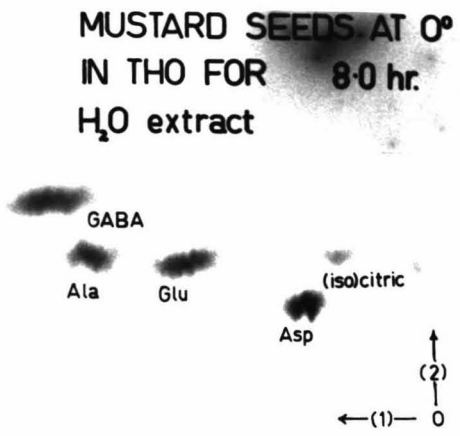
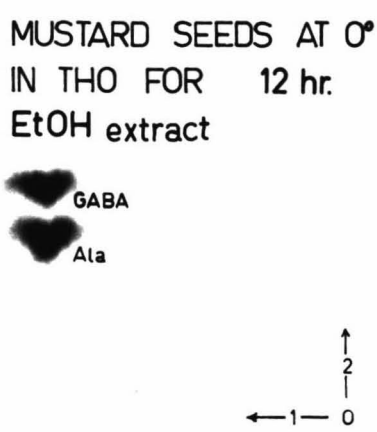
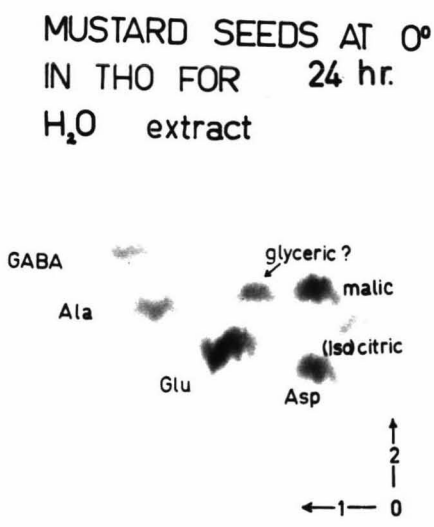

MUSTARD SEEDS AT 0°
IN THO FOR 4·0 hr.
H₂O extract
GABA
Ala
Glu
Asp
(iso)citric
(2)
(1) 0

PLATE VI

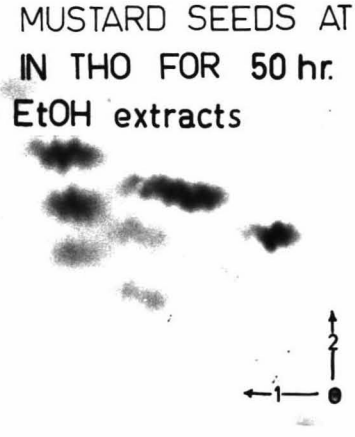

MUSTARD SEEDS AT 0°
IN THO FOR 8·0 hr.
EtOH extract
GABA
Ala
2
1
0


MUSTARD SEEDS AT 0°
IN THO FOR 8·0 hr.
H2O extract
GABA
Ala
Glu
(iso)citric
Asp
(2)
(1)
0


MUSTARD SEEDS AT 0°
IN THO FOR 12 hr.
EtOH extract
GABA
Ala
2
1
0


MUSTARD SEEDS AT 0°
IN THO FOR 24 hr.
H2O extract
GABA
glyceric ?
malic
Ala
(iso)citric
Glu
Asp
2
1
0


M''
GABA
Ala
U3
U4


MUSTARD SEEDS AT 0°
IN THO FOR 50 hr.
H2O & EtOH extracts
2
1
0

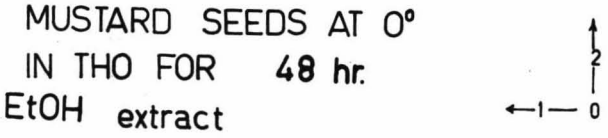
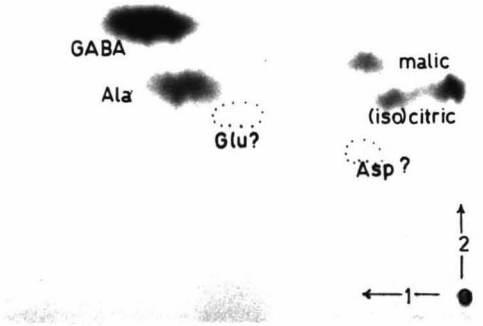
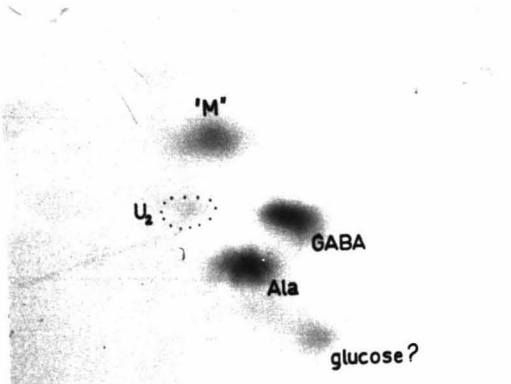

MUSTARD SEEDS AT 0°
IN THO FOR 48 hr.
EtOH extract
2
1
0

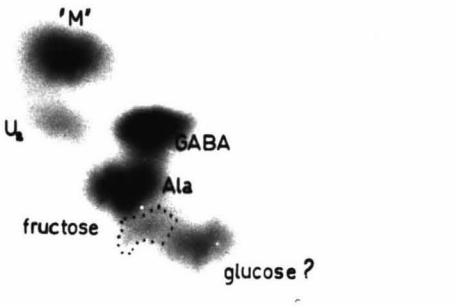
PLATE VII

MUSTARD SEEDS AT 0°
IN THO FOR 1·0 wk.
H_2O extract



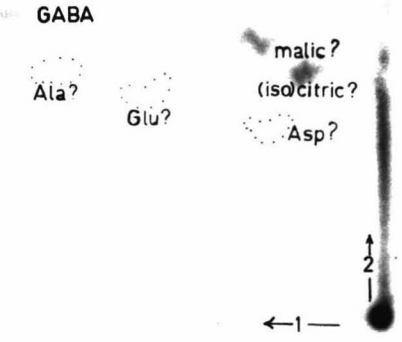


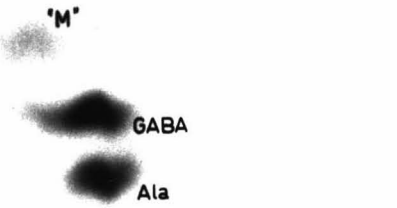
MUSTARD SEEDS AT 0°
IN THO FOR 2·5 wk.
EtOH extract ; 5 loads



MUSTARD SEEDS AT 0°
IN THO FOR 2·5 wk.
EtOH extract ; 20 loads

MUSTARD SEEDS AT 0°
IN THO FOR 4·0 wk.
H_2O extract





MUSTARD SEEDS AT 0°
IN THO FOR 4·0 wk.
EtOH extract

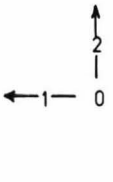
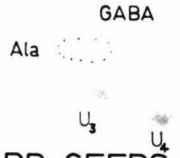
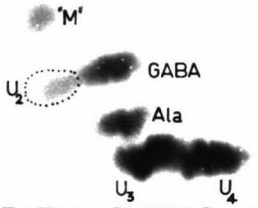


PLATE VIII

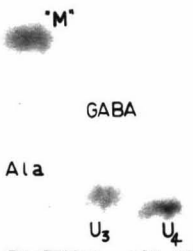


MUSTARD SEEDS AT 0°
IN THO FOR 10 wk.
EtOH extract , 5 loads

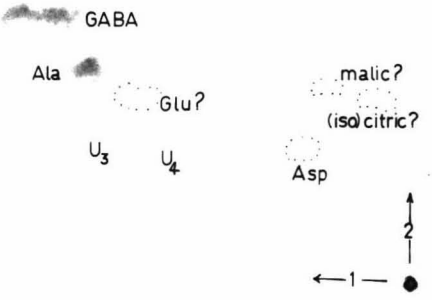


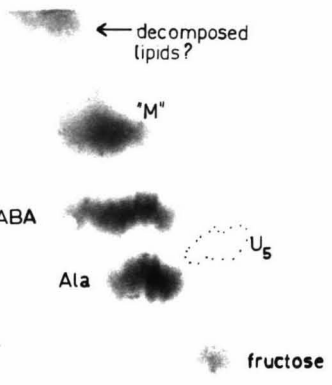
MUSTARD SEEDS AT 0°
IN THO FOR 10 wk.
EtOH extract , 20 loads

MUSTARD SEEDS AT 0°
IN THO FOR 16 wk.
H_2O extract



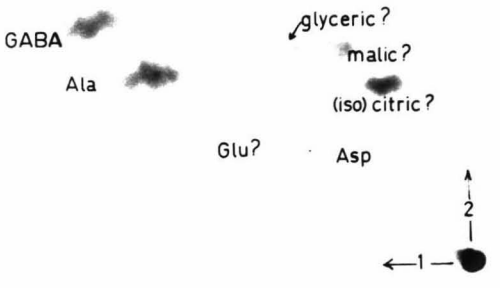
MUSTARD SEEDS AT 0°
IN THO FOR 16 wk.
EtOH extract





MUSTARD SEEDS AT 0°
IN THO FOR 26 wk.
EtOH extract

MUSTARD SEEDS AT 0°
IN THO FOR 26 wk.
H_2O extract



The Solid Residue

Spedding[1] did several experiments on the solid residue left after extracting, once with ethanol and once with water, five seeds which had been wet with THO for 1.0 hr. at room temperature. By scintillation counting, he found that an absolute ethanol extract of this solid residue was definitely tritiated. This is scarcely surprising in the light of the present writer's finding that chromatography of each of two ethanol and two water extracts showed appreciable amounts of tritiated compounds in all four extracts, as detected by the usual method (scintillation autography). Spedding's single ethanol and water extracts had not been made with the benefit of a tissue homogeniser, but only by the method of crushing the seeds with a glass rod. Therefore it is very likely that his solid residue contained significant remnants of the same tritiated compounds as had appeared in the two extracts.

However, Spedding also evaporated at 100° the ethanol extract of his solid residue. On re-dissolving the evaporated extract, he found its activity to have decreased considerably (but not to zero). He suggested that some THO might have been removed in this evaporation.

Hydrolysis of his solid residue with papain afforded Spedding some weakly labelled compounds which he chromatographed. One was identified as 4-aminobutyric acid; this and others were probably portions of the amino-acid content which had not been removed by the single extractions with alcohol and water. Also two other

tritiated compounds appeared, one at the origin and the other at R_f 1 in both solvents. These were not further investigated.

To test the hypothesis of non-metabolic incorporation of tritium into the solid residue, the present writer killed three batches of five seeds by these respective methods:

(a) storage in a desiccator over technical 98% sulphuric acid for four weeks

(b) heating, dry, at 110° for 4 hr.

(c) as (b), but preceded by autoclaving for an hour at 15 lb./in.^{2}, the seeds being open to the steam.

The batches of seeds were then treated with THO under the following respective conditions (all known to give tritiated metabolites in live seeds):

(a) 0°, 4 hr.

(b) 18°, 2 hr.

(c) 0°, 3 days

The usual extraction, chromatography and scintillation autography revealed in each batch no tritiated metabolites; but in each case the solid residue was very radioactive. This indicated that a good deal of the incorporation of tritium into the solid residue in the experiments on live seeds was non-metabolic.

Another highly important conclusion from these experiments was that non-metabolic labelling of those compounds which were extracted and chromatographed had <u>not</u> occurred. It had been thought possible that slow enolisation or perhaps some previously

unknown phenomenon akin to the Wilzbach synthesis[151] might cause non-metabolic incorporation of tritium. Heat-killed seeds imbibe water well[152]. Swollen beans and pumpkin seeds in D_2O exchanged a good deal of protium for deuterium, even in deeper layers of the seeds, within an hour. Killed seeds exchanged even better[155]. These experiments with killed seeds exposed metabolites, many of which would have survived the heating, to THO for quite long periods. But in no case was any non-exchangeable or very slowly-exchangeable label formed. In similar experiments, Edwards[2] killed some fungus spores with ethanol, evaporated the ethanol and then added THO. In 45 min. no incorporation of tritium occurred (no compounds became labelled). Again, treating with THO for 2 days at 20° portions, suitable for chromatography, of compounds such as glutamic acid and alanine which often become labelled <u>in vivo</u>, gave no detectable label in any of them after the usual washing and chromatography.

A similar conclusion was indicated by consideration of the fact that 10, 20, and 30 min. runs on live seeds, reported in table I, gave no detectably tritiated metabolites in ethanol or water extracts, but did give tritiated solid residues. It would require a very strange type of intermediary metabolism to give such results as those.

On the other hand, the incorporation of tritium into the solid residue did not seem to be entirely non-metabolic fixation

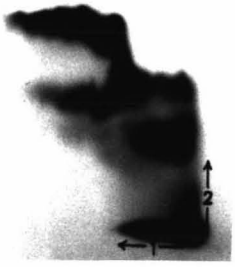
of tritium. The solid residues from two 16-week runs were hydrolysed with 10 ml. of 6 M 'Analar' hydrochloric acid in a sealed tube at 110-120° for 48 hr. To remove all the hydrochloric acid, which would have caused salting-out in chromatography, the mixture was then freeze-dried, and washed with de-ionized water several times, freeze-drying again between washings. The caramelised solid thus gained was extracted with ethanol and then with water. Chromatograms of these extracts are seen in Plate IX. The spots are much more highly tritiated than those which had been extracted from those same seeds immediately after killing (Plate VIII). Their R_f values do not seem to correspond with those of any metabolites met with in the rest of this thesis.

The solid remaining after that hydrolysis was only very weakly tritiated, and further hydrolysis under the same conditions for one week, followed by treatment as after the first hydrolysis, gave no tritiated spots on the resulting chromatograms. This proved that the first hydrolysis had been essentially as complete as possible with the reagent and temperature used.

It seemed possible that the activity of the solid residue might be, in part at least, owing to extremely small amounts of adsorbed metabolites having very high specific activities. An analogy was seen to a beaker contaminated with carrier-free ^{32}P-phosphate, which is very hard to remove with water only, but can be exchanged off with a large excess of inactive phosphate. Therefore ten mustard seeds were germinated in H_2O for two hr. at 0°, and

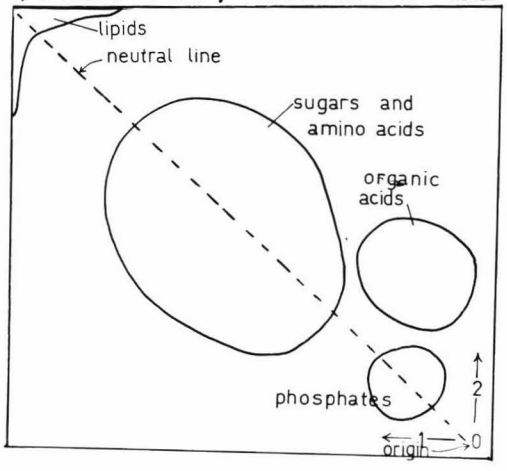
PLATE IX

MUSTARD SEEDS AT 0°
IN THO FOR 16 wk.
EtOH extract of hydrolysed solid residue



MUSTARD SEEDS AT 0°
IN THO FOR 16 wk.
H_2O extract of hydrolysed solid residue

'AREA MAP,' SOLVENTS 1&2



extracted in the usual way. The solid residue from five mustard seeds which had been treated with THO for 2 hr. at 0° was stirred several times over ¼ hr. in the alcohol extract of the ten non-tritiated seeds. The solid was then centrifuged down and washed similarly with the water extract of the non-tritiated seeds. The solid was again centrifuged down and subjected to scintillation autography alongside a sample of the original residue which had not been washed with the "carrier" solutions. No difference was detected between the activities of the two samples. The method of comparison was admittedly crude, but since the two samples were exposed side by side to the same film, it was estimated that the method would have been capable of detecting a halving in specific activity on washing the solid with the "carrier" solutions.

That experiment indicated that the postulated adsorption of metabolites was of, at most, minor importance in the labelling of the solid residue after two hr. at 0° in THO.

Spot "M" (see Plates VI-VIII)

This tritiated spot appeared in all runs of 48 hr. or longer. It was not reported by Spedding, despite the fact that his results up to 24 hr. of germination in THO at room temperature showed, in his longer runs, more tritiated spots than were ever found in this project at any stage up to 26 wk. at 0°.

There was no proof that all spots called "M" were the same compound, but that seemed the likeliest and simplest working

hypothesis; and if it were wrong, that fact would emerge as investigations advanced.

Since "M" was not reported from Spedding's work on normal germination, and since it occurred regularly in this work, its identification was considered likely to help in answering the question of why seeds do not germinate at low temperatures. "M" stands for the name "mystery spot", earned through its resistance to identification.

M was always extracted wholly by ethanol - it never appeared in water extracts.

Co-chromatography with proline, phenylalanine, leucine, methionine and methionine sulphoxide proved that M was stable during the elution and re-chromatography, and that it was definitely none of those amino-acids.

Its R_f values in solvents (1) and (2) (see Plate IX) indicate that M is neither strongly acidic nor basic; that it is not a sugar phosphate, lipid or oligosaccharide; and that it is not highly polar.

A sample of M was eluted as for co-chromatography but collecting the eluate overnight in a text-tube. The UV and visible absorption spectrum of this ethanolic solution was run on a Unicam SP700 double-beam recording spectrophotometer. The sole band was centred on 278 $m\mu$. The ethanol was then evaporated at room temperature and the solid left, which was just visible to the naked eye, hydrolysed, washed and re-chromatographed as for the

solid residues (see previous section). The result was two tritiated spots. This may indicate that M was tritiated at two positions, neither of which was labilised by the hydrolysis. Table II records data on the chromatograms of the hydrolysate. The whole experiment was duplicated, with no significantly different result.

Table II Products of Hydrolysis of Tritiated "M"

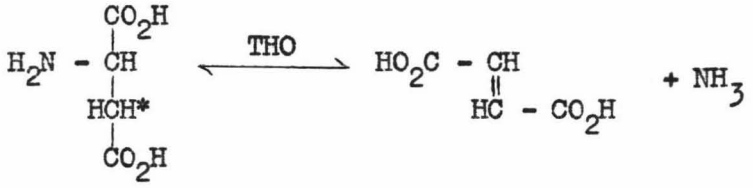
Spot no.	R_f's	Tritiated?	UV fluorescence
1	.74, .72	yes	nil
2	.08, .26	yes	nil
3	.08, .23	no	green

Spot no. 1 could possibly be unchanged M.

As the Introduction made clear, the "THO method" is well suited to the discovery of "new" metabolites. "M" may be one.

Position of Labelling of Amino-acids

The position of the tritium in an amino-acid molecule can help in inferring by what reaction it became labelled. For instance, transamination or deamination will give tritium on the α-C of aspartic acid; but in the "aspartase" reaction, 4.3.1.1 in the List,



the tritium will become attached to the β-C.

Spedding used repeated spraying with ninhydrin, alternated with scintillation autography, to examine the position of labelling of 4-aminobutyric acid, alanine, aspartic acid and glutamic acid. He found that after three separate sprayings with ninhydrin, these amino acids, which had become tritiated in mustard seed germination for 60 min. at room temperature, lost their activity.

The same compounds appeared on a chromatogram of the water extract from a 4-hr. run at 0°. This was sprayed with ninhydrin five times; between each two sprayings it was allowed to dry and another scintillation autogram made of it. Each exposure was of 7 days duration. Development was not precisely standardised but was by inspection till the citric acid spot's image reached an arbitrarily-decided density. This spot, being unaffected by ninhydrin, acted as an "internal standard". The result was a marked decrease (but not to zero) of the activity of each tritiated amino acid.

TREATMENT OF SEMEN

About $\frac{1}{4}$ ml. of bull semen, which had been stored frozen, was thawed at 0° and stirred with one drop of THO. After a further hour at 0° to allow diffusion of THO into the sperm cells, the semen was allowed to stand at room temperature for two hours. Minute samples were removed from microscopic examination at the start and end of this 2 hr. period, and revealed that at the start a very high proportion of the spermatozoa was motile, and at the end most were still motile.

The sperm were centrifuged down. Both the pellet and the supernatant were then processed as described above for seeds, but omitting the homogenising in the case of the supernatant.

No tritiated metabolites were detected on the chromatograms; but the solid residue was active.

These and the following negative results are recorded so that future workers may devise appropriately improved experiments. In the case of semen, it might be thought that, say, mannitol should have been added with the THO for maintenance of approximate isotonicity; but the high motility of the sperm after addition of pure THO seems to rule out osmotic damage as an explanation of the negative result.

TREATMENT OF FERN SPORES

To a few mg. of spores in the bottom of a centrifuge tube was admixed one drop of THO. Killing and subsequent procedures were as described for seeds. Even after 30 days at room temperature no tritiated metabolites were detected on the chromatograms.

The solid residue was active.

M. Lever (pers. comm.) has since followed the germination of several species of fern spores, including the same one used by the writer, by the method just described. He reports that Cyathea spores are not extracted by the usual method.

TREATMENT OF SAPWOOD

The freshly excised sample (see "materials", above) was treated with one drop of THO at room temperature for 30 min. Extraction, etc., was as for seeds. No tritiated metabolites were detected on the chromatograms; but the solid residue was tritiated.

Quite likely the THO had not permeated to all the living cells in the sample; and the number of these in the cube of side c. 2 mm might well be too few to give detectable amounts of tritiated metabolites. Also the use of pure THO may have caused osmotic damage to the cells.

DISCUSSION

The results condensed in Table I (p.143) allow, among others, the following conclusions:

(1) Mustard seeds, which do not germinate at 0°, metabolise 4-aminobutyric acid and alanine within two hr. of wetting with THO at 0°.

(2) Metabolism of aspartic and glutamic acids, and also malic and (iso) citric acids, is proceeding within four hr.

(3) At least one anomalous compound, the "mystery spot",[1] is being metabolised within 48 hr. It was not reported in germination at normal temperatures but becomes heavily labelled at 0°.

(4) Succinic acid, labelled fairly early in normal germination,[1] never becomes labelled at 0°.

(5) The order of labelling is, on the whole, similar to that in normal germination,[1] but labelling is about eight times slower.

(6) Lipids become involved in metabolism within 12 hr. (To correct to the sensitivity of detection of tritium in other types of compound, we should probably place the onset of detectable lipid labelling at 24 or 48 hr.)

(7) In contrast to results on metabolism early in germination of other seeds,[153,154] mustard seeds at 0° never (within months) inaugurate the Embden-Meyerhof glycolytic sequence. This result, which is of course to be read in conjunction with the sensitivity of the analysis (see start of part B of this thesis)[1] was also found

in normal germination. The absence of detectably tritiated phosphates also rules out the hexose monophosphate shunt.

(8) Metabolism of a free sugar, fructose, occurs after weeks.

(9) The solid residue remaining after extraction of metabolites in ethanol and water is always tritiated but at least a good deal of this is non-metabolic.

(10) These seeds wet at 0° inaugurate fewer metabolic reactions in months than they do in about four hours at 24°.

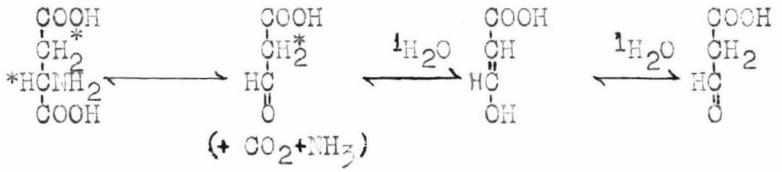
General Interpretative Remarks

What general scheme of the seeds' metabolism can we draw up from this survey? The most remarkable feature is the very narrow scope of the metabolic activity. Even given almost unlimited time, the seeds at sub-germination temperature inaugurate very few metabolic pathways indeed. Two reservations are needed to that statement. Firstly, other pathways could be labelling metabolites but below the detectable minimum. Secondly, as was pointed out in the Introduction, some metabolism cannot be detected by this method; e.g. hydrolysis of peptide or glycosidic links.

Labelling of amino and "Krebs" acids

In what reactions did the amino-acids become labelled? Spedding found the same ones tritiated at 24° as have been reported here, namely 4-aminobutyric acid, aspartic and glutamic acids and alanine. He concluded from the loss of tritium on repeated treatment with ninhydrin that the tritium must have been on the α-carbon

of the amino-acids. This reasoning is false. The type of mechanism for the ninhydrin reaction favoured by many, e.g. ref. 156, involves exchange of the α-hydrogen with protons of the solvent during that reaction. Spedding stated, on the contrary, that the α-hydrogen would not exchange during the ninhydrin reaction but, having become the hydrogen in the -CHO group of the aldehyde produced, was now slowly exchangeable by keto-enol tautomerism. The fact is, however, that the hydrogen of the -CHO group will not be exchanged in enolisation, but the hydrogen adjacent to it will be (if a double bond can form between what were the α and β carbons of the amino-acid). The correct analysis thus shows that both the β and the α hydrogens of an amino-acid are laid open for exchange with exchangeable hydrogen, when the amino acid is degraded by ninhydrin. For instance if aspartic acid were labelled at the α and β carbons, all the label could exchange out:



To show that all the tritium is removed from aspartic acid by the ninhydrin reaction proves, then, nothing at all about where the tritium was in the aspartic acid molecule. The same is true for alanine. In the case of glutamic acid, a complete lack of tritium in the product shows that the tritium had not been on C-4 of glutamic acid. If 4-aminobutyric acid's acivity is reduced to

zero, then the tritium cannot have been on C-2 of that compound, but must have been on C-4 or C-3. Non-zero radioactivity after treatment of a tritiated amino-acid with ninhydrin is ambiguous: either the tritium was in a position not laid open to exchange, or it was laid open ("labilised") but the rate of exchange out was too slow for the activity to drop below the detection minimum under the conditions used. In fact both interpretations could conceivably apply at once. Thus the result in this thesis (see "Results") that repeated spraying with ninhydrin, followed by drying each time, did not remove all the tritium, is of less use than had been thought.

The limitations in usefulness of the ninhydrin degradation were not seen until the practical work for this thesis had finished. It will be possible, however, to use some more informative degradation schemes.[144]

As far as it was meaningful, the degradation by ninhydrin indicated that 4-aminobutyric acid was tritiated at C-3 or C-4. Also 4-aminobutyric acid, the first compound labelled, was labelled relatively more heavily than it had been in the experiments at room temperature.[1] Furthermore, glutamic acid was labelled from four hours onwards. It seemed a plausible hypothesis that seeds might store α-amino-acids rather than the corresponding α-oxo-acids, because the latter would be prone to decarboxylation during prolonged storage. The α-oxo-acids of the Krebs cycle could be

supplied in germination by deamination and transamination of α-amino-acids. Production of α-oxo-acids by these reactions would label the amino-acids. This hypothesis finds supporting evidence in the fact that all the amino-acids which became labelled (Table I) were closely related to α-oxo-acids of the Krebs cycle: by transamination or deamination, alanine, aspartic acid and glutamic acid give respectively pyruvic acid, oxaloacetic acid and α-oxo-glutaric acid, all intermediates in the Krebs cycle. As for 4-aminobutyric acid, an enzyme is known (2.6.1c) to convert this compound to succinic semialdehyde, which might then enter the Krebs cycle by the valuably exergonic oxidation to succinate. Also, the most informative work before Spedding's on early reactions in seed germination pointed strongly to transaminations, carboxylations and at least some reactions of the Krebs cycle.[112] Operation of the Krebs cycle is at least a plausible supposition even in the absence of experiments, because seed germination involves many endergonic reactions, e.g. protein synthesis, and since no chlorophyll is present, exergonic reactions in the seed will be needed. The Krebs cycle is highly exergonic when coupled to the production of ATP by oxidative phosphorylation.[44] Barley soaked overnight was found[157] to contain in the embryo cytochromes a, b and c, identified by their absorption bands. These compounds, are of course, implicated in oxidative phosphorylation.

The Krebs cycle did not, however, appear to operate fully. Succinic acid, which must become labelled in the operation of the complete cycle, was identified by Spedding[1] among compounds tritiated within 24 hr. (and, with some doubt, within 90 min.) of normal germination. But in the present work succinic acid was never labelled.[19] It runs, in solvents (1) and (2), well clear of any of the compounds in Table I, so it would definitely have been noticed had its activity ever reached the detection minimum, 0.1 μC.[143] Other acids of the Krebs cycle which would become labelled during the operation of that pathway in THO were not found either at normal or sub-germination temperatures.[1] This might be owing to very low steady-state concentrations. It is not possible at present, then, to conclude finally that the whole or partial Krebs cycle exists in imbibed mustard seeds at sub-germination temperatures. The evidence certainly points that way, though. Also further investigations are suggested (see end of thesis).

Supposing that an important metabolic aberration in the imbibed seeds at 0° was curtailment of some Krebs cycle reactions, how could that be explained? One postulate centred on the "metabolic orphan" 4-aminobutyric acid. A plant would be considered odd if free of 4-aminobutyric acid,[158] yet metabolic roles of this compound are largely unknown. Assuming it is of little use to a plant, we now notice that is labelled first of all extractable compounds in the "attempted" germination at 0°, and it soon becomes heavily labelled. At least some of its label is shown by

the ninhydrin degradation to be on C-4 or C-3 and may therefore have been incorporated in the α-decarboxylation of glutamate, one of the very few known metabolic reactions in which 4-amino-butyrate is involved. It has seemed likely (see immediately above) that a fragmentary Krebs cycle exists in the imbibed seeds at 0°, and α-oxo-glutarate is probably supplied to that cycle from glutamate. Now if the postulated decarboxylation of glutamate to 4-aminobutyrate was slowed less in the temperature drop 24°-0° than the reaction supplying α-oxo-glutarate from what is assumed to be the same pool of glutamate, then the Krebs cycle may be curtailed owing to shortage of α-oxo-glutarate. It is highly probable that the rate-limiting step in the Krebs cycle is the oxidative decarboxylation to succinate of α-oxo-glutarate.[159] A shortage of supply of α-oxo-glutarate, owing to the "wasting away" of glutamate to the less important compound 4-aminobutyric acid, is therefore a possible explanation for the anomalous non-labelling of succinic acid.

This hypothesis is <u>not</u> presented as a confident conclusion. In fact it is based on several assumptions each one of which could be false. The hypothesis has, however, the great virtue of being experimentally falsifiable. It would predict that imbibed seeds at 0° might germinate if supplied with exogenous glutamate. It was found that 200 mustard seeds kept wet with distilled water at 0° for six months not only failed to germinate at 0°, but also germinated 0% when raised to 20° at the end. No mould was visible

to the naked eye. This experiment is consistent with the idea that the seeds at 0° become critically depleted of glutamate, though it does not, of course, prove that.

As a test of the hypothesis, 200 mustard seeds were kept wet at 0° for 3 months and then set at room temperature on filter paper wet with saturated aqueous glutamic acid (i.e. c.1% w/v at that temperature). A control batch of 200 seeds was treated exactly similarly except for the use of distilled water instead of glutamic acid solution. The result was, with no significant difference between the seeds supplied with glutamic acid and the control, 70% germination. There was not time to carry out the obvious next experiment, namely keeping the seeds at 0° for six 160 months before attempting to germinate them. It is known that exogenous glutamic acid is absorbed and metabolised by germinating Phaseolus seeds; though that work involved a 72-hr. germination period, so glutamic acid might conceivably not be absorbed in other circumstances or species. This is of course an example of the perennial permeability problem which arises in experiments where one is attempting to introduce an exogenous compound into a whole organism. No such problem is expected in the introduction of THO, though.

Labelling of lipids

Since the tritiated lipids were not fractionated by the method used, few detailed conclusions are possible about the reactions

in which they incorporated tritium. The degradation and synthesis of fatty acids in THO will tritiate lipids, as shown in the Introduction, p.108. Also synthesis of steroids is known from experiment, and very confidently expected from theory, to give tritiated lipids (p.113). The curious result (p139) that at least some of the tritiated material in the "lipid corner" of long-time experiments did not diffuse away into toluene solution requires some comment, especially as all "lipid corner" tritiated material was extracted from the seeds by ethanol. It is possible that some of the tritiated "lipid" material is tritiated carbohydrate bonded to non-tritiated lipid, e.g. tritiated galactose in a galactolipid.[161] This could break down on the chromatogram, resulting in some toluene-insoluble tritiated material (e.g. galactose) in the "lipid corner" of the chromatogram.

Labelling of fructose

Several reactions in the list of known enzymatic reactions are expected to incorporate tritium into fructose. Also passing-along of label, so often seen during the discussion of metabolic pathways in the introduction to this thesis, is possible. It would be desirable to know where the tritium is in the fructose molecule. The first experiment should be removal of the hydrogen on C-1.[162] Prolonged treatment with dilute alkali exchanges out that hydrogen, and gives an equilibrium mixture of fructose, glucose and mannose, which could be identified to give further confirmation of the identity of the labelled fructose. Another way

to labilise the hydrogen on C-1 would be oxidation of fructose to gluconic acid. The information from these experiments would tell whether the tritium in the tritiated fructose was on C-1 or elsewhere in the molecule.[60] It may be assumed that information on the exchange of hydrogen between fructose 6-phosphate and water[32] shows that the hexoses have 7 nonexchangeable hydrogens.

Some interesting speculations are possible to explain labelled fructose. For instance, a reaction like 4.1.1a could label fructose by decarboxylating the corresponding C_7 acid. Another possibility, apart from the obvious ones, is a reaction like 4.1.1.b. No decision will be possible between these speculations until the position(s) of the tritium in the fructose is (are) located. Aronoff[144] gives useful degration schemes.

Labelling of the Solid Residues

The results on this include evidence for non-metabolic labelling.[163] Some models of cellulose *in vivo* include bound water. Hysteresis in the hygroscopic equilibrium of rough rice could be interpreted in such terms.[164] Conclusive evidence was provided by an infra-red study of exchange of hydrogen between 99.7% D_2O and cellulose or viscose.[165] Each material failed to re-hydrogenate fully when kept in liquid ^1H_2O after 4 hr. in liquid D_2O. Changes in the percentage crystallinity were advanced as a means of binding water in cellulose.[166] THO vapour exchanged irreversibly with amylopectin, amylose, wood pulp, bacterial and algal cellulose, and

regenerated cellulose. Dried samples of these substances which had been exposed to THO vapour retained tritium for weeks when exposed to the water vapour of the air. Retention of tritium even after thorough washing in such cases is explained as follows: during drying, THO and/or T atoms on hydroxyl groups are trapped in crystalline regions of the polymers. In washing, not all the same regions "open up" for exchange so that a very large number of wetting and drying cycles will be needed to remove the tritium. There is thus good precedent for[165,166], the conclusion from the results herein, that some of the labelling of solid residues is non-metabolic.

Labelling of solid residues by adsorbed small molecules of high specific activity was shown to be of, at most, minor importance (p.152).

The hydrolysates of tritiated solid residues (plate IX) are at present uninterpreted, and are the main reason for not altogether subtracting labelling of the solid residue from the metabolic scheme being proposed for the seeds.

The most important task in this discussion is to answer the question posed at the very start of the introduction - how is the metabolism of imbibed seeds at 0° different from normal?

Firstly, we may conclude that the metabolism of the seeds at 0° is not hugely different from normal. Most of the reactions detected by the "THO method" at 24° are detected also at 0°, though they are much slower.

However, there are noticeable qualitative differences. Succinic acid, labelled at 24°, is not labelled at 0°. This could be owing to a block in the Krebs cycle, discussed above. Also, occurrence of the labelled "mystery spot" at 0° is a difference from normal. Unfortunately it has not been identified and so is at present of little help in answering the principal question.

The " THO method" has, then, been of considerable use in the problem. But its limitations have, as expected, prevented it from solving the problem "single-handed". Once again we see that this new biochemical method, for all its power and utility, is not to be thought of as replacing other methods. Rather is it complementary to others.

PART B

A PRELIMINARY STUDY OF TRITIUM INCORPORATION BY DRY SEEDS, SPORES AND POLLEN.

GENERAL INTRODUCTION

Seeds, spores and pollen exhibit the baffling phenomenon of "suspended animation". That is, they can be stored for very long periods (often many years - see refs. below) needing no food and apparently metabolising exceedingly slowly if at all. Then on wetting they grow in a very short period (a few hours or days) into rapidly metabolising tissues.

Conspicuously little progress has been reported in the literature in elucidating the pathways of intermediary metabolism of seeds, spores or pollen resting in this curious state of suspended animation. Two reasons are obvious for the lack of success of classical biochemical methods here. Firstly, the reactions in question proceed at rates many orders slower than those usually measured in biochemistry. Secondly, many classical methods are just not applicable to the problem. The speed with which seeds,[1,112] spores and pollen germinate[2] (in the chemical, molecular sense) on[1] wetting means that if it is desired to study resting metabolism one is debarred from the conventional method of making aqueous extracts and screening these for enzymatic activities. Very many investigators have not realised this, and have claimed to have extracted enzymes from resting seeds, using procedures which, we now know, began by germinating the seeds to an advanced stage!

To study unambiguously resting metabolism, then, was needed a method of analysis suited to following exceedingly slow reactions;

and it was also required that the organisms not germinate (in the molecular sense).

The principle of the method devised for this thesis was to expose the resting organisms to a fixed relative humidity of tritium water vapour. It was expected that THO would equilibrate with the organisms, the resting metabolism of which would then produce tritiated metabolites, in a way precisely comparable to their production in germination experiments (see part A of this thesis).

It was expected that the reactions in resting organisms might be so very slow as to correspond to a daily turnover of, say, a fraction of a micromole of each metabolite. Therefore the sensitivity of detection of a metabolic reaction by tritium incorporation was calculated.

The assumptions on which the calculation was based will now be given with relevant comments on each.

1 The reaction of 1 molecule of metabolite entails incorporation of 1 hydrogen atom from water.

This seems a conservative assumption, representing the lowest possible rate of incorporation (where any occurs).

2 Tritium is incorporated at the same rate as protium.

This assumption could be wrong by as much as 3 orders either way. See the discussion on the isotope effect in part A.

3 The specific activity of THO in the cells is that of the THO supplied, 5C/g, i.e. dilution by ^1H_2O already in the cells is negligible.

The specific activity of the THO will in fact be lowered by the unavoidable presence of $^{1}H_2O$ and exchangeable protium in the resting organisms before exposure to THO vapour. However, except in the case of seeds, the actual experimental quantities of THO and $^{1}H_2O$ (see "methods", below) justified this assumption. Also there is involved here the postulate of complete equilibration between THO vapour and that cellular water from which metabolites incorporate hydrogen.

4 To be detected as tritiated, a given metabolite will have to contain at least 0.1μC of tritium.

This is Wilson's figure[143] for the sensitivity of scintillation autography, which will be used to detect tritiation of metabolites.

5 Activity (rather than specific activity) of metabolites is the quantity governing the sensitivity of the method.

The threshold of 0.1μC (see assumption 4 above) was for a chromatogram spot 1 $cm.^2$ Spot area rises with the log of the mass of compound in the spot so multiplying the mass by 1000 only trebles the area of the spot. This holds, of course, only in the absence of overloading of the paper, streaking, etc.

6 Extraction is quantitative. This is an accurate enough assumption.

On these assumptions, it was readily calculated that only 10^{-9} mole of a metabolite would react before that compound became tritiated to an extent detectable by the method used. If the organism were in equilibrium with THO vapour for 100 days, a daily

labelling of 10^{-11} mole of a given metabolite would be detected. Even if the assumptions all erred badly, this calculation indicated that the method of assay would be sensitive enough to have some likelihood of success.

In striking contrast to many previous attempts at measuring resting metabolism, exposure to THO vapour perturbs the resting organism very little. The risk of radiation damage is lowered by the fact of low water content. [116]

Apart from its great intrinsic interest, the question of resting metabolism was also important in connection with the application of tritium incorporation in germination studies (see part A). The metabolism detected in germinating seeds, spores and pollen in liquid THO might have been, in part, accelerated resting metabolism. There was no doubt that germination had in fact been successfully studied; but the question was still unanswered as to its relation to resting metabolism.

It was expected that resting metabolism might prove qualitatively (as well as quantitatively) different from normal metabolism. New metabolic pathways might be discovered, involving, perhaps, previously unknown metabolites.

METHODS

Outline of Procedure

Resting seeds, spores or pollen were exposed to an atmosphere containing THO vapour, of fixed and known R.H. After the desired

period of time, the organisms were killed and analysed exactly as described in part A.

Exposure to THO Vapour

The requirement was for a closed vessel containing air at 1 atmosphere pressure, of sufficient volume to make it unlikely that the resting organisms would use up the oxygen to any important extent. The enclosed air was to have a fixed, known R.H. Samples of the resting organisms were to be withdrawn without affecting the remaining organisms. Sealing of the enclosure was to be good enough to preclude health hazards.

Accordingly, the apparatus shown in Plate X was designed. It looked somewhat like an octopus but had only five legs and so was called a "pentapus". Before the top extension was sealed, the four outer legs were supplied with pollen, seeds or spores as desired. To load a pentapus, it was held horizontal(i.e. at right angles to its position in Plate X). By turning about the axis of the centre leg, each outer leg could be loaded in turn, using a tube introduced through the extension of the centre leg.

The equilibrium R.H. above a saturated aqueous solution of a given salt is constant at fixed temperature, and almost any desired
199
R.H. can be obtained by appropriate choice of the salt. Therefore as soon as a particular pentapus had been loaded with the desired resting organisms, c. $\frac{1}{2}$ml. of liquid THO was pipetted into the centre leg, enough of the appropriate salt added to saturate it,

PLATE X

A Pentapus

the pentapus stoppered with a rubber bung, and the centre leg frozen in liquid air. The extension of the centre leg was then sealed (as in Plate X).

High R.H. in the sealed pentapus was desirable to maximise the equilibrium THO content of the resting organisms; but, as is discussed in detail below, growth of fungi is a danger and to preclude it the R.H. must be below about 60%. Suitable salts giving R.H. values in the acceptable range were[199] (values for 25°): LiCl 15%, K_2CO_3 43%, $Ca(NO_3)_2$ 51%, NH_4NO_3 62%.

Salts were dried by pumping on them at 5-8 μHg for two days, and transferred into pentapodes from the vacuum line as quickly as possible, to minimise uptake of water from the air.

Each loaded and sealed pentapus was kept in a thermostatted room (25 ± 1°) until it was desired to remove one sample of resting organisms, when the centre leg was again frozen in liquid air and one outer leg sealed off. Plate X shows a pentapus which has had one leg (in the position nearest to the camera) amputated.

Immediately on opening the detached leg, its contents were washed out with alcohol and homogenised. Any delay in this killing might allow de-labelling of tritiated metabolites: the THO in the organisms begins to be diluted by ^1H_2O from the air as soon as the leg is opened.

Extraction and subsequent procedures were exactly as described for the treatment of seeds in part A.

1 RESTING METABOLISM OF POLLEN

What Is Pollen?

Smith et al.[200] give a very clear account of the place of pollen in the life cycle of a gymnosperm such as a pine. Briefly: the pine tree, cells of which have 2n chromosomes, bears special branches (staminate strobili, commonly called male cones), about ½in. long and bearing many microspore mother cells. These cells divide meiotically; that is, the resultant cells, which are called microspores, have only n chromosomes each. Before shedding, each microspore divides into four. This group of four cells, each with n chromosomes, and surrounded by hard coats perhaps equipped with "wings" to help wind dispersal, is the pollen grain.

Its function is to fertilise an egg on the same or another tree. From the resultant zygote, which has 2n chromosomes, a seed develops.

Pollen is not homologous with a fungal spore; but in function they are similar in that both act as agents of dispersal, and both are able to "store life", in some cases for years.

Constituents of Pollen

The reviews by Paton,[201] Todd and Bretherick,[202] Lunden,[203] Visser,[204] and Johri and Vasil[205] reveal that pollens have been found to contain almost any biochemical one can think of: proteins, free amino acids; reducing and nonreducing sugars, starch, insoluble carbohydrates; hydrocarbons, long-chain alcohols, fats, sterols;

nucleoproteins; inositols, thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, biotin, folic acid, ascorbic acid, provitamin A , vitamin D, vitamin E, vitamin B_{12}; K, Mg, Ca, Cu, Fe, Si, P, S, Cl, Mn, Ti.

Pinus radiata pollen was studied for this thesis. Pine pollens have 0 - 1.61% reducing sugars, 6 - 12% sucrose, 7% starch, 12.6 - 16.6% protein, 2.8 - 5.5% ash, and 10 - 14.6% fats.[202] Gymosperms' pollens tend to have lower vitamin contents;[203] but on the whole, vitamins are more concentrated in pollen than other plant tissues.[203]

Pinus radiata pollen was found to be 13.45% protein, 1.80% ether extract, 13.92% carbohydrates, 11.25% water, 2.35% ash, and 57.23% undetermined.[202] The ash included (% ages of the whole pollen) 0.88%K, 0.30% P, 0.03% Ca, 0.11% Mg, 0.0015% Fe. The obvious insignificance of some digits does not detract from the qualitative value of these results.

Pinus silvestris was found to have a very much higher concentration of proline and free basic amino-acids in the pollen than in the leaves[206].

Resting Metabolism of Pollen-previous work

Only a handful of workers have attacked (let alone settled) this problem. Enzymes extracted from pollens have included amylase[201,221,222], invertase[201,221,222], catalase[201,219,222], succinic and lactic dehydrogenases[219], cytochromes[219,220], phosphatase[221], and an enzyme system capable of carrying out anaerobic

alcoholic fermentation[220]. The significance of these enzymes in the metabolism of dry pollen is hard to determine because some germinative processes, including perhaps enzyme formation or reactivation, are likely to occur very soon after one wets pollen at the start of conventional enzymological experiments.

Storage of Pollen

Pfundt[209] tested the viability of the pollen of 140 species at 17-22° and R.H. 0, 30, 60 and 90%. He found that low (0-30%) R.H. was best for maintaining viability in storage. This was confirmed by similar tests[210] on another 52 species at 17-22°C and 0, 27, 63 and 92% R.H. The broad conclusion was "storage at low humidities triples on the average longevity of those pollens which it affects". In none of the 52 species studied did pollen remain viable longer at room R.H. than at lower R.H.

On the evidence presented below in the review on storage of seeds, a maximum of about 60% R.H. was taken as applicable to prevent growth of bacteria and fungi in the pollen. Attack by fungi was found to destroy viability in pollen stored for long periods at R.H. 60% and higher[205].

Visser[204] presents data collected from several workers showing that P. radiata pollen has not been studied with regard to the effect of R.H. on viability; but four other Pinus species each stored well at 15-35% R.H., showing little decrease in viability after a year.

Pinus radiata pollen was collected by the writer and freed from at least some contaminants by the process of "surface sifting" discovered in the work on fern spores and described in "Materials", part A. The pollen was dried by storage over 98% technical H_2SO_4 for a day and then loaded into pentapodes.

Results on P. radiata pollen resting metabolism are summarised in Table III. The approximate figures for some R.H.'s result from the fact that the THC was not quite saturated in these cases. The salt, which had been present in excess at room temperature, dissolved in a week at 25°. It is not thought that the actual R.H. in these cases was more than a few% from the value for saturated solutions.

Table III

Expt. no.	1	2	3	4	5	6	7
See Plate no.	XI	XI	XII	XII			
R.H. (%)	c.43	c.43	c.51	62	15	15	62
Duration (days)	40	140	40	140	1	20	40
Metabolites tritiated?	yes	yes	yes	yes	no	no	no
Solid residue tritiated?	yes	yes	yes	yes	yes	yes	yes

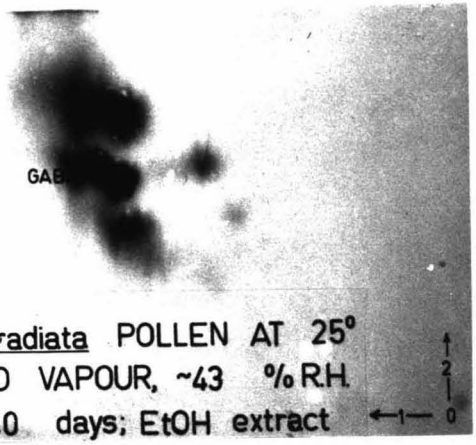
The last-mentioned experiment used pollen which had been stored for some months in a desiccator over technical 98% sulphuric acid. The other results, at c.43%, c.51% and 62% R.H.

indicated that 40 days' exposure to 62% R.H. should label metabolites if the pollen was viable. However, no tritiated metabolites resulted. No germination was observed when a sample of this same batch of pollen was placed on 10% sucrose solution, suitable for germinating fresh P. radiata pollen. It seems, then, that this pollen which had been stored over sulphuric acid for some months was no longer capable of conducting any metabolism detectable by this method. The tritiation of the solid residue in that experiment was probably non-metabolic. The same explanation can be advanced for the similar results in experiments 5 & 6. These were actually done to examine the possibility of labelling, in a brief experiment, by rapidly-metabolising microorganisms of unexpected resistance to low relative humidity. As well as ruling out that possibility of contamination, experiments 5 & 6 point to non-metabolic tritiation of the solid residue, though not so conclusively as the experiment (7) on inviable pollen. Experiment 7 also ruled out the possibility of non-metabolic labelling of metabolites.

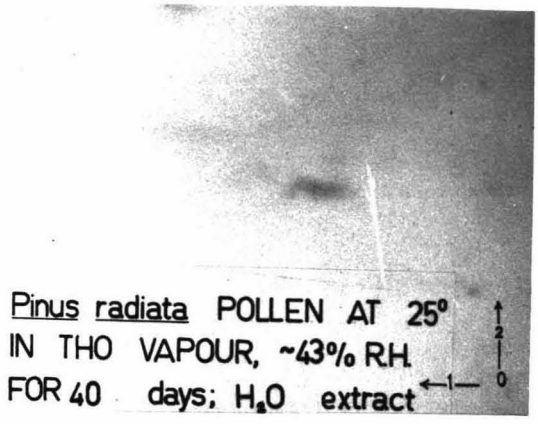
A sample of several score pollen grains was examined with a microscope immediately on removal from the pentapus in experiment 2. None had extruded a germ tube.

The solid residue from experiment 2 was hydrolysed with 10 ml. of 6M "Analar" hydrochloric acid for 30 hr. at 110-120 in a sealed tube. After repeated washing (as described in part A for hydrolysed seed solid residues) the solution was finally freeze-

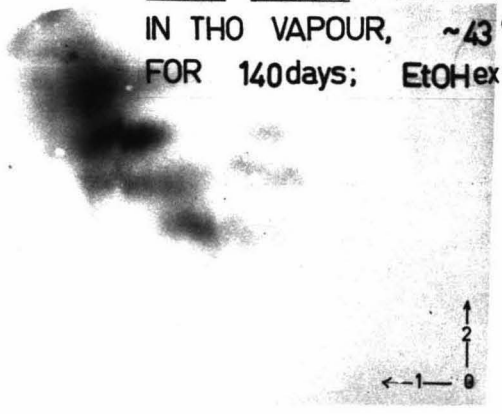
PLATE XI



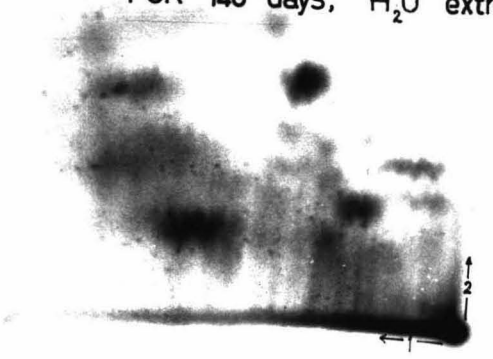
Pinus radiata POLLEN AT 25°
IN THO VAPOUR, ~43 % R.H.
FOR 40 days; EtOH extract



Pinus radiata POLLEN AT 25°
IN THO VAPOUR, ~43% R.H.
FOR 40 days; H_2O extract



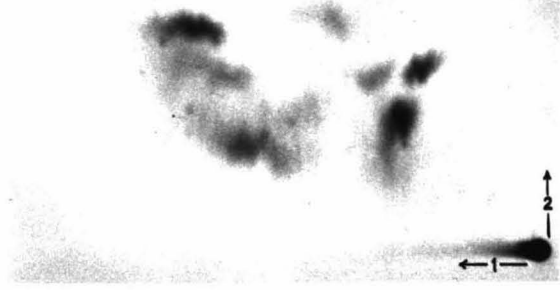
Pinus radiata POLLEN AT 25°
IN THO VAPOUR, ~43 %R.H.
FOR 140 days; EtOH extract



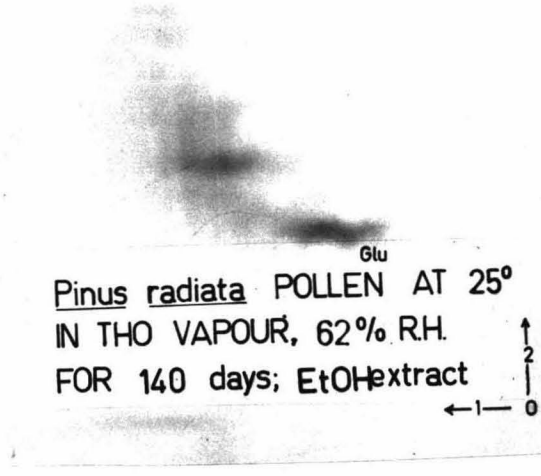
Pinus radiata POLLEN AT 25°
IN THO VAPOUR, ~43 % R.H.
FOR 140 days; H_2O extract

PLATE XII

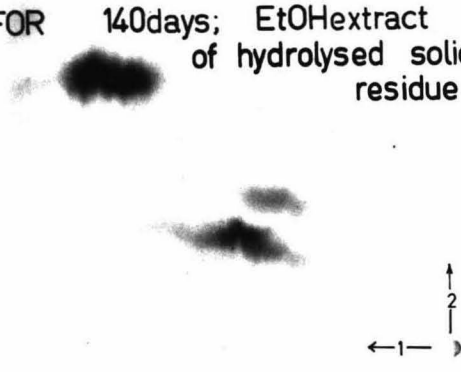
Pinus radiata POLLEN AT 25°
IN THO VAPOUR, ~51 %R.H.
FOR 40 days; EtOH extract



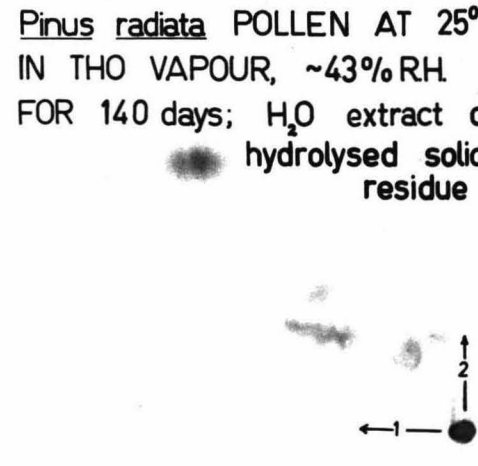
Pinus radiata POLLEN AT 25°
IN THO VAPOUR, ~51% R.H.
FOR 40 days; H_2O extract



Pinus radiata POLLEN AT 25°
IN THO VAPOUR, 62% R.H.
FOR 140 days; EtOHextract



Pinus radiata POLLEN AT 25°
IN THO VAPOUR, ~43% R.H.
FOR 140days; EtOHextract
of hydrolysed solid
residue



Pinus radiata POLLEN AT 25°
IN THO VAPOUR, ~43% R.H.
FOR 140 days; H_2O extract of
hydrolysed solid
residue

dried, and extracts of it chromatographed, revealing some tritiated compounds (Plate XII). These are probably remnants of those which had been extracted before hydrolysis. As with seeds, all four separate extracts were sometimes processed separately. When any tritiated compounds were present in the first alcohol or water extract, they also occurred in the second extract with the same solvent. Therefore the solid residue might well have contained unextracted portions of the same tritiated compounds.

The solid remaining after the hydrolysis just described was still tritiated. This was probably owing to incomplete hydrolysis. It was not further investigated.

The labour of co-chromatography of all the compounds tritiated by resting pollen will not be warranted until these compounds have been reproducibly obtained from repeated experiments. However, as shown in Plates XI and XII, 4-aminobutyric acid and glutamic acid have been identified (by co-chromatography in solvents (1) & (2) only). Many of the tritiated compounds do not correspond with any on the "standard map" of metabolites in solvents (1) & (2) (ref. 19). They may prove to be newly discovered metabolites. It is further speculated that one of the tritiated compounds extracted by ethanol from pollen resting in THO vapour is the "mystery spot" studied in part A of this thesis. This is surmised from its position on the chromatogram relative to 4-aminobutyric acid which has been identified.

DISCUSSION

Resting Pinus radiata pollen has been shown, by the experiments just described, to conduct several metabolic reactions. Two of the compounds have been identified and are common plant metabolites, and some others probably are also; in particular, three of the tritiated compounds are very likely to be citric, malic and succinic acids, which are characteristic of the Krebs (tricarboxylic acid) cycle. The adverse effect of high oxygen pressure on pollen in storage seems compatible with the hypothesis of the aerobic metabolism [204]. The Krebs cycle produces carbon dioxide and so a high pressure of that gas above resting pollen would be expected to slow the breakdown of stored foods. In fact pollen does remain viable longer under a high pressure of carbon dioxide [204].

Spedding [1] germinated Pinus radiata pollen for 75 min. in (liquid) THO. He did not identify any of the 12 labelled compounds produced; but comparison of that chromatogram with those from resting pollen shows that many of the compounds tritiated germinating pollen may be the same as in the resting pollen. It may well be, then, that the resting metabolism of pollen is not qualitatively different from its metabolism in early germination.

2 RESTING METABOLISM OF SPORES

The fungus spores studied were conidia of Pithomyces chartarum (Berk. et Curt.) M.B. Ellis, a saprophyte in the large class of imperfect fungi. The word "imperfect" actually refers to the state of botanical knowledge on the life history. Electron microscopy showed [207] that each spore contains c.6 cells. The concentration of ribosomes is very high, and mitochondria are numerous. These organelles need not, however, be metabolising in the dry spore.

The free amino acids extracted from these spores by ethanol/formic acid are [208] :- leucine, isoleucine, valine, tyrosine, alanine, proline, glutamic & aspartic acids, histidine, serine, phenylalanine, glycine, arginine and probably lysine and cystine.

Also relatively high levels of mannitol and malonic acid were found. The latter is a very well known competitive inhibitor of succinic dehydrogenase (from mammals). This enzyme catalyses one step of the Krebs cycle. It is conceivable that P. chartarum succinic dehydrogenase might be inhibited in the spores by the large concentration of malonate. This is a possible means for slowing of metabolism in the spores' state of "suspended animation".

Previous work

As with pollen, very little indeed has been reported previously on resting metabolism of fungus spores.

Longevity is usually less than that of seeds, [212] [213] but

dried spores of Aspergillus niger were stored for 10 years (at unspecified R.H.) at room temperature without great loss of viability.[211] A review on the subject mentions several early records of survival times up to 21 years. Like seeds, spores usually retain viability longest at low temperatures (around 0°) and R.H.[212]. However, a few cases have been reported[212] in which 100% R.H. was best.

Ascospores of Neurospora tetrasperma are dormant[214] (i.e. will not germinate when suitable conditions are supplied) but can be activated by heating at 50° for a few minutes. The writer finds it hard to understand how the species has survived if that treatment is necessary to break dormancy. Respiration after activation was greatly inhibited[214] by HCN or CO and this was taken as evidence for cytochromes' operation. Other inhibition experiments were taken to support the hypothesis that the spores were using the Meyerhof-Kiessling pathway (the scheme of alcoholic fermentation current in 1938).

The oxygen consumption of dry, ungerminated conidia (spores) of the brown-rot fungus Sclerotinia fructicola[218] is very high.

Methods and Results

The same batch of spores was used as for the germination studies[2]. They were known to be viable[2], but did not produce germ tubes in the experiments described here.

A few mg. of spores was loaded directly into each pentapus arm from storage at -20°. 15%, a very low R.H., was chosen

because some fungus spores can germinate, without liquid water, at 70% R.H.[215,216]. Although those studied in this work do not produce germ tubes at such R.H.'s, it must be realised that emergence of a growing hypha (i.e. germination in the conventional sense) is the end of a complex sequence of metabolic reactions, some of which might well occur at moderate R.H.'s. Yarwood's claim[217] that conidia of a powdery mildew, Erysiphe polygoni, germinated at about 0% R.H. seems incredible. Examination of his paper reveals that his method of providing 0% R.H. depended on keeping the spores and sulphuric acid (S.G. 1.84) in a Petri dish sealed only by a rubber band. Considerable growth of mycelium occurred. Very likely the sealing was poor and the R.H. well above zero.

Thermostatting of the resting spores, extraction and subsequent treatment were all exactly as described for pollen.

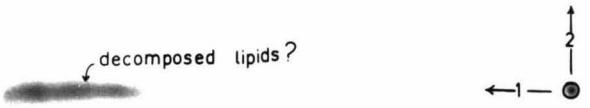
The result is shown in Plate XIII. On the chromatogram of the ethanol extract the lipid corner is of course missing, having been removed before autography and counted (see section A, "detection of tritiated lipids"). The count was many σ above background (i.e. toluene-soluble tritiated lipids were present).

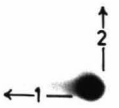
The tritiated streak labelled "decomposed lipids?" fluoresced light blue in UV light. A similar streak and the toluene-insoluble material at the solvent fronts were labelled in an exactly similar experiment for 4 months.

PLATE XIII

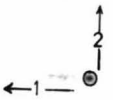
Pithomyces chartarum spores at 25° in THO vapour, 15% R.H., for 12 months; EtOH extract

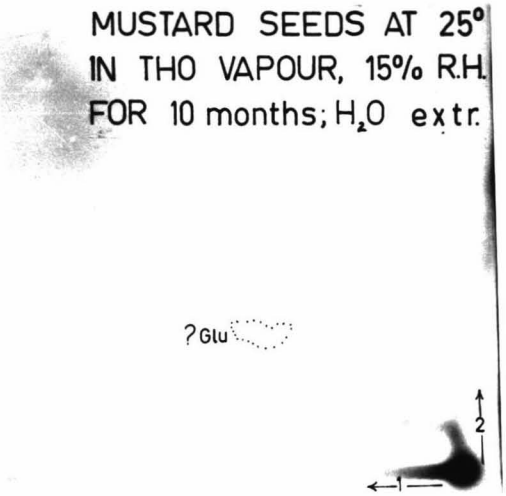
Pithomyces chartarum spores at 25° in THO vapour, 15% R.H., for 12 months; H_2O extr.





MUSTARD SEEDS AT 25° IN THO VAPOUR, 15% R.H. FOR 10 months; EtOH extr.





MUSTARD SEEDS AT 25° IN THO VAPOUR, 15% R.H. FOR 10 months; H_2O extr.

Tree fern spores (see "Materials", part A) exposed to 15% R.H. THO for 4 months at 25° tritiated only the toluene-insoluble material at the solvent fronts.

All solid residues were tritiated.

DISCUSSION

In contrast to pollen, P. chartarum spores resting at 15% R.H. conduct, according to the above results, metabolism extremely different from that in early germination. The lipid metabolism might be the same. Absent from the resting metabolism are those reactions which labelled glutamic, aspartic, citric and malic acids within 30 sec. after addition of liquid THO, and phosphates soon after.

The compounds labelled in resting metabolism are perhaps lipids. They will probably be best resolved by thin layer chromatography.

3 RESTING METABOLISM OF SEEDS

The problem of how seeds "store life" has resisted attack by conventional techniques. Although wheat thousands of years old, taken from Egyptian tombs, has never in fact germinated[234,235], some seeds several centuries old[237] have[236] germinated 100%. Many other cases of great longevity are listed in a recent book on the subject[238]. Ewart[239] proposed a classification according to longevity. He called seeds which retain viability less than three years "microbiotic"; 3-15 years, mesobiotic; and longer than 15 years, macrobiotic. However, as was pointed out by Crocker[240], discovery of better storage conditions for a given species' seeds can promote them to macrobiotic from mesobiotic or even microbiotic.

Conditions for Storage of Seeds

In general[238] low temperatures (around 0°) and low R.H. (below 60%) increase seeds' longevity in storage.

Some seeds have survived exceedingly drastic treatment. Seeds of Johnson grass were heated for 6 hr. at 100° and then germinated but grew to less vigorous seedlings than those of controls[241]. Radish seeds germinated 14% after 30 min. at 123° if their initial water content was 0.4% ; but if it was 4%, germination was 0% after 30 min. at 100°; and if 40%, no seeds survived 30 min. at 65° (ref. 242). "Well-dried" wheat, barley and oats germinated well after

30 hr. at 100° (ref. 243). White mustard seeds[244] kept at liquid air temperatures for two days later germinated normally.

Microflora of Seeds

Any experiment on the metabolism of resting seeds should be designed so that the results will in fact allow unambiguous conclusions about the seeds' activities. Now seeds almost always are contaminated with microflora[223]. Bacteria (including actinomycetes) and/or fungi occur externally and often internally too. It will be desirable, then, to ensure that these cannot metabolise appreciably in experiments on resting seeds.

The external microflora of seeds are mostly saprophytes. Dozens of species of moulds, yeasts and bacteria have been identified by the simple method of washing seeds briefly in very dilute detergent and examining the washings microscopically before or after culturing. It is probably impossible to find seeds occurring naturally free from external microflora.

Internal microflora of seeds are usually less abundant than the external ones. Parasites and saprophytes have been identified. Often it is not at all obvious from the outside that a seed is infected.

Only rotted or wounded seeds are penetrated by bacteria ,

which in any case require R.H. above 90%. Yeasts have a similarly high moisture requirement, the minimum R.H. for their survival being 88%. Proliferation of either bacteria of yeasts can therefore be prevented in resting seeds if the R.H. above the organisms is (allowing a margin for safety) below 80%.

Fungal hyphae have, in various species, been found in the pericarp, aleurone layer, endosperm and embryo of seeds. In the pericarp of wheat in storage, fungal hyphae are said[224] to be responsible for 95% of the CO_2 given off. But if fungi can be prevented from growing in or on the seeds, then not hyphae but merely spores can be metabolising in the seeds, apart from the seeds' own metabolism. If then only 5 seeds are used in an experiment, and they are not heavily contaminated with mould spores, we may expect the incorporation of tritium by the spores to be neglible - far below the detection level.

Fortunately, mould spores have a minimum requirement of R.H. in germination. For particular species the values have been found to fall in the range 62-99% R.H. For many species[226] the minimum is 75% R.H. Of the moulds occurring in or on seeds, only a few species can grow at lower R.H. than 75% (ref. 228). Mould spores' germination and growth in soybeans at room temperature increased with moisture content and caused lower germination percentage[225]. Mould counts in seeds increased logarithmically[227] from 75% to

100% R.H. Inoculation of wheat in storage at 85% R.H. with Aspergillus spp. and a Penicillium sp. caused lower germination when, 2 - 12 months later, germination tests were made. Little mould growth occurred in corn at 77.2% R.H., and it was 50% slower yet at 72.5%. Over long periods (more than a year) Aspergillus restrictus can[230] grow in wheat stored at 60% R.H. The graph of respiration rate versus moisture content of soybeans[226] and wheat[229] shows a slight rise up to 75% R.H., above which respiration rate sharply increases. It should be mentioned, however, that fungal infection was discounted as a primary cause of the loss of viability of cotton seed stored for many months at low moisture contents[232].

Various fungicides have been used[231, 246] on seeds in storage and in some cases have kept down mould without seriously lowering the seeds' viability. Such "differential poisoning" should probably be avoided when possible. There is, after all, no need to risk harming the seeds with foreign chemicals when fungi can be effectively controlled by keeping the R.H. below 60%. Many of the papers reviewed below on the metabolism of resting seeds have no mention of such measures to control fungi and are therefore somewhat unreliable.

Previous Work on Resting Metabolism of Seeds

This is reviewed here according to the type of experimental approach used.

1 Measurement of respiration

The R.Q. of dry barley[247] is 0.64; of Lupinus albus seeds[248], 0.89-0.65 at 18° and 0.66-0.40 at 8°; of several seeds dormant in a moist medium, 0.69 falling to 0.43 over 564 days[249]. These results are not much use for deducing the intermediary metabolism of the seeds.

CO_2 usually is given off by seeds in storage (e.g. ref. 244, and those above on R.Q.) but cottonseed in storage uses CO_2 (ref. 250).

Increased CO_2 content of the atmosphere increases seeds' longevity[251].

2 Changes in Constituent Compounds of Seeds in Storage

The reader is referred to the discussion in part A of this thesis of the ambiguous nature of negative results in this type of experiment. Positive results include decreases in concentration of protein[252], thiamine[253], ascorbic acid[254] and sucrose[255]; and increases in lactic acid[256] and fatty acids[257].

3 Identification of Enzymes in Extracts of Seeds

Many enzymes have been found in aqueous extracts of seeds. Whether they were operative in the dry seeds seems uncertain. The great temperature extremes through which seeds retain viability, discussed above under "Conditions for Storage of Seeds", might seem

likely to denature enzymes. Some enzymes found in aqueous extracts of seeds have been:- fumarase[258], hexokinase[259], carboxylase[260], NADPH-oxidase[261], hexose dehydrogenases[262], alcohol dehydrogenases[263] and glutamic decarboxylase (producing 4-aminobutyric acid).[264]

Also reported have been coenzyme A[265], NAD and NADP[266], and[269] cytochrome c.

Lipase activity has been found[267,268] in extracts of seeds which had been heated for an hour or more slightly above 100°C. Possibly these enzymes were synthesized during the soaking and extraction, rather than having been present all along. That possibility applies to all the enzyme studies mentioned in this section so far, but perhaps not to the lipases obtained[192,270] by macerating seeds in petroleum ether before making aqueous extracts.

4 Effects of Inhibitors

Barton[56] passed air containing 0.1% of a gaseous poison over seeds for periods from one to 960 min. HCN or H_2S had no effect, but NH_3, Cl_2 or SO_2 (potency was found to increase in that order) delayed or prevented germination when the seeds were moistened after treatment with the gas. The positive results are hard enough to interpret in metabolic terms, but the negative results are even harder, because no check was made as to whether the HCN and H_2S even got into the seeds.

METHODS AND RESULTS

Batches of 5 mustard seeds were exposed to THO vapour in a pentapus at 25°. The very low R.H. of 15% was chosen as a safeguard against proliferation of microflora in or on the seeds. Extraction and analysis of the seeds was exactly as described in part A.

After one day, and 20 days, no tritiated compounds except the solid residue were detected. But after 10 months the results were as shown in plate XIII. The toluene extract of the "lipid corner" was significantly tritiated, as was the solid residue.

DISCUSSION

These preliminary experiments suggest that the resting metabolism of seeds is different in kind from the metabolism in germination. The diffuse spots running near the lipid corner were not found in germination, and apart from the spot labelled "Glu?" (this is merely a guess) the areas near the origin are very different from those in typical germination experiments. It is very hard to say, looking at the standard map[19] of where biochemicals run in this chromatographic system, what the labelled spots may be. But we can conclude that germination is qualitatively different from resting metabolism, i.e. different pathways are operating.

SUGGESTIONS FOR FURTHER WORK

1 The labelled material at the "lipid corner" of chromatograms could be investigated in detail, e.g. by gas and/or thin-layer chromatography.

2 The "mystery spot" should be identified. Whether or not it proves to be a "new" metabolite, it is probably important, because it becomes labelled in mustard seeds germinating at 0° but not in normal germination at room temperature. Similar remarks apply to the unidentified spots "U_3" and "U_4".

3 Many seeds are dormant when shed from the parent plant, i.e. they require a period of after-ripening, wet or dry in different species, before they can be made to germination. This phenomenon of dormancy looks ideal for investigation by the methods of this thesis.

4 Apart from attack by microflora, seeds will in any case become inviable if kept long enough even under the temperature and R.H. which maximise longevity in storage. Introducing THO into such inviable seeds might provide clues as to which reactions or pathways have broken down, preventing germination. For instance, it might be found that the inviable seeds label amino-acids but not malate or citrate.

5 Many seeds can germinate (slowly) in an atmosphere containing only 5% oxygen[189,190]. Some even germinate under pure nitrogen or argon[191] ! It would be interesting to compare these species' germinat-

ion with others requiring normal or near-normal atmospheres for germination.

6 In view of the dominance of the Krebs cycle in our current theory of germination, other experiments to detect it should be made. For instance, one might germinate mustard seeds for (say) 4 hr., extract them with a suitable aqueous buffer, and screen the extract for the enzymes of the Krebs cycle.

7 Krahl[21] suggests that a fertilised sea-urchin egg, in the first 24 hr. of its development, changes from the pentose phosphate pathway to the Krebs cycle for metabolising glucose. This topic looks very suitable for examination by the THO method.

8 Analogous methods with other isotopes should be useful, though none would tell as much as tritium incorporation. Hydrolyses of peptide and glycosidic links, undetectable by the THO method because no hydrogen is attached to carbon from water, would entail incorporation of ^{18}O from water enriched in that isotope. This method would share most of the advantages of the THO method, and further, the kinetic isotope effect should be much smaller. However, the detection of stable isotopes is not as easy as scintillation autography. Many reactions of phosphates would entail incorporation of label from ^{32}P-orthophosphate, if that could be introduced into the living cell.

ABBREVIATIONS

AMP, ADP, ATP Adenosine mono-, di-, triphosphate respectively. Similar abbreviations are used for nucleotides containing cytidine (C), uridine (U), guanosine (G), and inosine (I).

FMN Flavin mononucleotide.

FAD Flavin adenine dinucleotide.

NMN Nicotinamide mononucleotide.

NAD Nicotinamide adenine dinucleotide (formerly DPN).

NADP Nicotinamide adenine dinucleotide phosphate (formerly TPN).

NAD(P) Either of the above two.

DNA Deoxyribonucleic acid.

RNA Ribonucleic acid.

CoA Coenzyme A.

T Tritium.

THO Water rich in tritium (sometimes called tritium water or tritiated water).

D_2O Deuterium oxide.

Ⓟ Orthophosphate, ionized as appropriate.

GABA 4-aminobutyric acid.

Ala α-alanine.

Asp	Aspartic acid.
Glu	Glutamic acid.
C	curie.
mC	millicurie.
μC	microcurie.
r.	roentgen.
rep	roentgen equivalent physical.
c.	approximately.
CA	Chemical Abstracts (U.S.).
JBC	Journal of Biological Chemistry.
BJ	Biochemical Journal.
JACS	Journal of the American Chemical Society.

References

1 Spedding: M.Sc. thesis, 1962, Victoria University of Wellington.

2 Edwards: M.Sc. thesis, 1963, Victoria University of Wellington.

3 Shukuya & Schwert: JBC 235 1658 (1960).

4 Pearson & Harper: "The World's Hunger", Cornell Univ. Press, 1945.

5 Scheibe & Staffeld: Fortschr. d. Landw. 6 364 (1931).

6 Pauli: Trans. Kansas Acad. Sc. 66 255 (1963), from CA 59 14504.

7 Buduryan & Kandina: Vopr. Fiziol i Biokhim. Kul't Rast., Akad. Nauk Moldavsk. SSR, Inst. Fiziol i Biokhim. Rast. 1962 (1) - three papers, pp. 33, 45 & 71.

8 Mayer: Penguin Sc. News 29 440 (1953).

9 Evenari: Symp. Soc. Exptl. Biol. 11 21 (1957).

10 Emerson: Plant Physiol. 29 418 (1954).

11 Tang: J. Gen. Physiol. 15 87 (1932).

12 Fukui & Nikuni: Mem. Inst. Sc. & Ind. Res., Osaka Univ. 13 187 1956; from CA 50 15762.

13 Klatzkin, Norris & Nokes: Abstr. 1st Internat. Congr. Biochem. (1949) pp. 26 & 490.

14 Feldheim: Ehrnährungsforschung 2 97 (1957); from CA 51 18174.

15 Radhakrishnan, Vaidyanathan & Giri: J. Ind. Inst. Sc. 37A 178 1955; from CA 50 5095.

16 Crocker & Barton: "Physiology of Seeds", Waltham U.S.A., 1952.

17 Krebs: in Sumner & Myrback (eds.), "The Enzymes", vol. 2, p. 499. Acad. Press N.Y., 1951.

18 Mandels: in Halvorson (ed.), "Spores", publication no. 5, Am. Inst. Biol. Sc., Washington D.C. (1957).

19 Bassham & Calvin: "The Path of Carbon in Photosynthesis", Prentice-Hall 1957.

20 The International Union of Biochemistry: Report of the Commission on Enzymes. Pergamon, 1961.

21

22 Vennesland & Westheimer, in McElroy & Glass: "The Mechanism of Enzyme Action", Johns Hopkins Press, Baltimore, 1954.

23 Ogston: Nature 162 963 (1948).

24 Fisher, Frieden, McKee & Alberty: JACS 77 4436 (1955).

25 Englard & Colowick: JBC 221 1019 (1956).

26 Farrar, Gutowsky, Alberty & Miller: JACS 79 3978 (1957).

27 Englard & Colowick: JBC 226 1047 (1957).

28 Pearson & Dillon: JACS 75 2439 (1953).

29 Mandeles, Koppelman & Hanke: JBC 209 327 (1954).

30 Graves & Vennesland: JBC 226 307 (1957).

31 Vennesland et. al.: Prog. Stereochem. 3 299 (1962).

32 Topper: JBC 225 419 (1957).

33 Alexander & Schwenk: JACS 79 4554 (1957).

34 Kosower: "Molecular Biochemistry", McGraw-Hill, 1961.

35 Konikova, Kritzmann & Teiss: Biokhimiya 7 86 (1942).

36 Konikova, Dobbert & Braunstein: Nature 159 1107 (1947).

37 Hilton, Barnes, Henry & Enns: JBC 209 743 (1954).

38 Longenecker & Snell: Proc. Nat. Acad. Sc. U.S. 42 221 (1956).

39 Metzler, Ikawa & Snell: JACS 76 648 (1954).

40 Oshima & Tamiya: BJ 78 116 (1961).

41 Harley-Mason: Arch. Biochem. Biophys. 93 178 (1961).

42 E.H. Fischer, Kent, Snyder & E.G. Krebs: Abstr. 4th Internat. Congr. Biochem., paper 4-61, p.45 (1958).

43 Rose & Rieder: JBC 231 315 (1958).

44 Fruton & Simmonds: "General Biochemistry", 2nd edn., Wiley (New York), 1958; p. 468.

45 Tatum & Shemin: JBC 209 671 (1954).

46 Englard: JBC 232 1003 (1958).

47 McLennan: pers. comm. (1964).

48 Glaser: JBC 235 2095 (1960).

49 Flavin & Slaughter: JBC 235 1112 (1960).

50 Rose: JACS 80 5835 (1958).

51 Oesper & Meyerhof: Arch. Biochem. 27 223 (1950).

52 Nicholson: "Metabolic Pathways chart", L. Light, England, 1963.

53 Kent & Brink: Science 106 547 (1947).

54 Kawahara & Talalay: JBC 235 PC 1 (1960).

55 Popjak et al.: Biochim. Biophys. Res. Com. 4 138 (1961).

56 Barton: Contrib. Boyce Thompson Inst. 11 181 (1940).

57 Schoenheimer et. al.: JBC 114 381 (1936)
121 235 (1937)
133 713 (1940).

58 Thompson: JBC 197 81 (1952).

59 Cline: Plant Physiol. 28 717 (1953).

60 Aronoff & Choi: Arch. Biochem. Biophys. 102 159 (1963).

61 Foster & Bloom: JBC 238 888 (1963).

62 Goldsby: PH.D. dissertation, Univ. of Calif., Berkeley (1960).

63 Glascock & Duncombe: Proc. Biochem. Soc. 51 xl (1952).

64 Wiberg: Chem. Revs. 55 713 (1955).

65 Melander: "Isotope Effects on Reaction Rates", the Ronald Press, New York (1960).

66 Lewis: Science 79 151 (1934).

67 Pratt & Curry: Am. J. Bot. 24 412 (1937).

68 Luler: Arkiv. Kemi, Mineral, Geol. 24A no. 13, 15pp. (1946); from CA 42 6893.

69 Brun & Tronstad: Kgl. Norske. Videnskab. Selskabs Fork. 7 171 (1935); from CA 29 5137.

70 Macht & Davis: JACS 56 246 (1934).

71 Meyer: Science 79 210 (1934).

72 Plantefol & Champetier: Compt. rend. 200 423 (1935); from CA 29 3361.

73 Weinberger & Porter: Arch. Biochem. Biophys. 50 160 (1954).

74 Eyring & Sherman: J. Chem. Phys. 1 345 (1933).

75 Thompson & Ballou: JBC 206 101 (1954).

76 Krichevsky et. al.: JBC 236 2520 (1961).

77 Marcus & Vennesland: JBC 233 727 (1958).

78 Bové, Martin, Ingraham & Stumpf: JBC 234 999 (1959).

79 Ochoa: Advances in Enzymol. 15 183 (1954).

80 Fisher, Conn, Vennesland & Westheimer: JBC 202 687 (1953).

81 Dixon & Webb: "Enzymes", 2nd edn., Longmans (1964).

82 Kosicki & Srere: JBC 236 2566 (1961).

83 Halford & Anderson: JACS 58 736 (1936).

84 Thorn: BJ 49 602 (1951).

85 du Vigneaud et al.: JACS 74 5941 (1952).

86 Bowen & Thick: Radiation Res. 13 234 (1960).

87 Spalding, Langham & Anderson: Radiation Res. 4 221 (1956).

88 Brues et al.: Proc. Soc. Exptl. Biol. Med. 79 174 (1952).

89 McDonough & Wood: JBC 236 1220 (1961).

90 Overath, Kellerman & Lynen: Biochem. Z. 335 500 (1962).

91 Rose & O'Connell: JBC 236 3086 (1961).

92 Lack: JBC 236 2835 (1961).

93 Krebs & Lowenstein: in "Metabolic Pathways", ed. Greenberg, Academic Press N.Y. & London, (1960); p. 134.

94 Martius & Schorre: Z. Naturf. 56 170 (1950).

95 Anet: JACS 82 994 (1960).

96 Gawron & Fondy: JACS 81 6333 (1959).

97 Nakil & Bressler: JBC 236 1643 (1961).

98 Zelitch: JBC 234 3077 (1959).

99 R.Henwood: pers. comm.

100 E. Zilch: pers. comm.

102 Belleau & Burba: JACS 82 5751 (1960).

103 Curry, Pratt & Trelease: Science 81 275 (1935).

104 Hughes, Bennett & Calvin: Ann. N.Y. Acad. Sc. 84 763 (1960).

105 Gross & Harding: Science 133 1131 (1961).

106 Wilson & Dinning: Biochim. Biophys. Acta 53 223 (1961).

107 Evans & Stanford: Nature 197 551 (1963).

108 Pollard & Powell: Radiation Res. 2 109 (1955).

109 Lea et al.: Parasitology 36 110 (1944).

110 Guild: Phys. Rev. 83 230 (1951).

111 Tetenyi: Agrokemia es Talajtan 8 219 (1959); from CA 54 796.

112 Haber & Tolbert: Plant Physiol. 34 376 (1959).

113 Govindjee: Naturwiss. 44 183 (1957).

114 Jennings & Brues: ANL-4333 136 (1949) quoted by Thompson: Nucleonics 12 31 (1954).

115 Porter & Knauss: Plant Physiol. 29 60 (1954).

116 Hevesy & Forsberg: Proc. 3rd Internat. Congr. Biochem. 479 (1955).

117 Stoklasa et al.: Biochem. Z. 194 15 (1928); from CA 22 2581.

118 Stoklasa: Ehrnähr. Pflanze 30 299 (1934); from CA 28 7294.

119 de Caramay & Champy: Compt. rend. soc. biol. 121 750 (1936); from CA 30 3856.

120 Valasyuk & Grodzinskii: Doklady Akad. Nauk SSSR 106 562 (1956).

121 Dale: Proc. 3rd Internat. Congr. Biochem. 494 (1955).

122 Foster, Dewey & Gale: Nucleonics 11 14 (1953).

123 Ehrenberg: Svensk Kem. Tidskr. 67 5 (1955).

124 Haber, Carrier & Foard: Am. J. Bot. 48 431 (1961).

125 Biebl: Proc. 2nd U.N. Conf. on the Peaceful Uses of Atomic Energy 27 299 (1958).

126 Bacq: Proc. 3rd Internat. Congr. Biochem. 494 (1955).

127 Savulescu, Becerescu, Mihalcu & Abromovici: Proc 2nd U.N. Conf. on the Peaceful Uses of Atomic Energy 27 286 (1958).

128 Saric: ibid. p. 233.

129 Scully: Agr. Expt. Sta. Kansas State Coll., Rept. no. 4 242 (1953); from CA 48 4638.

130 Shiroishi: Agr. Biol. Chem. 25 611 (1961).

131 Broda: "Radio Isotopes in Biochemistry", Elsevier (1960).

132 Wilson: Ph.D. dissertation, Univ. of Calif., Berkeley, (1954).

133 Wilson & Calvin: JACS 77 5948 (1955).

134 P.O. Whimp: pers. comm.

135 Jeanes, Wise & Dimler: Anal. Chem. 23 415 (1951).

136 Cerbulis & Taylor: Anal. Chem. 35 114 (1963).

137 Moffat & Lytle: Anal. Chem. 31 926 (1959).

138 Davison et al. (eds.): "Data for Biochemical Research", Clarendon Press, Oxford (1958).

139 Trevelyan, Proctor & Harrison: Nature 166 444 (1950).

140 Anet & Reynolds: J.Chem. Soc 174 930 (1954).

141 R.W. Bailey: pers. comm.

142 Kallmann & Furst: Phys. Rev. 79 857 (1950).

143 Wilson: Biochim. Biophys. Acta 40 522 (1960).

144 Aronoff: "Techniques of Radiobiochemistry", Iowa State Univ. Press (1956).

145 U.K. At. Energy Authority Health Safety Code no. H.1.2., issue no. 1, 15 pp. (1961).

146 Crow (president, Genetics Soc. of Amer.): in "Survival", ed. Fowler; MacGibbon & Kee (1960); p. 99.

147 Cajola: Rivista ital. essenze profumi 12 xxii (1930); from CA 25 1637.

148 Fischer, Parsons & Morrison: Nature 161 764 (1948).

149 Horder (ed.): "The Ilford Manual of Photography", 5th edn. (1958); p. 259.

150 Bassham: Ph.D. dissertation, Univ. of Calif., Berkeley (1949).

151 Wilzbach: JACS 79 1013 (1957).

152 Mayer & Poljakoff-Mayber: "The Germination of Seeds", Pergamon Press (1963).

153 Stumpf: JBC 182 261 (1950).

154 Hatch & Turner: BJ 69 495 (1958).

155 Lebedev: Doklady Akad. Nauk SSSR 128 632 (1959).

156 Filippovich: Nauch. Doklady Vysshe Shkoly, Khim. i Khim. Tekhnol. (1959) 110.

157 James: Proc. R.S. B141 289 (1953).

158 Fowden: Endeavour, January (1962).

159 Krebs: Enzymologia 12 88 (1946).

160 Sivaramakrishnan & Sarma: BJ 62 132 (1956).

161 Benson: pers. comm. to A.T. Wilson

162 Speck: Adv. Carbohydrate Chem. 13 63 (1958).

163 M. Probine: pers. comm.

164 Breese: Cereal Chem. 32 481 (1955).

165 Mann & Marrinan: Trans. Far. Soc. 52 481 (1956).

166 Lang & Mason: Can. J. Chem. 38 373 (1960).

167 Gutmanis: Latvias PSR Zinatnu Akad. Vestis (1959) no. 1, p. 55; from CA 54 6887.

168 Tits: Bull. sci. acad. roy. Belg. 12 545 (1926); from CA 21 942.

169 Soldatenkov & Mazurova: Biokhimiya 21 573 (1956).

170 James: New Phytol. 39 133 (1940).

171 Mayer, Poljakoff-Mayber & Appleman: Physiol. Plantarum 10 1 (1957).

172 Pett: BJ 29 (1935).

173 Fukuba: Symposia on Enz. Chem. (Japan) 8 85 (1953); from CA 51 15641.

174 Funahashi, Uritani & Akazawa: J. Agr. Chem. Soc. Japan 27 684, 842, 849 (1953); from CA 48 12253.

175 Albaum & Cohen: JBC 149 19 (1943).

176 Smith & Williams: Arch. Biochem. Biophys. 31 366 (1951).

177 Leonard & Burris: JBC 170 701 (1947).

178 Sisakyan & Vasil'eva: Biokhimiya 19 730 (1954); from CA 49 6389.

179 Kornberg & Beevers: Nature 180 35 (1957).

180 Losada et al.: Biochim. Biophys. Acta 73 646 (1963).

181 Heydeman: Nature 181 627 (1958).

182 Marcus & Velasco: Biochim. Biophys. Acta 38 365 (1960); also JBC 235 563 (1960).

183 Brown & Goddard: Am. J. Bot. 28 319 (1941).

184 Schmidt: Z. Pflanzenernähr. Düngung. 4B 162 (1925); from CA 19 3558.

185 Kidd & West: Ann. Bot. 31 457 (1917); from CA 12 1205.

186 Thornton: Boyce Thompson Inst. Contrib. 13 355 (1944).

187 Dilling: Ann. Appl. Biol. 13 160 (1926).

188 Mitchell & Marth: Bot. Gaz. 107 408 (1946).

189 Heydecker: Nature 181 1140 (1958).

190 Siegel & Rosen: Physiol. Plantarum 15 437 (1962).

191 Siegel et al.: Nature 197 329 (1963).

192 Singer & Hofstee: Arch. Biochem. 18 229 (1948).

193 Sivaramakrishnan & Sarma: BJ 62 132 (1956).

194 McConnell: Can. J. Biochem. Physiol. 35 1259 (1957).

195 Stiles & Leach: Proc. Roy. Soc. B113 405 (1933).

196 Evenari, Neumann & Klein: Physiol. Plantarum 8 33 (1955).

197 Smith: Gt. Britain DSIR Food Invest. Bd. Rept. (1935) p. 132.

198 Pringsheim, Jedlitschka & Gorlich: Planta 19 653 (1933).

199 International Critical Tables.

200 Smith et al.: "A Textbook of General Botany" 5th edn. Macmillan, New York, 1953; pp. 490-500.

201 Paton: Am. J. Bot. 8 471 (1921).

202 Todd & Bretherick: J. Econ. Entomol. 35 312 (1942).

203 Lunden: Svensk. kem. Tidskr. 66 201 (1954).

204 Visser: Meded. Landb. hogesch., Wageningen 55 1 (1955) (in English).

205 Johri & Vasil: Bot. Rev. 27 325 (1961).

206 Virtanen & Kari: Acta Chem. Scand. 9 1548 (1955).

207 Dickson: N.Z. J. Bot. 1 381 (1963).

208 Gumbley: p. comm. to Edwards (ref. 2 above).

209 Pfundt: Jahrb. Wiss. Bot. 47 1 (1910).

210 Holman & Brubaker: Univ. Calif. Pub. Bot. 13 179 (1926).

211 Zobl: Arch. Mikrobiol. 13 191 (1943).

212 Cochrane: "Physiology of Fungi" (Wiley, 1958).

213 Novoselova: Trudy Vsesoyuz. Nauch-Issledovatel. Inst. Konditerskoi Prom. (1958) no. 12 p.33; from CA 54 816.

214 Goddard & Smith: Plant Physiol. 13 241 (1938).

215 Sawyer: Am. J. Bot. 16 87 (1929).

216 Armolik & Dickson: Phytophath. 46 398 (1956).

217 Yarwood: Phytophath. 26 845 (1936).

218 Frampton & Marsh: Phytophath. 31 9 (1941).

219 von Euler et al.: Arkiv. Kemi, Mineral. Geol. 19A no. 4 (1945), 26A nos. 21,22,30 (1948).

220 Okonuki: Acta Phytochim. 13 93 (1943).

221 Haeckel: Planta 39 431 (1951).

222 Elser & Ganzmuller: Z. Physiol. Chem. 194 21 (1930).

223 Anderson & Alcock (eds): "Storage of Cereal Grains and their Products", Am. Assoc. Cereal Chem., St Paul, Minnesota.

224 Oxley & Jones: Nature 154 826 (1944).

225 Tervet: Phytophath. 35 3 (1945).

226 Milner & Geddes: Cereal Chem. 23 225 (1946).

227 Bottomley, Christensen & Geddes: Cereal Chem. 27 271 (1950).

228 Snow, Chrichton & Wright: Ann. App. Biol. 31 102 (1943).

229 Milner, Christensen & Geddes: Cereal Chem. 24 182 (1947).

230 Christensen: Cereal Chem. 32 107 (1955).

231 Matz & Milner: Cereal Chem. 28 196 (1951).

232 Arndt: Phytopath. 36 30 (1946).

233 Hummel, Cuendet, Christensen & Geddes: Cereal Chem. 31 143 (1954).

234 Luthra: Current Sc. (India) 4 489 (1936).

235 Zimmer: Nature 132 469 (1933).

236 Ohga: Am. J. Bot. 13 754 (1926).

237 Libby: Science 114 291 (1951).

238 Barton: "Seed Preservation and Longevity", Interscience, New York, 1961.

239 Ewart: Proc. R.S. Victoria 21 1 (1908).

240 Crocker: Bot. Rev. 4 235 (1938).

241 Harrington & Crocker: J. Agric. Res. 14 525 (1918).

242 Waggoner: Am. J. Bot. 4 299 (1917).

243 Atanasoff & Johnson: J. Agric. Res. 18 379 (1930).

244 White: Proc. R.S. B81 417 (1909).

245 Semeniuk, Nagel & Gilman: Iowa Agr. Exptl. Sta. Res. Bull. 349 255 (1947).

246 Milner: Science 104 463 (1946).

247 James & James: New Phytol. 39 145 (1940).

248 Tang: J. Gen. Physiol. 15 561 (1932).

249 Barton: Ann. N.Y. Acad. Sc. 46 185 (1945).

250 Simpson: Bull. Univ. Tenn. Agric. Exp. Sta. no.228 (1953); from ref. 238.

251 Lewis: John Innes Hort. Inst. (London) Ann. Rept. for 1952 43 14 (1953).

252 Jones, Divine & Gersdorff: Cereal Chem. 19 819 (1942).

253 Bayfield & O'Donnell: Food Res. 10 485 (1945).

254 Hoover: Food Res. 20 469 (1955).

255 Jones: Bot. Gaz. 69 127 (1920).

256 Wyttenbach: Landbw. Jb. Schweiz 4 161 (1955); from ref. 238 p.152.

257 Many refs. on p.152 of ref. 238.

258 Das & Sen Gupta: Ann. Biochem. Exptl. Med. 1 117 (1941).

259 Saltman: JBC 200 145 (1953).

260 Cohen: JBC 164 685 (1946).

261 Vennesland et al.: JBC 194 143 (1952).

262 Miyake & Kurasawa: Rept. Japan Assoc. Adv. Sc. 16 559 (1942); from CA 44 3099.

263 Bach: Compt. Rend. Soc. Biol. 127 175 (1938).

264 Rohrlich: Getreide u. Mehl 7 89 (1957); from CA 53 20593.

265 Seifter: Plant Physiol. 29 403 (1954).

266 Riggio-Bevilacqua: Atti accad. nazl. Lincei, Rend., Classe sci. fis., mat. e nat. 18 214 (1955); from CA 49 16074.

267 Guillemet: Compt. Rend. Soc. Biol. 108 779 & 781 (1931).

268 Iwamoto: J. Soc. Chem. Ind. Japan 33 409 (1930); from CA 25 2019.

269 Butler & Norris: Arch. Biochem. Biophys. 87 31 (1960).

270 Longenecker & Haley: Arch. Biochem. 18 229 (1948).

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SUMMARY

PART A

1 The metabolism of mustard seeds wet at sub-germination temperatures has been studied, using tritium incorporation as an index of metabolism. The theory and scope of the method are discussed.

2 Improvements have been made in the chromatography procedure.

3 At 0°, many of the normal germination chemical reactions proceed, but about one tenth as fast as at 24°. Amino-acids are being metabolised within 2 hr. of wetting the seeds, and malic and citric acids within 4 hr. Within 24 hr. lipids and fructose are undergoing reactions.

4 An unidentified compound, not reported in normal germination, is being metabolised within 48 hr. Another aberration from normal is the absence of detectable succinate metabolism.

5 Labelling of the solid residue (insoluble in ethanol and in water) always occurs. It is shown to be largely non-metabolic. Control experiments showed that this was the only non-metabolic labelling.

6 A theory is proposed to explain the non-germination of

seeds at temperatures near 0°. It is suggested that the Krebs cycle is qualitatively altered, perhaps by "wasting away" of glutamate to 4-aminobutyrate instead of its routing into the Krebs cycle as α-oxoglutarate.

PART B

7 A method has been developed for studying the metabolism of dry seeds, spores and pollen by exposure to THO vapour.

8 Dry Pinus radiata pollen has been shown to label many compounds. A few have been identified and are common metabolites. It may be that the metabolism of dry pollen is not qualitatively different from its germination reactions.

9 Dry mustard seeds and spores of the fungus Pithomyces chartarum give, in contrast to pollen, patterns of incorporation very different from those in early germination.

10 Suggestions are made for further applications of tritium incorporation from THO as an index of metabolism.